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PhD THESIS

Insects: the new challenge of animal farming for a more sustainable feed and food production.

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"Domestication of insects is a really good idea. I believe it is crucial because it will allow local communities to produce insects so as to increase their availability and, at the same time, the increase in production will lead to an increase in their income. The domestication of insects therefore represents a double advantage. Insects will be produced sustainably and at the same time the livelihoods of rural communities will continue to improve. " [1]

1 Ousseynou Ndoye, 2014, FAO (Cameroon).

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ABSTRACT

Population growth and rapid urbanization have increased the global demand for animal feed and protein sources. Therefore, the traditional production of animal feed should be increased through the use of alternative nutrient sources. Insects as feed are starting to meet this need. One such insect is the black soldier fly, *Hermetia illucens* L. (Diptera: *Stratiomyidae*). However, in order to mass-produce the black soldier's fly (BSF) more effectively, a better understanding of its thermal biology is needed. Therefore, the aim of one of the five contributions of this thesis was to evaluate the impact of age, size and sex on thermal preference for adult black soldier flies. The thermal preference of adult black soldier flies was determined by exposing the flies to a thermal gradient with a range of surface temperatures and monitoring their location over time. An aluminum plate was used to create a linear thermal gradient in which surface temperatures ranged from 15 to 60 ° C. The flies were distinguished by age (1 day after emergence vs 7 days after emergence), size (large vs small) and gender (male vs female) to assess whether thermal preference differed from specific life history traits. Thermal preference for 7-day post-emergence adults was significantly lower (19.2 ° C) than for 1-day post-emergence adults (28.7 ° C), respectively. Similarly, small adults selected significantly cooler temperatures (21.1 ° C) than large adults (26.9 ° C). There were no significant differences in thermal preferences between thesexes, regardless of age or size. Indeed, males and females had a similar thermal preference of 23.8 °C and 24.2 ° C, respectively.

Due to the high sustainability of insect farming, the possibility of breeding insects as a food and feed source appears to be very promising. Reusing and enhancing food waste is possible by using it as a substrate for the growth of insects. In this context, BSF can grow on a wide range of substrates turning it into valuable biomass. In the second contribution, four different substrates were used and evaluated for their suitability for the reproduction of the larvae. *Hermetia illucens* larvae (five days old) were raised on chicken feed (control diet), a vegetable diet (V100), a 50% vegetable diet + 50% butchery waste (V50 + B50) and a vegetable diet 75% + 25% of butchery waste (V75 + B25) to assess their suitability. Ten kg of substrate and 6,000 larvae made up each replicate (9 per group). The larvae were weighed and measured every two days until 25% developed into prepupae. Mortality and larval growth rates were calculated. Ash substrates, larvae and chemical composition were analyzed. The oxidative state and stability of the larvae were measured in the hemolymph and in the body. V100 larvae exhibited the lowest live weight, length, thickness, and growth rate, but hada low mortality rate, high substrate reduction rate, and protein conversion ratio. The V100 larvae hadsimilar proteins and lower lipids than the control ones while the V50 + B50 and V75 + B25 larvae contained higher lipids and lower proteins than the others. Although plant wastes, to varying degrees,reduced the reactive oxygen species content of the hemolymph, the V100 diet reduced growth performance and should be avoided. Butchery waste may be suitable, but it must be well combined with other ingredients to balance the high lipid level and low protein content, and vegetable waste may be an appropriate candidate. Vegetable and butchery waste is easy to find and collect and, in thepresent study, showed interesting potential for BSF larvae growth by producing insects with interesting chemical characteristics at 22 days of age. The use of plant waste reduced the level of reactive oxygen species in the insects' hemolymph, suggesting a positive effect on the welfare of the

larvae. In recent years, several studies have focused on the use of insect larvae meal as an alternative to soybean meal in poultry diets. In this regard, it is essential to try to understand all the possible aspects related to the chemical-nutritional characteristics, the effects on animal health and welfare, and the impact on food safety of the various insect flours.

Another contribution of this project aimed to evaluate the production of volatile fatty acids in the caecum, the intestinal morphometry and the enzyme activity of the brush rim of hens fed with increasing levelsof *Hermetia illucens* maggot meal. To evaluate the effects of feeding a *Hermetia illucens* (HI) larvaemeal on different intestinal tracts of hens and to determine the concentration of trace and toxic elements in insect meal and diets, 162 hens were divided into three age groups between 16 and 40 weeks. The control was fed a cornmeal and soybean meal (SBM) diet; the HI25 and HI50 groups were fed two diets in which 25% and 50% of the dietary protein were replaced by the HI protein, respectively. The height of duodenal and jejunal villi and villi / crypt were higher ($P < 0.01$) in the SBM than in the HI groups. Ileal villus height was greater ($P < 0.05$) in the SBM and HI25 groups compared to HI50. The HI50 group had inferior duodenal maltase activity. IAP decreased linearly inthe duodenum and jejunum with increasing inclusion of the insect meal in the diet. Ileal -GT activitywas greater in SBM than in both insect groups ($P < 0.05$). The HI50 group had higher acetate and butyrate than SBM, with potential positive effects on gut health. Levels of toxic elements such as Cd,Pb, Hg, and as in diets and insect meal were lower than the maximum levels of heavy metals set bythe EU Commission for feed.

The forth contribution, conducted for this PhD project, was done on a total of 120 Japanese 12-week-old quail females, who were divided into 4 groups (6 replicas of 5 birds each). The controlgroup (CON) fed a corn and soy diet; in the other 3 groups, *Tenebrio molitor* grub meal (TML)replaced 5, 10 and 20% of the soy protein (T5, T10 and T20). Spawning performance and egg qualitywere studied for 54 days. The data was processed by a one-way ANOVA; Orthogonal contrastanalysis was performed to test linear, quadratic, and cubic effects between the means. Egg-laying rateand egg mass decreased linearly ($P < 0.01$) as the level of inclusion of TML in the diet increased. Eggweight and feed conversion ratio increased linearly from control to T20 diet ($P < 0.01$) whiledigestibility of dry matter, organic matter and crude protein decreased linearly ($P < 0.05$). Egg whiteand yolk weight showed a linear increase ($P < 0.01$) due to the inclusion of TML in the diet, whileeggshell weight showed the opposite ($P < 0.05$). Estimated Δ9-desaturase (C16: 0), 5 + Δ6-desaturaseactivity on both n-6 and n-3 polyunsaturated fatty acids increased linearly ($P < 0.05$) as influenced bydietary LMT. The lightness of the boiled yolk (L *) showed higher values in the T5 and T10 groups(quadratic contrast, $P < 0.01$). The yolk redness index (a *) showed lower values in T5 and T20compared to the control and T10 groups (cubic contrast, $P < 0.01$). The albumen indices L *, a * andb * showed a significant quadratic contrast effect ($P < 0.05$). Furthermore, the albumen index b *showed a significant effect of the cubic contrast ($P < 0.01$). Total lipids showed the highest values (cubic contrast, $P < 0.05$) in the T10 and T20 groups. Total monounsaturated fatty acids increasedlinearly ($P < 0.05$) based on the increase in dietary LMT. The best inclusion level of the TML defattedmeal for laying quail appears to be 1.4% of the diet, corresponding to the T5 diet.

The latest contribution of this PhD project was aimed at proposing the larvae of the queen of honeybees, discardedfrom the production of royal jelly, as a possible food supplement in animal nutrition. To this end, thechemical characteristics, chitin content, amino acids, fatty acids and mineral profile (including toxicelements) were determined on pooled samples of queen bee larvae. Queen bee larvae meal is rich inchitin, proteins, essential amino acids and some essential minerals such as

phosphorus and magnesium; it is also relatively low in fat, and with negligible levels of toxic elements such as Cd, Pb, As and Hg. However, its fatty acid profile showed a very low amount of polyunsaturated fatty acids and the flour was low in Ca and other trace elements compared to the more common insect flours used in feed. Queen bee larvae have a standard royal jelly diet and this could be a great advantage for use in animal production. However, the collection of the queen bee larvae does not allow to give high quantities of final product both for the low quantity of collected larvae (on average
58.9 g / hive / month) and for the relatively low yield of flour (on average 23, 12%) registered. Therefore, queen bee larvae meal cannot be considered an alternative protein source in animal production but could represent a potential dietary supplement to be inserted at low doses to exploit the possible modulating activities of the intestinal microbiota due to the high levels of chitin.

The use of insects for feeding farmed animals represents a promising alternative because of the nutritional properties of insects and the possible environmental benefits, given the sustainability of this type of farming. Although rapid development is expected, insects remain underutilized in the animal feed industry mainly due to technical, financial, and regulatory barriers.

INTRODUCTION

INSECTS: A NOVEL FOOD BETWEEN FOOD RISKS AND NUTRITIONAL ASPECTS

Population growth as well as increasing urbanization have surged up the global demand for food, and in particular for animal protein sources. For this reason, the traditional production of animal feed such as fishmeal, soy and cereals, needs to be further intensified or to be replaced through the use of alternative nutrient sources. It is estimated that in 2050 more than 10 billion people (FAO, 2017) will have to be fed, in addition to the billions of farm and companion animals. Moreover, it must be taken into account that alarming phenomena, such as water and land pollution caused by the intensive production of livestock as well as deforestation caused by excessive grazing, will contribute to climate change with further possible negative impacts on the environment.

The insect breeding could be a way addressing or at least limiting these problems. Insects live everywhere, reproduce quickly, have a high rate of growth and food conversion and a low environmental impact throughout their life cycle. Being very nutritious, they can integrate traditional feed sources, given their high content in proteins, fats and minerals; they can be raised on food waste, eaten whole or reduced into powders and / or pastes to be then incorporated into other types of food. The large-scaleuse of insects as potential food is technically feasible and industries in various parts of the world arealready engaged in this production.

The insects with the highest potential for large-scale feed production are: the black soldier fly (*Hermetia illucens*), the common house fly (*Musca domestica*) and the flour worm (*Tenebrio molitor*), but also other insect species are designed for this use. Chinese, South African, Spanish and American producers already breed large quantities of flies with the bioconversion of organic waste, in order to use them as feed for aquaculture and poultry farming. Insects, in addition to being an alternative source of food, are important for various other reasons: they provide important services to ecosystems, such as their fundamental role in pollination, biological control and decomposition of organic material, as well as their abilities to reduce livestock manure (for example pig feces) and to mitigate bad odors.

It is well known that insects have always inspired human progress, leading to the creation of a real science, biomimicry, which imitates the attributes and processes of living organisms to give rise to innovative ideas. This new scientific branch has used, among other things, the characteristics of hives, spider webs and termite nests to inspire new products or production systems. Still, insects have been part of traditional medicine for thousands of years, just think of the use of fly larvae to clean wounds from dead tissue, or the increasingly widespread use of hive products such as propolis, royal jelly and honey, which have always been used for their healing properties. The importance of insects is not limited only to these uses; their natural color has been used for centuries by different cultures: already the ancient civilizations, as the Aztecs used for various purposes the red color produced by cochineals; even today this product is widely used as a dye in the food and cosmetic industry.

Returning to the use of insects for food and feed production, it can be said that they have various advantages for the environment, for health and for improving the social condition and livelihoods of various populations.

As for the benefits for the environment, it can be said that insects:

• they can be raised on organic waste and by-products which are thus recycled and converted.

• they require very little water to be raised. For 1 kg of chicken, 2300 liters of water are needed, for 1 kg of pork 3500 liters are needed, for 1 kg of beef 22000-43000 liters are needed, differently many insects can survive several days without water;

• farmed insects emit much less greenhouse gases (carbon dioxide and methane);

• they can be reared in small spaces with a consequent reduction in soil consumption;

• the Life Cycle Assessment (LCA), which assesses the environmental impact associated with all the life stages of a product, for edible insects was estimated as extremely inferior to that of proteins obtained from traditional animal farms;

• they help in diversifying agricultural activities in a sustainable way. Many insect breeders and producers, in fact, give the possibility to other breeders (in the poultry and pig sector, for example) to take care of the insect larvae, grow them and feed them to the necessary stage, and then return themto the initial producer who will provide to turn them into feed.

• they have a high efficiency of nutritional conversion, since they are cold-blooded animals. Nutritional conversion rates for meat (i.e. how much feed is needed to produce a 1 kg weight gain of an animal) vary widely depending on the type of animal and the farming practices used. On average, insects can convert 2 kg of food into 1 kg of mass;

• they produce a probably lower amount of greenhouse gas than that of conventional livestock. For example, pigs produce 10-100 times more greenhouse gases per kg of weight than that produced by mealworms;

• they have higher conversion efficiency. For example, in converting from food substrate to meat, cricket is twice as efficient as chicken, five times than pork, and 12 times more than cattle; you eat almost all of the insects. In fact, they provide a higher percentage of edible fraction than traditionally bred animals due to the lack of bones, cartilage and fur / hair;

• they can feed on organic waste such as remains of food and human products, compost and animal sewage, and can transform them into high quality proteins which can in turn be used for animal feed;

• they use less water than conventional livestock. Mealworms, for example, are much more resistant to lack of water than cattle.

• they need less land than conventional livestock.

As regards the health benefits for both humans and animals, it is widely established that they:

• provide high quality protein and nutrients comparable to those provided by meat and fish;

• are particularly important as dietary supplements for undernourished children, because many species have a high amount of fatty acids (comparable to that provided by fish). They are also rich in fiber and micronutrients such as copper, iron, magnesium, manganese, phosphorus, selenium and zinc;

• have a low risk of transmitting zoonoses such as H1N1 (avian flu) or BSE (mad cow disease).

In January 2012, the Commission of technicians and experts, trained to evaluate the potential of insects for food and feed production, indicated the following key areas for research and development:

1) Mass production technologies: increasing innovation in mechanization, automation, processing and logistics to reduce production costs to a level comparable to that of other sources of food and feed; develop tables for the breeding of insects and for the nutritional value of growth substrates; conduct more in-depth research on the life cycles of a wide range of insect species to allow comparison with traditional food and feed; maintain a wide genetic diversity to avoid the collapse of farming systems.

2) Food and feed safety: investigate possible allergic reactions to insects and the digestibility of chitin (the main component of their cuticle); increase data on the nutritional value of edible insect species and their contribution to human and animal health; carry out research on the risk of possible zoonoses, pathogens, toxins and contamination with heavy metals (with the use of waste streams) caused by the consumption of insects; develop means to increase the shelf life of products.

3) Legislation: develop voluntary codes and regulations at national and international level to manage the production and consumption of food and feed of entomological origin in the context of human health and animal welfare (see for example the Codex Alimentarius); improve the methods for assessing the risks associated with mass farming and harvesting in nature, to avoid the introduction of alien and invasive species into natural insect populations.

4) Consumer education: support entomophagy in cultures where it is already present; conduct comprehensive research on the ecology of species potentially suitable for consumption or for breeding; inform consumers about the benefits of entomophagy; develop new ways to integrate insects into the diets of a wide range of consumers by creating insect-based products; advocate the use of insects as supplements to pet feed.

Finally, as regards the advantages for livelihood and social condition, it can be said that:

• the collection and breeding of insects can offer important strategies for a differentiation of livelihoods; insects can be directly and easily collected in nature; the collection and basic equipment for breeding require simple techniques and a minimal capital investment.

• insects can be collected from the wild, raised, prepared and sold by the poorest members of society such as women and landless workers in rural or urban areas. These activities can directly improve diets and provide cash income by selling excess production in popular markets, thereby providing business opportunities in developed, transition and developing economies.

• insect can be prepared as food or feed relatively easily. Some species can be eaten whole, others can also be made into doughs or ground before being consumed or their proteins can be extracted and used separately.

Insects

Insects are a class of animals belonging to the phylum of the Arthropods, very close, from the phylogenetic point of view, to the Crustaceans. This class represents the largest of the groupings of living beings that populate the planet, including about one million described species, equal to five sixths of the entire animal kingdom. Thinking that a quarter of the known species are made up of Beetles, it is easy to understand that the Earth is populated for the most part by insects.
Not all species of insects are known, moreover many of them (some estimates make them around 4-5 million), become extinct even before being discovered mainly due to the massive deforestation carried out by man. Insects not only have a total biomass that far exceeds the one of vertebrate animals, especially in tropical regions, but also have a great biodiversity, which is the reason why they are implicated in all terrestrial food chains.
They represent a large part of the nutrition of many animals then used by man, and recently, just as reported by in-depth studies conducted by FAO in various emerging and third world countries, they have also become part of human nutrition. The success of insects is to be attributed to their extraordinary ability to adapt: in fact, they occupy all terrestrial environments, various freshwater environments as well as the air environment; they also adapt to all temperatures, from the rigid ones of the poles to the very high ones of the Equator. They feed on everything from mushrooms to meat, from debris to wood, from fabric to plants. Their small size, ranging from 0.2 to 120 mm, resulted in the occupation of limited ecological niches, the reduced need for nutrition for each individual and the possibility of forming large populations in small habitats. The external covering of their body, the exoskeleton, a sort of rigid shell covered with waxy substances, is perhaps the most important factor of success as it protects them from mechanical damage by limiting the loss of internal fluids and makes them also able to colonize dry environments.
This "shell", hindering growth, requires a periodic replacement process (the so-called moult) which in some cases culminates in a complete metamorphosis of the body (for example, from the caterpillar-larva stage it then passes to the adult insect). This characteristic, which could appear as a limitation, has instead allowed different stages of development (young and adult) to occupy separate ecological niches avoiding competition phenomena. Another success factor of insects is their ability to fly, which allows them to disperse, to escape from terrestrial predators and to search more quickly for both food and partners to mate with. However, it must be emphasized that since ancient times, the history of man has been influenced by insects both positively, as we have seen above, and negatively: an example could be the swarms of locusts that have caused various famines, such as they reported the sacred texts, or, being often vectors of diseases and epidemics, the devastating black plague, (caused by a bacterium transmitted to man by the rat flea), which killed a third of the European population in the fourteenth century. Not only examples from the past, even malaria, transmitted by the Anopheles mosquito, still threatens over 40% of the world population, especially those residing in tropical countries.
Insects can be contaminated with different types of bacteria and parasites, even of clinical relevance. Furthermore, they can contain and accumulate toxic substances of an endogenous or exogenous nature capable of causing problems in the consumer, especially in cases of ingestion of large quantities or habitual consumption. The scarcity of data deriving from insect farms, the total absenceof a real production chain, the reduced chemical analyzes on insects raised with different diets and the potential problems deriving from environmental contamination with metals, pesticides and

possible pharmacological treatments in phase of breeding, require further studies and a precautionary approach before being able to define insects as safe food.

According to a study by the Food and Agriculture Organization of the United Nations, carried out in collaboration with the University of Wageningen in the Netherlands, there are already over 1900 insect species in the world that humans eat. The insects most commonly used as food belong to the Beetles (31%), Lepidoptera (caterpillars, 18%), bees, wasps and ants (Hymenoptera, 14%) followed by grasshoppers, locusts and crickets (Orthoptera, 13%), cicadas, leafhoppers, scale insects and bedbugs (Hemiptera, 10%), termites (Isoptera, 3%), dragonflies (Odonata, 3%), flies (Diptera 2%) and other orders (5%).

The consumption of insects for human and animal nutrition is today strongly encouraged by FAO, which has promoted various conferences on the subject and which stimulates research from the point of view of the regulation of laws for breeding and consumption at national and international level (FAO, 2015). In the perspective of an ever-increasing demand for new food sources to support the growth of the world population in the coming decades, insects can therefore represent an adequate response both as a source of animal protein for livestock farming and as direct human nutrition. More easily acceptable to numerous populations of developing countries. The need to raise the most suitable insect species on a large scale to ensure healthy food can also encourage the development of various types of business initiatives in the future.

The pressing need to identify new food sources capable of feeding the ever-growing global population, and to produce high-quality animal proteins with greater respect for the planet's limited resources and animal welfare, require innovative strategic guidelines regarding choice of new animal species to be bred and farming methods. The food use of insects, both for human consumption and for the production of meal for animal feed, has been indicated by FAO as the most promising way for sustainable food production (FAO, 2015).

The proposal to use insects as food for humans and as feed for animals is now accepted, not only by FAO, but also by a large number of organizations, academies, private production sectors, and the media. Global food production, in fact, cannot grow further if radical technological innovation is not introduced in the current production system, as already anticipated for some time in some forecasts, such as those of the Club of Rome Report in 1972. It is necessary to take into account however, that food must also be produced for about one billion pets and nearly 100 billion farm animals. A crucial factor for achieving global food security is the need to more efficiently produce an adequate amount of animal protein to support a healthy growing world population, with an environmental impact lower than that of the current industrial production of animals. The possibility of breeding insects on organic waste is currently the most promising alternative to obtaining a new source of animal protein. The introduction of insects as safe food in the agendas and promotional campaigns of food agencies will require a more in-depth knowledge of the nutritional value and food safety of a greater number of edible insect species. In particular, more knowledge is needed about their effect on consumer health, food safety, environmental impact and a proper risk assessment in the introduction of insects into the food chain.

Lawmakers and policy makers are now called upon to take a stand and include insects as food and feed in national policies and in the legal framework regarding food, health and the use of animal feed. The European regulatory framework regarding the possibility of marketing insects for food use is quite clear and binding. In fact, insects fall within the definition of Novel Food (Reg. 258/1997) which collects all foods not consumed to a significant extent in the European Union before May 15, 1997

(date of entry into force of the regulation) and falling into one of the categories defined by the regulation itself. The field of application of this device is certainly vast if we consider that at the date of entry into force, it also applied, among others, to GMOs, food from cloned animals and nanotechnologies. Although the objectives were probably different, it also regulates insect-based products and allows their marketing only upon submission of a safety dossier pursuant to Recommendation 97/618/EC that contains scientific data sufficient to demonstrate safety of the foods for which authorization is requested, considering any groups most vulnerable to the identified potential adverse effects. This dossier must be approved by the competent national authority and possibly by the European Food Safety Authority (EFSA) (Belluco *et al*., 2013). Since the requisites necessary to satisfy the dossier are numerous and complex (among other things, each species of insect or its product can be identified as a different Novel Food), to date no one has faced this path in a convinced way and alternative routes have been preferred. Some EU member states have interpreted Reg. 258/1997 literally and have excluded whole insects from the Novel Food definition, allowing their distribution in their territory of competence, following summary risk assessments (FASFC, 2014; Netherlands Food and Consumer Product Safety Authority, 2014). Other states have chosen more precautionary approaches, postponing the decision to the European institutions, which have asked EFSA for a formal opinion on the possible health risks deriving from the consumption of insects. A new version of Reg. 258/1997 relating to the consumption of insects in human nutrition, was then amended with EU regulation 2015/2283.

This proposal provides for the certain inclusion of insects among the Novel Foods but allows for a more streamlined authorization procedure for products traditionally consumed in third countries. In this context, the experiences of traditionally entomophagous countries would be allowed to support the wholesomeness of new foods, always subject to an appropriate scientific safety assessment.

With regard to the use of insects in animal nutrition, the European Commission has currently undertaken a process of modification of the annexes of EC regulation 999/01 and of the annexes of regulation (EU) 142/201 to allow the use of processed animal proteins (PAPs) from insect, in the feeding of aquaculture animals, but under very specific processing, storage, transport and use conditions.

The application of the legislation, initially, left to the Member State the decision to allow the use of live insects / whole carcasses for feeding some animals, it being understood that Regulation (EC) 999/2001, in article 7, it prohibited its use for ruminant species. The Ministry of Health authorizes its use for feeding pets or animals not reared for food production, such as ornamental, fur, zoo or laboratory animals or for uses other than food (technical uses). Instead, with the support of EFSA, the current legislation has defined a positive list of insect species that will be allowed the productionof PAT for aquaculture, including *Hermetia illucens* (*H. illucens*) and *Tenebrio molitor* (*T. molitor*). Specifically, with the note of the Italian Ministry of Health 11399 of 05/05/2017, indications were provided on the correct application of the current legislation regarding the breeding and use of insects for feed production, in order to prevent non-compliant conduct by of operators. These measures were issued by following the growing demand for protein raw materials in the feeding of farm animals, which has proportionally increased the interest in the breeding of insects as the latter are able to supply high quantities of proteins to high biological value with rapid breeding cycles and using, as nutritional sources for their production, materials derived from the food industry, also favor the use of waste products. Therefore, terrestrial invertebrates and insect PATs can be considered as feed materials. Predictably, insects that can be used as feed must not belong to pathogenic species, must

not be vectors of pathogens for humans, animals or plants and must comply with environmental criteria for the protection of native species. Insects are also subject to compliance with the microbiological criteria and the limits of contaminants and undesirable substances provided for by the Community legislation for feed materials. With the new EU Regulation 2017/893 it will instead be possible to use, for the feeding of aquaculture animals, the transformed proteins derived from insects and the compound feeds that contain them.

Currently, fish and soybean meal are the main protein sources used and precisely due to the increase in demand for these raw materials, there has been a drastic increase in their price. Furthermore, their production exploits resources also used for human nutrition and has a major impact on the environment (Pelletier and Tyedmers, 2010; Steinfeld and Gerber, 2010). This is the main reason that forced the identification of alternative protein sources and that saw insects as a concrete and valid alternative (Van Huis *et al.*, 2013).

As previously mentioned, an important criterion to consider in determining whether a given source can represent a valid raw material for the production of feed is the protein content. Numerous studies, summarized in recent publications (Makkar *et al.*, 2014; Sanchez-Muros *et al.*, 2014), have shown that many insect species have a high protein content, comparable to that found in fish or soy meal. These proteins have excellent nutritional quality as they are rich in essential amino acids, including lysine, methionine and leucine, usually lacking in conventional vegetable protein sources. To this it should be added that insects have a high conversion efficiency of ingested food, far superior to that of cattle, pigs or sheep (Van Huis, 2013; Van Huis *et al.*, 2013), in addition to the aforementioned advantages such as the fact that their breeding does not require large spaces, which requires low water consumption and which determines reduced greenhouse gas and ammonia emissions (Oonincx *et al.*, 2010; Oonincx and De Boer, 2012). Additionally, some insects are scavengers and can grow on decaying organic matter, thus turning waste material into a source of animal protein.

This aspect is particularly interesting because it has been estimated that one third of the product of the world agri-food chain (equal to about 1.3 billion tons per year) is now lost or eliminated as waste (Gustavsson *et al.*, 2011) and the enhancement of this enormous quantity of organic matter through its biotransformation into animal proteins with high nutritional value is certainly a stimulating prospect. There are several species of insects considered interesting for feed production. Studies have been carried out on them, and others are still in progress, to better understand their biology (Jones and Tomberlin, 2020; Macavei *et al.*, 2020; Park *et al.*, 2016), evaluate their growth capacity on different substrates and analyze the quality of the flour obtained in terms of macro- and micronutrient content and hygienic safety (Makkar *et al.*, 2014; Bovera *et al.*, 2018; Secci *et al.*, 2018; FAO, 2021).

The breeding of edible insects is one of the activities that will increasingly grow in the future, in order to produce, also in Europe, alternative and sustainable animal proteins. In some non-European countries, where the climate and culture have favored the development of these breeding practices, it is easier to find examples of breeding edible insects. Companies have recently sprung up in Europe and America that have taken inspiration from these breeding practices by re-proposing more mechanized and controlled models. Their purpose is to produce an excellent source of animal proteins in relatively small spaces, with less use of water and feed compared to normal intensive farming of common animals reared for human nutrition. Edible insect farms represent a new page in the breeding of food animals in Europe and also in Italy. To keep up with the times, it is necessary that the practices in place are efficient, the environments and materials used safe and controlled. New technologies,

vertical farming, advanced sensors are essential elements for any farm that aspires to become a next generation farm of edible insects.
In recent years, breeding of edible insects is growing in many countries of Europe, North America, Asia.
According to International Platform of Insects for Food and Feed (IPIFF), the profile of European operators dealing with insects is varied. About 80% of companies are micro-enterprises, including innovative start-ups, as we would define them in Italy. According to the IPIFF survey, most companies have invested less than 0.5 million Euros, less than 20% of companies have made investments up to 5M Euros, and only 6% have received investment rounds of 10 M of Euro and up. The profile of the operators is also very different: some companies breed insects, others transform them into food, only some breed and transform them. The main sales channel is e-commerce.
FAO in December 2020 published a document that aims to be a guide for the management of sustainable insect farms (Hanboonsong and Durst 2020). The document contains indications on the suitability of the place where it is possible to start the activity, on the type of shed, on the way to manage the rooms and internal materials, on the type of feed to be used for crickets, on hygiene practices to follow, on the necessary training for the operators, on the methods of transformation of insects. The guide appears complete and detailed, but certainly the breeding described does not represent the best model for a breeding of edible insects to be reproduced in Italy or in Europe. For example, in our latitudes it is unthinkable to be able to host an edible insect farm in unheated and insulated sheds. The feed used could not be different from what is foreseen by the European Regulations on feeding stuffs. Even materials used in breeding, in the slaughterhouse, in the processing areas, EU regulations would lead to a very different management of the entire process. The breeding methods vary according to the species of insect that you want to breed. The cricket, for example, cannot be raised as a mealworm and vice versa. In fact, insects, like other farm animals, have different life cycles depending on the species, and the environments and equipment are chosen by the breeder accordingly.
Recently, the IPIFF published a very useful document for operators. This guide gives detailed indications on good hygiene practices to be followed in the various stages of breeding and processing, regardless of the species you want to breed. The legal basis to be followed in the case of breeding for animal feed and human consumption purposes is therefore distinguished. The breeding of insects represents a valid sustainable alternative both to produce food for humans and feed for farmed animals, but also a new source of income in a still unexplored market (Madau *etal*., 2020; FAO, 2021). Great opportunities are also represented by the production of pet food, alternative energy (biogas) and agricultural fertilizers. Europe is increasingly projected towards this new horizon. In fact, EFSA (EFSA, 2021) has recently expressed a positive evaluation on a product derived from insects, the *Tenebrio molitor* larva proposed as a human food. Another dozen questionsregarding other insects awaiting evaluation are also being examined.

The authority responsible for controlling the safety of animal feed in the USA, is the Federal Food and Drug Administration (FDA), which collaborates with the Association of American Feed Control Officers (AAFCO) in the area of feed regulation, particularly in addressing new feed ingredients (Lahteenmaki-Uutela *et al*., 2018). AAFCO is composed of state, federal, and international regulatory officials who are responsible for the enforcement of state laws regulating the safe production and

labeling of animal feed. Edible insects are considered to be food additives in the United States (Lahteenmaki-Uutela *et al*., 2018), and the annual AAFCO Official Publication, which contains the most complete list of feed ingredients with their definitions, includes the list of approved food additives, as well as the list of generally recognized as safe (GRAS) substances. Today, only HI has been included as an ingredient for animal feed in both forms (dried whole larvae and HI meal), and its use is limited to aquaculture (i.e., salmonid fish).

In Canada, the Animal Feed Division, Animal Health Directorate, of the Canadian Food Inspection Agency (CFIA) is the authority in charge of managing the Food Act and Feeds Regulation of 1983; it also registers feed and feed ingredients and develops feed-related policies (Lahteenmaki-Uutela *et al*., 2017). In Canada, insects are considered to be novel feeds, which are those ingredients that do not have a history of safe use. Each registration proposal must detail the insect species, their specific rearing condition, and the substrate on which the insects were grown and fed. In 2016, the use of HI larvae was authorized for chicken feed, and in 2017, it was authorized for use in aquaculture. In 2018, the authorization was extended to all poultry.

In several Asian countries, insects have been historically considered food and feed and used as a good source of protein. In China, there are no specific laws for their regulation. Insects can also be used as feed additives, and in this case, producers must respect the rules collected in the Administrative Measures for Feed and Feed Additives (Lahteenmaki-Uutela *et al*., 2017). Another example is the different approaches of North Korea (Democratic Republic of Korea) and South Korea (Republic of Korea). These two countries, despite having the same history (almost untilthe twentieth century), language, culture, and food culture, currently have a completely different approach to insects as food and feed. According to Jo and Lee (2016), in North Korea, there are legalproblems that affect the use of insects as feed, because insects are considered to be animal-based protein, and these are banned for use in animal feed. On the other hand, in South Korea, insects are considered to be a historical component of the human diet and are included in animal feed (Han *et al*., 2017). There are no specific rules about insects as food and feed because of the deregulation of legislation concerning insects decided by the South Korean government in 2015 feed (Han *et al*., 2017).

Breeding insects is more sustainable than traditional animal husbandry. On a nutritional level, insects can be a source of three macronutrients: proteins, fats and chitin whichcan be used not only as a food source but also for energy production, biodiesel and bioplastic; to theseis added the frass, insect droppings valid as a fertilizing product. The insect can be used as it is, live,dried, ground or on the contrary divided into its components, each of which can have specific applications. As for human nutrition, the fractionation of nutrients allows the various components tobe incorporated into the food in such a way that the insect is not visible to the Western consumer, who is still reluctant to feed on insects. As far as the feed sector is concerned, the fractions make it possible to obtain more balanced feed from a nutritional point of view.
There are several edible insects that can be farmed. The first among them is the soldier fly (*Hermetia illucens* or black soldier fly). The larvae of this dipteran consist of 37% lipids, 32% proteins and 9% chitin and develop in a very short time (1-2 weeks). Among the other candidates are the yellow

mealworms *Tenebrio molitor*, *Alphitobius diaperinus* and *Zophobas morio* and the crickets *Acheta domesticus*, *Gryllodes ceraatus* and *Gryllus assimilis* and many others.

Referring to the policies, regulations and novel food in the European panorama, in 2012 FAO evaluated insects as a potential food source for humans and animals. Since 2015, in Europe, edible insects and products containing them are considered Novel Food (Reg Eu 2015/2283).

A "new" food whose marketing, however, is not yet permitted.

Numerous dossiers were then submitted to the European Commission to request marketing authorization and on 13 January 2021, EFSA published the first positive opinion on the flour larva, *Tenebrio molitor*. An important recognition that will hopefully soon lead to final authorization.

There are other types of restrictions in the feed industry. First of all, regarding to the breeding substrates, according to the EC regulation n. 767/2009 animals reared in the European Union can only be fed with safe products. Excrements and contents of the digestive tract, kitchen waste or foodstuffs no longer intended for human consumption, unprocessed that contain meat or fish are prohibited.

In fact, the legislation at European level is not very clear and its complexity pushes many people not to enter the market despite the prospects are very attractive for the future, even if insect farms are becoming real entrepreneurial realities in Europe in general, but also in Italy. Small and medium-sized enterprises producing for both the feed and food markets.

The black soldier fly (*Hermetia illucens*)

PHYLUM Arthropoda

CLASS Insecta

ORDER Diptera

FAMILY Stratiomyidae

GENUS *Hermetia*

SPECIES *Hermetia illucen*

Fig. 1. Hermetia illucens: larvae (left) and adult (right).

It is a medium-sized Diptera native of the tropical, subtropical and temperate regions of America, now widespread in all regions of the Earth between the 45th parallel north and the 40th parallel south (Makkar *et al*., 2014).

In fact, in recent decades it has spread to all continents, practically becoming a cosmopolitan species. In Europe, the black soldier fly has been reported in the Iberian Peninsula, southern France, Croatia, Malta, the Canary Islands and Switzerland. In Italy it was reported for the first time in the middle of the last century. Currently it is widespread throughout the peninsula and in the major islands it is supplanting indigenous dipters with similar ecological niches as a sarco-scavenger degrader. This rapid spread can be explained on the basis of its biological characteristics: *Hermetia illucens* (*H. illucens)* is, in fact, a voracious competitor and predator of other diptera and is also able to inhibit the development of other species through allomones. This species is also not attacked by parasitoids and is extremely resistant to chemicals and insecticides.

The body, 15-20 mm in size, is predominantly black in color, with metallic reflections ranging from blue to green on the chest and sometimes with the extremity of the abdomen of a reddish hue. The second abdominal tergite has translucent areas, from which the specific epithet derives. The head is broad, with very developed eyes, and is equipped with antennae about twice the length of the head.

The legs are black with whitish legs. The wings, of the membranous type, in the resting phase are superimposed and folded horizontally on the abdomen (Fig.1).
The eggs of this insect, which hatch in about 4 days, measure about 1 mm in length and chromatically range from creamy white to pale yellow.
The post-embryonic development passes through 6 stages, the development times of which are well known. At the sixth stage we observe the reduction of the mouth parts and the accentuation of the inclusions of calcium carbonate on the cuticle, which hardens, forming a real case that is indispensable for pupation.
The larva, in the first stages, is 1.5-2 mm long, apoda and eucephalus, with a cylindrical-fusiform body, markedly segmented. The integument is strongly sclerotic with a cuticle containing calcium carbonate inclusions with hexagonal crystals forming a characteristic microsculpture (Fig. 1). The larvae of this species have a predominantly scavenger diet and are extremely voracious (in fact they consume daily a quantity of food substrate equal to double their weight), moreover they are found in various decaying organic substrates, both vegetable and animal, including composted organic waste, tree bark, wet soils and litter, manure, vertebrate corpses, agro-food industry and agricultural process waste, livestock manure and urban wet waste, significantly reducing their mass (Diener *et al*., 2009, Kalova and Borkovcova, 2013; Sheppard *et al*., 1994; St-Hilaire *et al*., 2007; Van Huis *et al*., 2013). It has also been shown that larvae fed on manure are able to modify its microflora, reducing the load of bacteria such as Escherichia coli and Salmonella enterica (Erickson *et al*., 2004): this is probably linked to the ability of these insects to produce antimicrobial peptides particularly effective against different bacterial strains (Makkar *et al*., 2014).
The development cycle of this insect is relatively fast: in ideal conditions of temperature and humidity the larval stage has an average duration of 2 months, the pupal stage of 2 weeks, while the adult mates 48 hours after the flicker and oviposes within a few days (Makkar *et al*., 2014). The pupa evolves within the exuvia of the last larval stage, a characteristic common to all *Stratiomyomorpha*.
Two other interesting features of this insect are the fact that the adult does not appear to be a vector of diseases and does not need to feed, keeping only with the nutrients accumulated during the larval stage. Unlike other scavengers, the adults of the *Stratiomidae* have no relationship with the growth substrate of the larvae, except for the oviposition phase. Numerous studies have shown that the flour obtained from the prepupal stage of this insect has a high content of proteins with high nutritional value, comparable to that present in fish and soy meal (Makkar *et al*., 2014). These flours have been pioneered as a raw material for feed production for the breeding of different animals such as chickens, pigs, rainbow trout, catfish, tilapia and salmon with absolutely satisfactory results (Makkar *et al*., 2014). In the case of the feed used for aquaculture, very encouraging data are reported since, using fishmeal replacement percentages of 25, 50 and 100%, the farmed species showed good growth performance and no variation in the histological indices or in the fillet quality (Makkar *et al*., 2014).
The biology of *H. illucens* has been extensively studied for its potential ecological importance as well as for the benefits that could be drawn from an economic, environmental and energy point of view.

The Miller's Tenebrio (*Tenebrio molitor*)

PHILUM Arthropoda

CLASS Insecta

ORDER Coleoptera

FAMILY *Tenebrionidae*

GENUS Tenebrio

SPECIES *Tenebrio molitor*

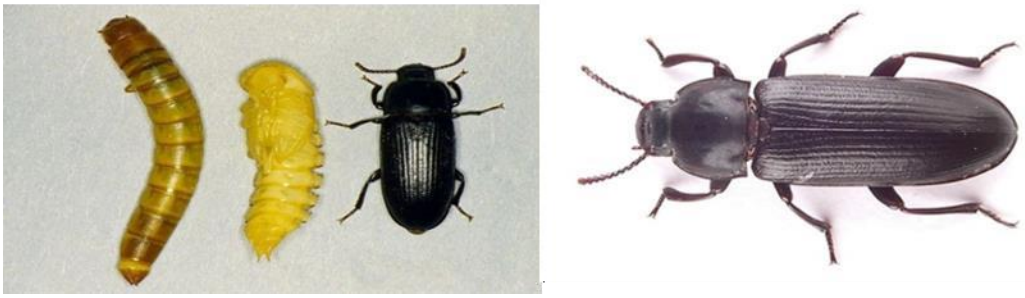

Fig. 2 Tenebrio molitor: larvae (left), adult (right).

It is an insect of the order of the beetles and of the *Tenebrionidae* family commonly known as the mealworm. It is a native species of Europe, however it is now present all over the world, with greater diffusion in the temperate regions and in the northern hemisphere; however, it is unable to reproduce in the tropics. In recent decades, its presence in the UK appears to be in decline.

The adult is dark brown or black (more rarely light brown), 12 to 20 mm long and 4.5 to 6 mm broad (Fig. 2); it is similar in appearance to a carabid, from which it differs above all for the clypeus protruding on the sides of the head, which partially covers the antennae, which are formed by 11 antennomers that swell towards the tip. The prothorax is broad and the elytra are crossed by evident longitudinal dotted striae under which membranous wings unfold, which allow it to fly.

The larva is roughly cylindrical in shape, initially whitish and subsequently, with the growth, of a bright rusty yellow color; it has short legs in correspondence of the thorax and can reach 30-32 mm and 130-160 mg of weight.

Similarly, the pupa is also white at first, and turns yellow over time. Reproduction is sexual, but cases of parthenogenesis have been reported by Frederikse (1924); the eggs are laid every year in spring but the development of the larvae is irregular, as it depends on the availability of food and environmentalconditions.

Each female can produce from 275 to 600 eggs, averaging 30-40 per day, which hatch after about 10-14 days. The larva is virtually omnivorous: in nature it develops in rotting wood, while in anthropized environments it prefers cereal-based products, which is why it has been called "mealworm"; however, it also feeds on residues of kernels, bran, remains of other vegetables and meat, tobacco, dead insects,

fertilizers, feathers and bird feces, so much so that it can proliferate, as well as in pantries and warehouses, even in the nests of bird or bat and even in old carpets and stuffing.
It is a very resistant larva, since it can resist for a long time in conditions of food shortage and adapts well both to dry environments with a temperature above 23-26 ° C, and to long periods of cold (even three weeks at -15 ° C). Under normal conditions, the larva pupates after 6-7 months, without cocoon and sometimes even moving away from the place where it grew. It should also be said that the larvae are raised and sold (often live, but also dried or powdered) in large quantities as food for birds, reptiles, amphibians and fish, as well as bait for fishing. Breeders sometimes feed the larva with a hormone that prevents its transformation into an adult, causing it to grow to more than 20 mm in length and 200 mg in weight.
The adult comes out after 7-24 days, in a time span between May and September; unlike the carabids, it walks rather slowly, and makes up for this lack thanks to a marked aptitude for flying. It also appears to be attracted to domestic lighting. For this reason, it appears to be a strongly synanthropic species, which is therefore found above all in homes, in particular in pantries; in the larval state, the flour moth is infesting and harmful, since it develops within foodstuffs, in particular those based on cereals such as flour, bread, pasta and biscuits, intended for human consumption. The food is practically contaminated by its excrement, thus taking on an unpleasant taste. Although it is one of the species most likely to attack foodstuffs, its control and disinfestation are relatively simple and its number is therefore limited; flour moth spread can be effectively prevented by storing supplies in clean, airtight spaces and properly disposing of waste.
In addition to the economic damage deriving from the infestation of foodstuffs, it has been shown that this moth also involves the onset of some diseases: the inhalation of fragments of *T. molitor*'s exoskeleton can activate immunoglobulins E, causing the onset allergies, a situation that presents itself as an occupational disease especially among those who work in contact with cereals.
These insects are widespread all over the world and have a rather long development cycle, which can last up to several months. However, the breeding of these species is relatively simple and excellent growth results have been obtained using waste plant material as a substrate (Makkar *et al*., 2014). It has been shown that the flour obtained from these insects can be successfully used for the breeding of poultry and fish (catfish, sea bream, sea bass, trout) (Makkar *et al*., 2014).

Honey bees (*Apis mellifera*)

PHYLUM Arthropoda

CLASS Insecta

ORDER Hymenoptera

FAMILY *Apidae*

GENUS *Apis*

SPECIES *Apis mellifera*

SUBSPECIES *Apis mellifera ligustica*

Fig. 3 Apis mellifera: queen bee and nurse bees (left), royal jelly cells (right).

The European bee or western bee (*Apis mellifera* Linnaeus, 1758) is the most widespread species of the genus *Apis* in the world.

Apis mellifera (*A. mellifera*) is native to Europe, western Asia and Africa. From the 17th century it was introduced by man to other continents, and now it can be found all over the world, including East Asia, Australia and the Americas, except in Antarctica.

In general, honey bees (*A. mellifer*a) are red-brown in color with black bands and orange-yellow rings on the abdomen. They have a lot of hair on the chest and less on the abdomen. In the hind legs there are "pockets" for collecting pollen. The paws are mostly dark brown-black in color.

The ligustica bee is native of Italy. It is the most widespread subspecies in the world among honey bees, due to the appreciation it has among beekeepers, as it has proved to be adaptable to most climates from subtropical to temperate, even if it has shown less adaptation to tropical humid climates.Italian bees evolved in the warm Mediterranean climate; they can withstand the harsh European winters and the cool, humid springs of the northernmost latitudes. They consume a significant amount of reserves in winter. The tendency to brood in autumn increases the consumption of honey. Having evolved in Italy, in a geographical position that favors the growth of a wide range of nectar plants and poor in honey predators, the Italian bee tends to be docile and very hardworking.

There are two castes of females in the bee community: nurse bees and queen bees (Fig. 3). The nurse bee is a normally infertile female due to the particular diet at the larval stage and due to the inhibitory

pheromones emitted by the queen. Each hive contains from 30,000 to 80,000 individuals who are almost all worker bees. They are smaller (they measure 10-15 mm) than the queen and their reproductive systems are present even if atrophied and only in some cases due to orphanage are they able to produce haploid eggs from which only male individuals will be born. The nurses who lay eggs are called "daughter-makers". Over the course of their life, nurse bees perform different tasks according to their age, up to 21 days they do not leave the hive and perform different functions: cleaners, nurses, wax producers, stockers, fans. After 21 days, the cerigen glands atrophy and for this they leave the hive becoming foragers collecting: nectar, pollen, propolis, water. This cycle is not the same for all bees, as there are bees that come to forage without having carried out the activities mentioned. Some appear to mature prematurely, as others may under certain conditions rejuvenate. The queen bee, on the other hand, is the only fertile female in the family and is distinguished from the others by its longer abdomen and larger chest. Their body dimensions are about of 18-20 mm (Fig.3). It leads almost its entire life inside the hive to lay eggs which will then give life to the workers and drones. The queen bee is born from a fertilized egg laid by an adult queen in a royal cell, unlike the others, this cell is larger and is shaped like a dome with the hole facing downwards. There are several reasons that push bees to raise a new queen, one of these is swarming, that is a process by which bees from a family are able to create two or more. Another reason is the need to replace an old queen, not very fertile and performing, which does not meet the survival needs of the family. Unlike worker bees, which are fed royal jelly only for the first three days, the queen bee will continue for life, and it is precisely the properties of this noble food that allows it to increase its size, even double it. than female workers, and to always be healthy for spawning. In her first days of life, the young queen matures sexually and performs the so-called orientation flights, after which she will enter heat and will begin to produce pheromones that will attract the drones, then the wedding flight will take place, that is the moment when the queen will mate for his first and last time with the drones. After fertilization the queen will begin to lay from 2,000 to 3.00 eggs per day, she can lay two types of eggs, the fertilized and the unfertilized ones, the first will give life to the operatives and possible future queens, from the second instead the drones will be born. After three / four years of spawning, the queen being no longer provided with spermatozoa, she will produce only male eggs; before this happens, usually, the worker bees try to breed new queens, considering that the queen bee, through the production of pheromones, regulates all the activities inside the hive and therefore, the death of the latter it would destroy the delicate balance of the hive.

To produce royal jelly (RJ), it is necessary to ensure that the bees raise queens, because as it is known, they are fed with royal jelly throughout their life cycle, unlike the workers who are fed with RJ only for the first two days of life (Fig.3). From an orphan family, also commonly referred to as a starter, cells are introduced with the queen bee larvae inside. Subsequently it is necessary to get a honeycomb with a very young fresh brood; the smaller the larvae, the less they will eat, so the quantity of royal jelly that will then be extracted will be greater. This process can be useful for two functions, both for the production of RJ and for the production of queens. Having taken the honeycomb, go to insert the larvae, which possibly must be less than a day old (maximum 36 hours), they are easily recognizable, they look like small white commas. Through a picking, the larvae will be extracted from the honeycomb and inserted one by one in each dome, and on the bottom of this, it will be necessary to put a little royal jelly, even of poor quality, in order to place the larva on top and make sure you don't get dehydrated. This poor-quality baby food will then be removed by the worker bees once the domes are accepted inside the hive. The collection of RJ will be carried out on the third day, through a

compressor that will suck the royal jelly from each cell, in each of these up to 250 mg of product will be taken, so it takes no less than 4 cells to accumulate a single gram of RJ. It is just before the RJ is taken that the queen larvae will no longer be useful for breeding and will therefore be discarded.

Goals and strategies for breeding "food of the future"

The available data on *H. illucens* are certainly important and encouraging, but they represent only the starting point. To make a correct assessment of the potential of insects as biotransformers, from which to obtain the raw material for the production of feed, it is first of all necessary to keep in mind the possibility of breeding the species of interest on a large scale, which must therefore have some fundamental characteristics, such as:

• ability to grow on a food substrate of low value, deriving from waste from production processes;

• high conversion rate of the food substrate into biomass;

• low mortality rate in youth stages;

• short development cycle;

• high reproductive potential;

• ability to live at high density;

• high nutritional quality;

• reduced vulnerability to disease.

Other factors, no less important, which must be considered in the case of farms on an industrial scale, are:

• the ability to farm based on automation processes, reducing the use of labor and reducing production costs;

• the development of genetic improvement plans for the species to obtain highly productive lines;

• the definition of containment strategies if the species is reared in non-endemic areas.

To meet the growing demand in the animal feed sector, there are already examples in Europe of companies that have developed the breeding of *H. illucens* on an industrial scale, while on the research front several projects have been funded by national agencies or programs of the European Union. In Italy, in recent years the scientific community has been interested in the possible useof insects in the production of feed for farmed animals, mainly fish and poultry species. Different Universities and Research Centers are involved in projects in which *H. illucens* and *T. molitor* are mainly used.

The main objectives of this PhD project are:

• The standardization of *H. illucens* breeding on waste material of vegetable origin, deriving from the fruit and vegetable sector. This will allow to identify the optimal conditions to obtain the best insect growth, the maximum waste reduction and the best substrate conversion efficiency. It should be borne

in mind that in most of the studies carried out so far for the breeding of this insect, organic waste material such as manure or urban wet waste has been used, while the information on the possibility of breeding *H. illucens* on original waste is limited. For example, as reported by Kalova and Borkovcova (2013) biodegradable waste represent a growth substrate characterized by a better hygienic-sanitary profile.

• The identification of morphological, molecular and functional markers to be used as diagnostic markers to monitor the growth, development and health of insects in large-scale farming. This research activity would allow to obtain on the one hand basic information on the biology, physiology and development of this insect, currently rather limited but indispensable in order to fully exploit its potential for application purposes, and on the other hand to build a solid platform of knowledge to be used in improving current farming methods.

• Production of insect meal and evaluation of its nutritional and microbiological qualities.

• Exploitation of residual biomass from the *H. illucens* breeding for the production of soil improvers.

The residue of vegetable waste not consumed by the larvae and enriched by insect droppings and their exuvia will be analyzed for its possible use in vermiculture in order to obtain fertilizers. This PhD project therefore had the ambitious goal of evaluating the possible creation of a closed-cycle production system, with a complete reuse of waste.
The studies carried out so far on edible insect species and on the flours derived from them have shown a certain variability in the composition of macro and micronutrients in relation to the stage of development used and the substrate on which they are raised; this makes it difficult to generalize about their nutritional value and their possible use in feed production.
It is therefore possible to affirm that the detailed knowledge of the biology of a significant number of insects with the previously listed characteristics, of their nutritional value, of the food safety of flours in terms of the presence of pesticides, heavy metals, toxins, pathogens, allergens, and the identification of the best waste substrates on which it is possible to fine-tune the breeding of them, will represent a solid scientific basis to be able to exploit the enormous biodiversity of insects and thus allow the development of a new industrial sector.
In order for this goal to be achieved, the promulgation of laws governing the use of insects as a raw material for the production of feed is fundamental and at the same time urgent. Given their high food value and, according to the knowledge acquired so far, to the greater efficiency compared to conventional farmed animal species in terms of conversion of food into body weight, there is a growing interest in mass farming of insects as a food source. alternative, not only for humans but above all for feed production for zootechnical use.
Although the numerous positive aspects that the use of insects as food would entail are now known, the literature on the subject is still small and lacking. Furthermore, also the evaluation of environmental performance is on the whole in an embryonic stage, even though a reduction of the environmental impact has been estimated compared to others sources of dietary proteins.

The topics of this PhD thesis are organized in the following five chapters:

Chapter 1 aimed to evaluate the impact of age, size, and sex on adult black soldier fly thermal preference. The following scientific article has been published:

Addeo NF, Li C, Rusch TW, Dickerson AJ, Tarone AM, Bovera F, Tomberlin JK, (2021). Impact of Age, Size, and Sex on Adult Black Soldier Fly (*Hermetia illucens* L. (Diptera: Stratiomyidae) thermal preference. *Journal of Insects as Food and Feed*, in press, https://doi.org/10.3920/JIFF2021.0076

Chapter 2 focused on the evaluation of the potential use of wastes obtained from vegetable markets and from butcheries as substrate for black soldier fly larvae, comparing the larvae growth performance and chemical traits with those obtained with a standard poultry diet. The following scientific article has been published:

Addeo NF, Vozzo S, Secci G, Mastellone V, Piccolo G, Lombardi P, Parisi G, Asiry KA, Attia YA, Bovera F, (2021). Different Combinations of Butchery and Vegetable Wastes on Growth Performance, Chemical-Nutritional Characteristics and Oxidative Status of Black Soldier Fly Growing Larvae. *Animals*, 11, 3515. https://doi.org/10.3390/ani11123515

Chapter 3 aimed to evaluate the effects of feeding a black soldier fly larvae meal on different intestinal traits of hens and to determine the trace and toxic elements concentration in insect meal and diets. The following scientific article has been published:

Moniello G, Ariano A, Panettieri V, Tulli F, Olivotto I, Messina M, Randazzo B, Severino L, Piccolo G, Musco N, **Addeo NF**, Hassoun G, Bovera F, (2019). Intestinal Morphometry, Enzymatic and Microbial Activity in Laying Hens Fed Different Levels of a *Hermetia illucens* Larvae Meal and Toxic Elements Content of the Insect Meal and Diets. *Animals* 9, 86. https://doi.org/10.3390/ani9030086

Chapter 4 aimed to study the effect of low inclusion levels of a *T. molitor* defatted larvae meal on laying performance and egg physical and chemical characteristics of quails. The following scientific article has been published:

Secci G, **Addeo NF**, Rodriguez LFP, Bovera F, Moniello G, Parisi G, (2021). In vivo performances, ileal digestibility, and physicochemical characterization of raw and boiled eggs as affected by *Tenebrio molitor* larvae meal at low inclusion rate in laying quail (*Coturnix japonica*) diet. *Poultry Science* 100:101487. https://doi.org/10.1016/j.psj.2021.101487

Chapter 5 aimed to propose an additional income to royal jelly production, using the removed queen bee larvae as nutritional supplement for animal production. The following scientific article has been published:

Addeo NF, Roncarati A, Secci G, Parisi G, Piccolo G, Ariano A, Scivicco M, Rippa A, Bovera F, (2020). Potential use of a queen bee larvae meal (*Apis mellifera ligustica* Spin.) in animal nutrition: a nutritional and chemical-toxicological evaluation. *Journal of Insects as Food and Feed* 7, 173 – 186. https:// doi.org/10.3920/JIFF2020.0079

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CHAPTER 1

Impact of age, size, and sex on adult black soldier fly (*Hermetia illucens* L. (Diptera: Stratiomyidae) thermal preference.

1. Introduction

Population growth and urbanization are increasing and consequently creating shifts in the composition of global food demand, especially for production of animal protein (e.g., fish) (Msangi and Rosegrant, 2011). However, one alternative food that is emerging is insect-based biomass. Thus, the traditional production of food and feed from fish meal, soybeans, and cereals, could be increased or augmented through the use of insect sources to meet the increasing demand. The Food and Agriculture Organization (FAO) of the United Nations estimates that the human population will reach 10 billion by 2050, to which the billions of animals bred for food, work, and pets must also be added (FAO, 2017). Furthermore, the daily energy intake per individual human also is predicted to increase from 2770 kcal to 3070 kcal by 2050 (Alexandratos *et al*., 2012). Together, these factors will require an increase in both feed and food production, which will put more pressure on land use and renewable water, futher exaserbating environmental challenges associated with climate change (e.g., drought) (Alexandratos *et al*., 2012; FAO, 2017).

Insects as an alternative protein source are not meant to replace existing animal and plant protein sources. Rather, they are to be integrated as adititives with traditional feed sources to reduce enviromental damage and economic concerns while still providing a diet full of proteins, fats, and minerals (Cutrignelli *et al*., 2018). The large-scale potential use of insects as an additive or alternative feed and food source is already being realized as some corporations (e.g., Protix in the Netherlands, Ynsects in France, and EVO Conversion Systems and Enviroflight in the USA) are beginning to mass produce and sell insects for such purposes.

Among the insects currently mass reared for feed, the black soldier fly, *Hermetia illucens* L. (Diptera: Stratiomyidae) is one of the most studied (Tomberlin *et al*., 2002; Tschirner *et al*., 2015; Cammack *et al*., 2017). The black soldier fly is a medium-sized Diptera that has a New World origin (Ståhls *et al*., 2020). The black soldier fly has spread globally due in part to technology allowing for indoor production (Tomberlin and Van Huis, 2020) and global transportation. Its rapid spread is also due to the associated benefits of recycling organic wastes while suppressing associated pest arthropod populations (Sheppard, 1983). For example, black soldier fly larvae regularly exclude house fly *Musca domestica* L. (Diptera: Muscidae) larvae from food sources (e.g, manure), which reduces local house fly populations (Sheppard, 1983). Thus, the ability of black soldier fly larvae to outcompete house fly larvae can be viewed and implemented as a form of biological control, as the house fly is a vector for many pathogens and considered a pest in many systems (e.g., livestock) (Bradley and Sheppard, 1984; Furman *et al*., 1959; Sheppard *et al*., 1994). Further, black soldier fly larvae can be used as a feed due in part due to their high protein content (40-44% crude protein) (Makkar *et al*., 2014; Bovera *et al*., 2018) and are regularly used as a feed ingredient for chickens (Bovera *et al*., 2018; Loponte *et al*., 2017; Secci *et al*., 2018; Marono *et al*., 2017) and fish (Makkar *et al*., 2014).

Most of the current research on the black soldier fly examines the larval stage as it is responsible for recycling organic material, including food waste, and producing the biomass used as feed (Surendra *et al*., 2020; Lalander *et al*., 2019; Barragan-Fonseca *et al*., 2017; Diener *et al*., 2011). Consequently, less is known about adult biology (Jones and Tomberlin, 2020; Macavei *et al*., 2020; Park *et al*., 2016), which is the obvious stage responsible for reproduction (i.e., egg production) and thus regulates population levels in colony (Sivanantharaja and Gnaneswaran, 2018). Without a viable adult population, the process of mass producing black soldier fly larvae is simply not possible.

Several abiotic factors are known to impact black soldier fly life-history traits as related to reproduction (Holmes *et al*., 2012). For instance, temperatures below 27°C resulted in reduced adult activity and subsequently reduced mating and egg production (Tomberlin and Sheppard, 2002). Of course, such results may be population specific (Ståhls *et al*., 2020), but without further research, widespread effects of temperature remain unknown. Similarly, biotic factors such as age also affect physiological processes and stress responses, with most organisms becoming less resilient with age (McHugh and Gil, 2018). Consequently, the negative effects of heat stress on an aging insect typically intensifies with time (Rikke and Johnson, 2004; Conti, 2008). Although temperature is recognized as an important abiotic factor affecting black soldier fly fitness (e.g., temperature sensitive development rates see, Tomberlin *et al*., 2009; Harnden and Tomberlin, 2016; Holmes *et al*., 2016), the black soldier fly industry lacks knowledge on basic adult thermal biology that likely impacts reproduction as observed in other species (Chia *et al*., 2018). For instance, variation in black soldier fly egg production is often observed but difficult to explain (Jones and Tomberlin, 2020). One possible, but little explored, explanation is that such fluctuations in egg production are due to minor fluctuations in environmental temperature. However, if black soldier flies were provided thermal resources (e.g., heat lamps or cooling pads) in rearing facilities where they could seek out desired temperatures that may vary over time or with different body conditions (e.g., gravid vs non-gravid), they could better stabilize their body temperatures to optimize various temperature sensitive performances such as mating and oviposition (Chia *et al*., 2018). In fact, some black soldier fly studies have demonstrated individual preferences for warmer environments in the range 27˚ C -30°C, which are likely optimal conditions for such physiological (Harnden and Tomberlin, 2016) and behavioral processes (Gligorescu *et al*., 2018; Shumo *et al*., 2019). Therefore, the purpose of this study was to quantify the thermal preference of adult black soldier flies across age, size, and sex, as preferred temperatures are often correlated with optimal temperatures.

2. Materials and Methods

2.1 Colony maintenance

Colony phenotype and history are provided as recommended by Bosch *et al*. (2020). Black soldier fly adults were maintained in a single colony cage (3.4 L × 1.68 W × 1.68 H m) located in a greenhouse at the Forensic Laboratory for Investigative Entomological Sciences (F.L.I.E.S.) Facility at Texas A&M University (College Station, TX, USA). The greenhouse was maintained at ~30°C with natural sunlight, heater, and a water wall. This colony was established in January 2014 from eggs received from Phoenix Worm, Inc., Tifton, GA, USA, which originated in 1998 from a laboratory colony maintained at the Coastal Plains Experiment Station, University of Georgia, Tifton, GA, USA.

Colonies were manipulated following a modified method outlined by Cammack and Tomberlin (2017). A bullet® (Tomberlin *et al*., 2021) consisting of ~10,000 7-d-old larvae was received weekly

from EVO Conversion System, LLC, Texas, USA (Fig. 1.1). The larvae were transferred to plastic pans (60 L x 40 W x 12 H cm) and fed once with 8 kg Gainesville diet (50% wheat bran, 30% alfalfa meal, 20% corn meal) (Hogsette, 1992) at 70% moisture plus 1 L dry Gainesville diet as a surface/border treatment to prevent larval escapes from plastic pans (Fig.1.1). The dry diet was placed directly on the moist feed, along the lateral edges of the plastic pans as a means to prevent larval escape.

Fig. 1.1 Bullet® (Tomberlin et al., 2021) consisting of ~10,000 7-d-old larvae (left), Gainesville diet (right).

Additionally, the dry substrate became saturated over time and was consumed by the larvae as well. Another 2 kg Gainesville diet at 70% moisture was added on day 14 as supplemental feed. Mature larvae were sifted (Fig. 1.2) and transferred to plastic shoe box (30 L x 15 W x 15 H cm) forpupation on day 19 (see Tab. 1.1 for detailed feeding schedule).

Fig. 1.2 Sifted larvae

The larvae and pupae were reared in a walk-in environmental chamber set to 30.2 ± 0.5°C, 60.0 ± 5.1% RH, and a 14:10 L:D cycle. Emerging adults were transferred to the colony cage kept in the greenhouse (described above) to reproduce (Fig. 1.3).

Fig. 1.3 Emerging adults of H. illucens.

Adult flies were provided only with water, by providing a 75 x 75 cm linen cloth hanging vertically in the center of the cage that was moistened with an automated irrigation system for 10 minutes every 6 h (Fig. 1.4).

Fig. 1.4 H.illucens cage with automated irrigation system.

An oviposition site consisting of a plastic container (30 cm × 15 cm × 15 cm) with ~1 kg of Gainesville diet at 70% moisture inoculated with ~5,000 black soldier fly larvae was placed on a 20 cm tall wooden bench located centrally in the cage but not directly under the moistened cloth. The container was covered with a plastic lid which had a 2 cm x 8 cm opening covered with screen so that odors could emanate from a focal point and attract gravid females. Eight layers of corrugated cardboard (~10 cm × 5 cm × 3 cm) were bound together with masking tape over the screened opening.

The shoebox with larvae and diet was replaced every week (Fig. 1.5). Eggs were collected daily and used for maintaining the colony as well as conducting this research (Fig. 1.6).

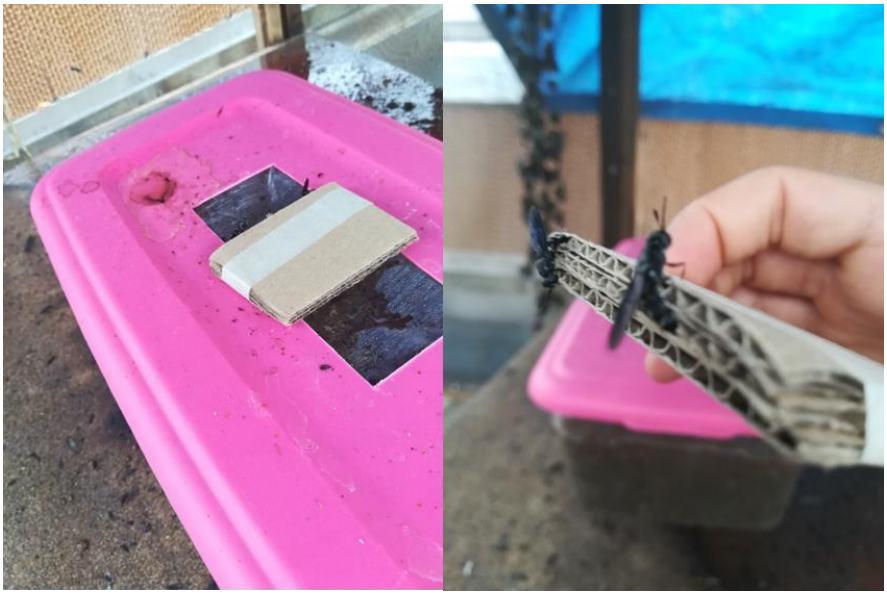

Fig. 1.5 Oviposition site.

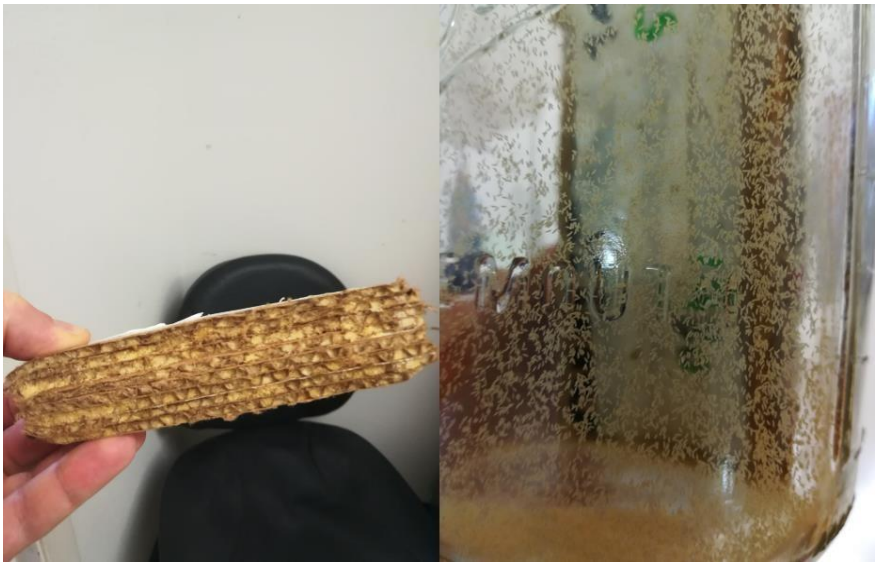

Fig. 1.6 H.illucens eggs (left), H.illucens newly hatched larvae (right).

To obtain adults with two distinctive sizes (large and small) for experiments, newly hatched larvae (< 24 h) were used for experiment. Following modified methods from Jones and Tomberlin (2019), the neonates were used to generate adults at two size classes (i.e., large adults were 0.062 ± 0.0026 g and small adults were 0.035 ± 0.0011 g). 500 and 2000 newly hatched larvae (< 24-hr-old) from the eggs collected from the colony were counted and transferred by hand to separate 532 ml plastic cups containing 40 g of Gainesville diet at 70% moisture (20 g of diet dry mixed with 34 mL of water) for the first four-days of rearing (days 1-4) (Fig. 1.7). On day five, 14 g of Gainesville diet were added to the plastic cups. On days six and eight, 54 g of the 70% moisture Gainesville diet was provided. On the ninth day, the 532 ml cups were emptied into larger cylinder plastic containers (16 D x 11 H

cm). Subsequent feedings were 54 g wet Gainesville daily until ~40% of the larvae developed into prepupae (Table 1.2 for feeding schedule) (Fig.1.7).

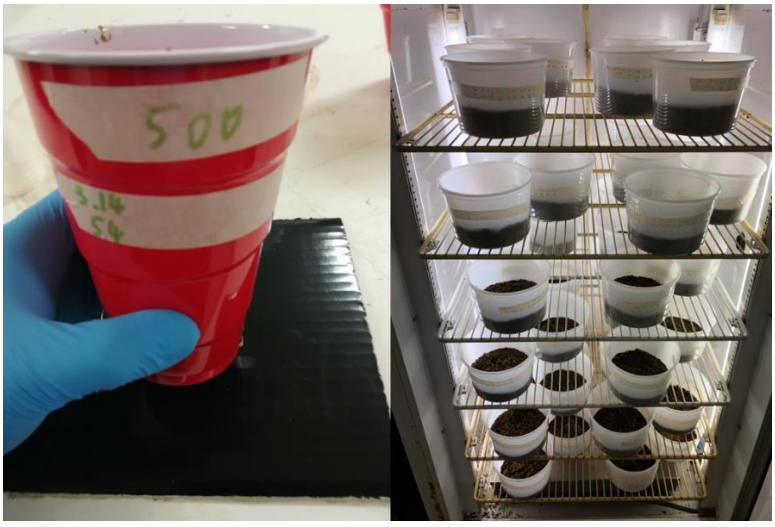

Fig. 1.7 532 ml plastic cups containing larvae and Gainesville diet(left), cylinder plastic containers (right).

Prepupae and the remaining larvae were sieved to remove the frass and undigested food, returned to the empty container, which in turn was covered with mesh netting to prevent flies from escaping. Pupae were monitored daily for adult emergence. Emerging adults were sorted by sex based on morphological traits (Tomberlin and Sheppard, 2002) and transferred to separate plastic cylinder container previously described (n = 60 flies of one sex per container) and covered with a breathable mesh lid. Adult flies were held in a walk-in environmental chamber set to 27.0 ± 0.5°C, 60.0 ± 5.1% RH, 14:10 L:D cycle, and provided water *ad libitum*. No food was provided to adults prior to, or during, the experiments.

Tab. 1.1. Feeding schedule for black soldier fly colony maintenance in FLIES Facility a Texas A&M University.

Day	**Activity**
1	Egg hatched and managed in EVO
7	Received as Bullet®
	Add 8 kg wet Gainesville diet plus ~0.7 kg dry Gainesville diet
14	Add 2 kg wet Gainesville diet
19	Sift out frass for pupation

Tab. 1.2 Feeding schedule for black soldier fly larvae used in the experiment.

Day	Activity
1	Egg hatched, and 40 g wet Gainesville diet added
4	14 g wet Gainesville diet added
6	54 g wet Gainesville diet added
8	54 g wet Gainesville diet added
9 and after	54 g wet Gainesville diet added daily until ~40% pupation

2.2 Thermal gradient design

For measuring the preferred temperature of adult black soldier fly, we followed similar methods of Dillon *et al*. (2009) and used the same thermal gradient design described by Malaway *et al.* (2020). An aluminum plate (85 cm x 20 cm with a thickness of 0.039mm) was used as a thermal gradient by folding 10 cm from each end at 90° to create a platform with "legs" under each side of the platform. To modify and control the surface temperature of the aluminum platform (i.e., the thermal gradient), each leg of the platform was submerged into a separate water bath. One water bath was heated to 85°C (WB02A11B Digital General purpose water bath, 2 L capacity, 120 V / 60 Hz, PolyScience®, IL, USA) (Fig. 1.8), while the other water bath (a styrofoam cooler with ~20 cm^3) was chilled to 0°C using ice and liquid water. Consequently, a thermal gradient of 15-60°C was obtained on the surface of the aluminum plate (Fig.1.8). During experiments, the temperature of the gradient remained constant by refilling the hot water bath (due to evaporation) and adding ice to the cold-water bath (due to ice melt) as needed. The room lights (Ecolux w/Starcoat, F32T8, SPP41, ECO, Hg E, 32W, 4100K, General Electric, Boston, MA, USA) were also left on during experiments to stimulate daytime activities. To prevent flies from leaving the surface of the thermal gradient, a transparent plastic box (53 cm x 18 cm x 4 cm) was placed on top of the gradient, creating an experimental arena for the flies. The adults stood directly on the aluminium thermal gradient. The plastic section functioned as a cover to precent the flies from escaping. Flies that did not stand on the gradient (on the plastic cover) were excluded from data collection and resulting analysis. Note, the side walls of the plastic box had air vents drilled into them and were covered with a mesh to allow air flow while also preventing flies from escaping. We recorded the surface temperature of the thermal gradient using four k-type thermocouples (model A0188598; Gain Express Inc., Kowloon, Hong Kong, China); two placed at the ends of the gradient (2 and 51.5 cm) and two towards the middle of the gradient (17 and 37 cm).

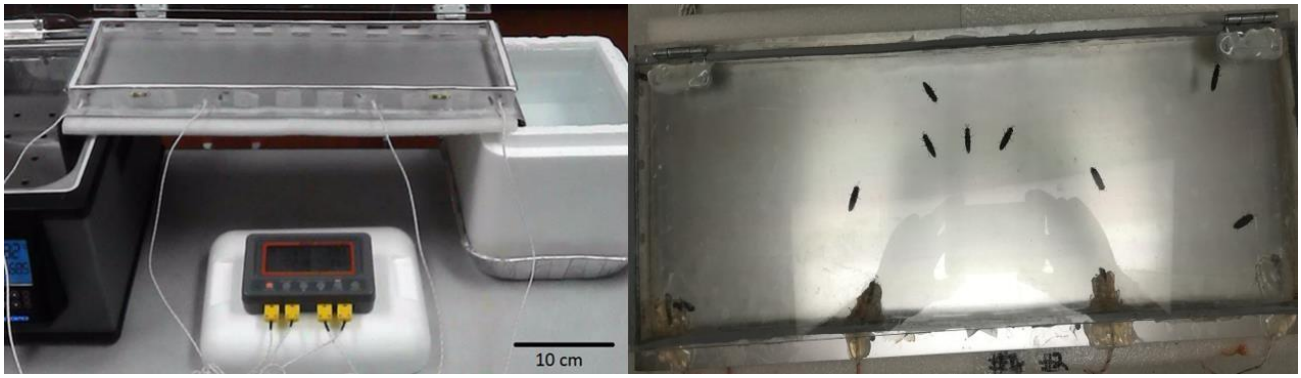

Fig. 1.8 Thermal gradient design, used to assess thermal preference of H. illucens adults. Note the thermal gradient represented by the arrow pentagon created by a water bath and an ice bucket placed in each extremity of the metal plate, providing a temperature range of 15-60 °C (± 2°C). The temperatures of the gradient were recorded using four equidistant fine-wire fast-response digital thermocouples affixed to the aluminum plate.

2.3 Experiment design

Ten adult flies were randomly selected for a specific sex, age (1-d-post-emergence vs 7-d-post-emergence), or size (large and small) and released to the center of the thermal gradient where they were allowed to explore and seek out desired thermal conditions for 20 min. After the initial 20 min

exploratory period, the spatial positioning of each fly was recorded at 5 min intervals for one hour with a camera [iPhone® 8 (Apple Inc, CA, USA), 12-megapixel wide-angle f/1.8 camera] placed 60 cm directly above the center of the thermal gradient. A second order polynomial equation was derived by fitting a trendline through four points obtained from the position of the four thermocouples (x-coordinates) and their associated temperatures (y-coordinates) to estimate the slope of the thermal gradient. This equation was then used to estimate the surface temperature at each fly's location at any point on the thermal gradient. The x-coordinates of the spatial position of each fly were recorded via a Cartesian coordinate superimposed on digital images taken during experiments at 5 min interval. All images were post cropped down to the area of the thermal gradient with Cam Scanner (INTSIG information Co., Ltd, Shanghai, China). A grid with calibrated cells was applied on top of each cropped image digitally with GridOnPhoto (Andreas H. Lengauer, Schwarzhorngasse, Vienna) and the position of each tested individual on the thermal gradients were read out manually. Three groups of 10 (n = 30) flies were examined for each sex, age, and size (n total = 2 for sex * 2 for age * 2 for size * 3 replicates * 10 flies each = 240 flies). Two trials (i.e., unique generations) were conducted for this experiment (n = 240 * 2 trials = 480 total flies tested).

2.4 Statistical analysis

The thermal preferences of flies that did not stay on the surface of the thermal gradient were removed from analyses as we did not measure the temperatures of the side walls or lid and could thus not estimate the surface temperatures they selected. Data were established based on the median of the thermal preferences of all individuals (n = 10) in each measurement. Mean and standard error of those median data was used for statistical analysis. Based on our a priori hypotheses, a linear mixed-effects model (Bernal-Rusiel *et al*., 2013) was used to test the main effects of sex, age, size, time, and trial as well as the three-way and two-way interaction effects among sex, age, and size (tables 3-5). Because ten flies were tested simultaneously in each replicate, and repeated measurements were taken every 5 min over a one hour period, the group ID was nested within time as a random effect in the model. An alternative variance structure (restricted residual maximum likelihood, REML) was specified in the model since the data were non-normally distributed and contained heterogeneity. Analyses were conducted in R version 4.0.3 (R Core Team 2015) with the significance level set at $p < 0.05$.

3. Results

All values reported are means ± standard error of the mean (SEM). The first model (temperature ~ trial + time + age * size * sex) was tested based on our priori hypothesis and revealed a trial effect ($F = 6.78$, $df = 1$, $p = 0.013$) with adult thermal preferences in trial 2 (25.7 ± 0.5 °C) being higher than in trial 1 (22.3 ± 0.4°C) (Fig. 1.9). Therefore, a second model (temperature ~ trial + time + age * size * sex + trial * size + trial * age + trial * sex) was run to check whether there was an interaction effect between trial and age, size, or sex. No two-way interaction was found between trial and age ($F = 1.97$, $df = 1$, $p = 0.169$), size ($F = 1.41$, $df = 1$, $p = 0.243$), and sex ($F = 0.56$, $df = 1$, $p = 0.460$). Subsequent analysis were separated for each trial with the following model: temperature ~ time + age * size * sex.

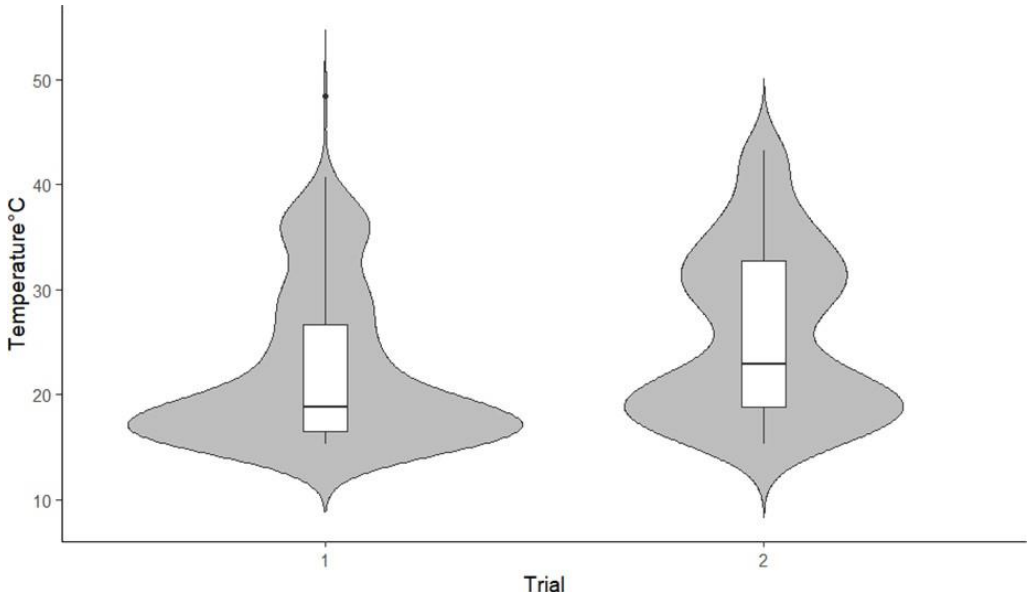

Figure 1.9 Thermal preferences of adult black soldier flies in trial 2 (25.6℃ ± 7.90） were higher than trial 1.

In trial 1, the two-way interaction between age and size was the only significant interaction (F = 0.8.68, *df* = 1, *p* = 0.0095). The large adults preferred 32.2 ± 0.7℃ when they were 1-d-post-emergence. The thermal preference dropped to 19.9 ± 0.7℃ when large adults were 7-d-poste-emergence. A similar trend was also observed for small adults, but with a much smaller difference between young (1-d-post-emergence) (20.1 ± 0.7℃) and old flies (7-d-post-emergence) (17.0 ± 0.2℃) (Fig.1.10). The main effects of sex (F = 0.1187, *df* = 1, *p* = 0.7349, Fig.1.11) and time (F = 1.43, *df* = 1, *p* = 0.1594, Fig.1.12) were not significant descriptors for adult thermal preference (see Tab.1.3 for specific p-values), though the thermal preference showed certain decrease (from 23.7 ± 1.5℃ to 21.7 ± 1.4℃) within such one-hour measurement (Fig. 1.12).

Tab. 1. 3 Estimated regression parameters, degree of freedom, F-values and p-values of lme for adult black soldier fly thermal preference differ in age, size, and sex in trial 1.

	Df	**F value**	**P value**
Intercept	1	803.4206	<0.0001
Time	11	1.4304	0.1594
Age	1	23.4218	0.0002
Size	1	21.7087	0.0003
Sex	1	0.1187	0.7349
Age : Size	1	8.6823	0.0095
Age : Sex	1	0.4744	0.5008
Size : Sex	1	0.9654	0.3405
Age : Size : Sex	1	0.0005	0.9818

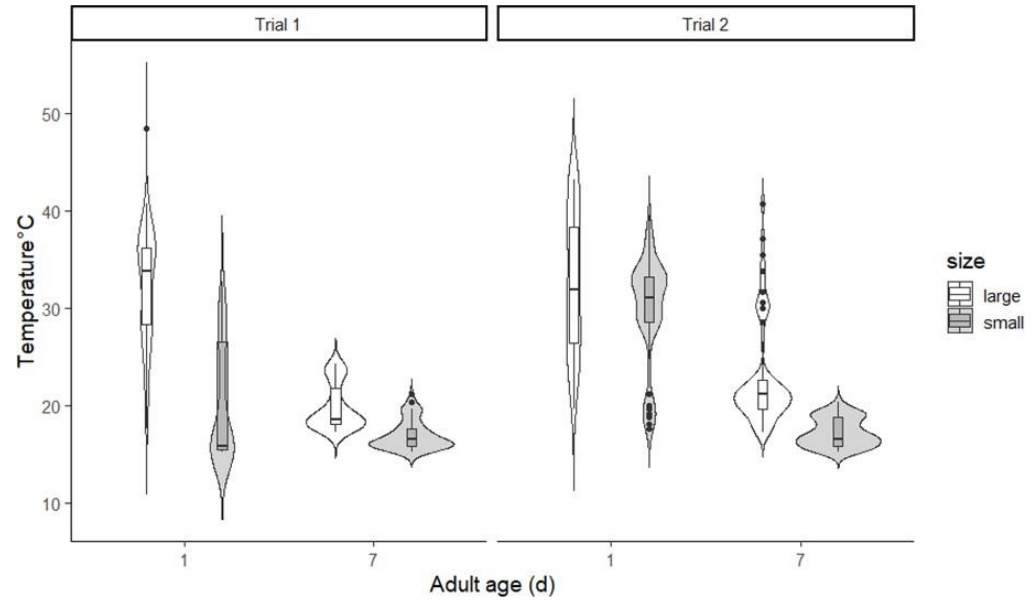

Figure 1.10 Effect of age and size on adult black soldier fly thermal preferences in trial 1 and 2. (Interaction effect was significant between age and size in trila 1, but not in trial 2).

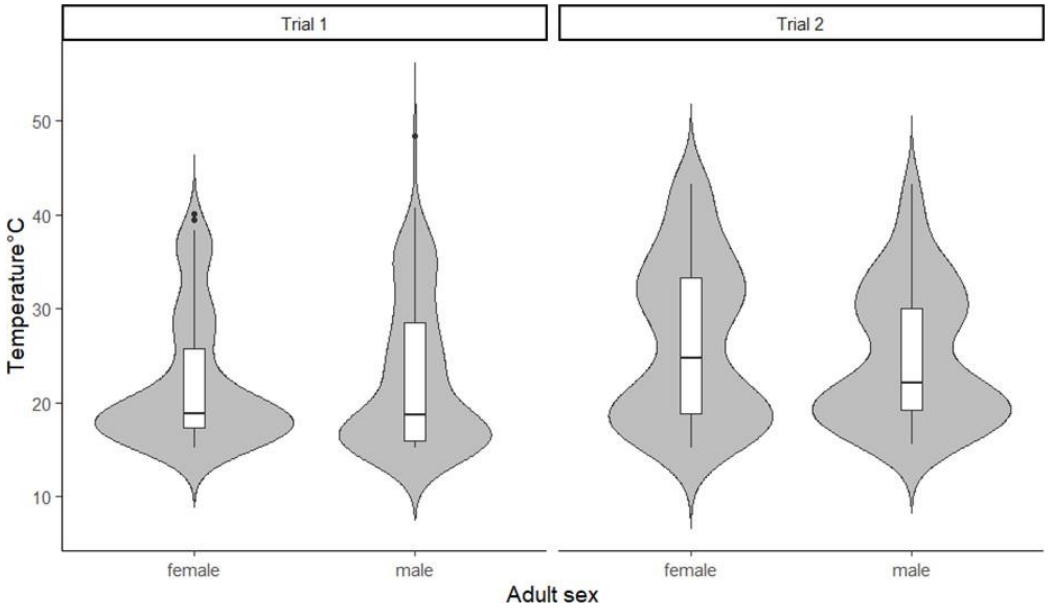

Figure 1.11 Effect of sex on adult black soldier fly thermal preference in both trial 1 and 2.

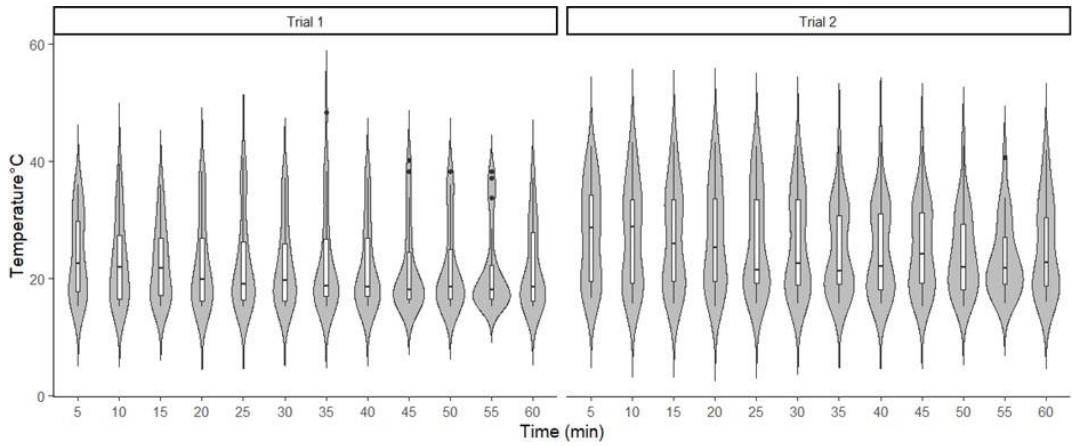

Figure 1.12 Effect of time on adult black soldier fly thermal preferences of the black soldier fly in trial 1 and 2.

In trial 2, neither the three-way interaction (F = 0.1869, *df* = 1, *p* = 0.6713), nor the two-way interactions among age, sex, and size (all two-way interactions resulted in p-value > 0.5, see Tab. 1.4 for specific p-values) were significant. The main effects of age (F = 38.13, *df* = 1, *p* < 0.001, Fig. 1.10), size (F = 5.55, *df* = 1, *p* < 0.0315, Fig. 1.10), and time (F = 1.92, *df* = 1, *p* = 0.0371, Fig. 1.12) significantly impacted adult thermal preference. Young (1-d-post-emergence) adults preferred 31.2 ± 0.5℃, while old (7-d-post-emergence) adults preferred 20.1 ± 0.4℃ (Fig.1.10, Tab. 1.5). Large adults preferred 27.7 ± 0.7℃, while small adults preferred 23.6±0.6℃ (Fig. 1.11, Tab 1.5). Thermal preference dropped from 27.5 ± 1.7℃ to 25.1 ± 1.6℃ during the one-hour measurement (Tab. 1.5).

Tab. 1.4 Estimated regression parameters, degree of freedom, F-values and p-values of lme for adult black soldier fly thermal preference differ in age, size, and sex in trial 2.

	Df	**F value**	**P value**
Intercept	1	787.5253	<0.0001
Time	11	1.9214	0.0371
Age	1	38.1259	<0.0001
Size	1	5.5515	0.0315
Sex	1	0.6100	0.4462
Age : Size	1	0.5035	0.4882
Age : Sex	1	4.2376	0.0562
Size : Sex	1	0.3744	0.5492
Age : Size : Sex	1	0.1869	0.6713

Tab. 1.5 Adult black soldier fly thermal preference in each group (mean ± SEM).

Trial	**Age (d)**	**Size**	**Sex**	**Mean**	**SEM**
1	1	Large	Female	30.6	1.10
1	1	Large	Male	33.8	0.78
1	1	Small	Female	20.0	1.05
1	1	Small	Male	20.3	0.93
1	7	Large	Female	19.3	0.30
1	7	Large	Male	20.4	0.43
1	7	Small	Female	18.0	0.24
1	7	Small	Male	16.0	0.09

2	1	Large	Female	35.1	1.09
2	1	Large	Male	30.2	1.18
2	1	Small	Female	32.6	0.45
2	1	Small	Male	27.0	1.01
2	7	Large	Female	20.8	0.46
2	7	Large	Male	24.9	1.04
2	7	Small	Female	17.1	0.21
2	7	Small	Male	17.5	0.27

4. Discussion

The fact that the world's population is continuously increasing, while land available for growing food is finite, has led to an urgent need to find supplemental feed and food sources to conventional plant and meat products. Insects represent a promising alternative as human food and animal feed worldwide (van Huis *et al*., 2017). Insect growth, development and distribution are regulated by several biotic and abiotic factors (Harrison *et al*., 2012). Among abiotic factors, temperature is considered one the most influential factors impacting insect development rate and seasonal occurence (Logan *et al*., 1976; Ratte, 1985). Furthermore, temperature can also directly affect various aspects of insect biology, such as survival, lifespan, fertility, sex and growth (Summers *et al*., 1984;Schneider, 2009). However, insects are not entirely at the mercy of their thermal environment and seemingly have preferred temperatures of which they seek out given choices (i.e., seek out temperature specific microcllimates), presumably to optimize temperature specific performances (Dillon *et al*, 2009; Angilletta, 2009; Harrison *et al*., 2012).

The current study determined that thermal preferences of adult black soldier flies varied by age and size, but not sex for the given population and experiment design. Young black soldier fly adults (1-d-post-emergence) preferred a higher average temperature (28.7 ± 0.5°C) than older adults (7 d-post-emergence) (19.2 ± 0.2°C) (Fig. 1.10, Tab. 1.5). Therefore, it is hypothesized that 7-d-post-emergence adult preference for lower temperature might be due to the reduced availability of energy reserves for their basal metabolism as energy reserves deplete over time and black soldier fly adults are not fed in colony and therefore are not able to replenish bodily reserves (other than water). Consequently, selecting cooler temperatures as they age reduces energy depletion, which likely increases longevity and mating potential (Chia *et al*., 2018). Conversely, the 1-d-post-emergence adult thermal preference of higher temperatures might be related to temperature specific performances, such as mating, that are often optimized at warmer temperatures (Cook, 1994). Furthermore, differences in thermal preferences between 1 d post emergence and 7 d post emergence flies may be due to the fact that the body composition chances over time. In fact, younger (1-d-post-emergence) black soldier flies contain more energy reserves (e.g., lipids), accumulated during the larval stage, than older flies (7-d-post-emegence), that select progressively cooler temperatures, to preserve their energy reserves as fasted (Gobbi *et al*., 2013; Tomberlin *et al*., 2009; Sheppard *et al*., 2002). This phenomenon of younger flies selecting warmer temperatures than older flies was also observed in Malawey *et al*. (2020) and by Nyamukondiwa and Terblanche (2009). Furthermore, recently fed flies by Nyamukondiwa and Terblanche (2009) were more resistant to extreme temperatures, indicating body reserves influence thermal sensitivity to which organisms may alter their thermal preferences.

Small black soldier fly adults showed a lower thermal preference (21.1 ± 0.4°C) compared to larger adult black soldier flies (26.9 ± 0.5°C) (Fig. 1.10, Tab. 1.5). It may be assumed that this different

thermal preference might be related to the influence that body size-temperature interactions has on black soldier fly body reserves, such as water and lipids. Indeed, larger insects seem to be able to tolerate warmer temperatures for longer periods of time, because they contain more body reserves. Moreover, they have a smaller surface area to volume ratio, that allows both to reduce the loss of evaporative water to the environment and to select warmer and presumably more optimal temperatures at a lower water cost.

In this study, both large and small flies were observed for 60 min. Their overall thermal preference decreased over time (within a trial) from 25.6 ± 1.1°C at 5 min to 22.4 ± 1.0°C at 60 min, regardless of sex, age, or size (Fig.1.12). It is possible that flies may have moved towards the cool end of the gradient later in the trial as they likely became dehydrated over time and cooler temperatures may allow for water conservation (see discussion below). Moreover, some water became available on the cold end of the gradient due to condensation buildup. Consequently, flies may have been attracted to the water and artificially appeared to select lower temperatures over time. If true, it can be supposed that there was a tradeoff of resources; suboptimal cooler temperatures with water for optimal warmer temperatures. This result highlights that the other abiotic factors, such as the presence of resources, my ineract with temperature and influence fly thermal preferences. Indeed, Prince and Parsons (1977) studied three *Drosophila* (Diptera: *Drosophilidae*) species (*Drosophila melanogaster* Meigen, *Drosophila simulans* Sturtevant, and *Drosophila immigrans* Sturtevant) on a thermal gradient (16.5-36.5°C) for 12 h at 0% and 100% relative humidity (RH). At 0% RH flies moved to the cold end of the gradient after 6 h, whilst at 100% RH, flies presented a stable distribution around 29-32°C. Therefore, they demonstrated that flies prefer lower temperatures under dry conditions likely to minimize water loss (Prince and Parsons, 1977) and increase survival (Parsons, 1979). Moreover, Gunn and Cosway (1938) reported the existence of a tight relationship between temperature and relative humidity, showing that *Blatta orientalis* L. (Blattodea: Blattidae) preferred lower temperatures when kept in a dry environment. However, even if the flies in our study moved to the cooler end of the gradient for water rather than thermal resources, they did not move back to the warmer end of the gradient after replenishing water. Therefore, it is not likely that the flies reduced their thermal preference over the course of a trial solely due to the presence of water resources, but rather did so at least in part for thermoregulatory purposes. Of course, the duration of the experiment may have not been long enough to detect such a response.

Males and females had similar thermal preferences of 23.8 ± 0.5°C and 24.2 ± 0.5°C respectively, without exhibiting effects of age or size (Fig. 1.11, Tab. 1.3-1.5). Such information could be used for optimizing adult longevity and mating success, which affects fertile egg production needed for larval digestion and consumption of organic waste to produce the insect biomass that can be used as feed and food. Chia *et al*. (2018) showed that adult female black soldier flies were able to reproduce in the temperature range of 20-35°C, with the highest fecundity at 30°C. Even though in this study the reproductive activity of adult female black soldier fly was not explored, their thermal preference had a value of 24.2 ± 0.5°C, which is within the optimal temperature range for reproduction (Chia *et al*., 2018). This behavior can be linked to the fact that insects tend to select specific temperatures that maximize their fitness. In fact, insects' thermal preferences are generally close to body temperatures that maximize many physiological performances (Dillon *et al*., 2012). Intriguingly, Martin and Huey (2008) demonstrated that this optimal temperature is often lower than the temperature at which a performance is maximal for a given species in part due to the costs of thermoregulatory error being greater at elevated temperatures compared to cooler temperatures. For instance, being 3°C over

optimal is more costly than being 3°C below optimal, as is explained through Jensen's inequality (see Martin and Huey 2008 for a detailed explanation). So cooler suboptimal temperatures are actually optimal (or safer) for an insect's physiology.

Chia *et al*. (2018) also showed that the lifespan of both male and female black soldier flies was inversely proportional to temperature, decreasing when temperature gradually increased from 15 to 37°C. This shift might be due to energetic expenditure, especially if adult black soldier flies are not feeding. While multiple studies have investigated the effects of environmental temperature on different performances (e.g., development of the different stages; larvae, prepupae, pupae and adults) of *H. illucens* (Chia *et al*., 2018; Shumo *et al*., 2020), there is only one study on black soldier fly adult thermal preference (Malawey *et al*., 2020). Consequently, less is know about adult thermal preferences, which can vary depending on which phenotype (e.g., metabolic rate) an adult black soldier fly is attempting to optimize (Terblanche *et al*., 2008; Lachenicht *et al*., 2010). Furthermore, based on data generated in the current study, adjusting temperatures as black soldier fly age could be critical in order to optimize reproduction. Malawey *et al*. (2020), for example, found young (< 24-h-old) black soldier fly adult males and females had different temperature preferences, but as they aged their thermal preferences converged. Differences between these data and those generated in the current study could be due to Malawey *et al*. (2020) not considering size as a possible reason for the observed differences in thermal preference. And, as a side note, the temperature range (15°C-60°C) used in the current study differed from Malawey *et al*. (2020) (e.g., 10°C-50°), which could have resulted in less, or greater, resolution. Herein, differences in adult black soldier fly thermal preferences between sex were not observed, since both unmated males and females likely attempted to increase their longevity and, thus, they sought out the same temperature, in order to optimize their performances. Similar results were determined for males and females of other insect species, such as *Rhodnius prolixus* Stäl (Hemiptera: Reduviidae) (Schilman and Lazzari 2004), *Drosophila tripunctata*, L., (Diptera: Drosophilidae) (Dillon *et al*., 2009), *D. immigrans* (Yamamoto, 1994), *D. simulans* (Krstevska and Hoffmann, 1994), and *D. melanogaster* (Yamamoto and Ohba, 1984; Sayeed and Benzer, 1996). However, further studies focused on thermal preference differences between mated and unmated black soldier flies should be realized.

5. Conclusion

The results of this study reveal that black soldier fly adults actively seek out thermal microclimates via behavioral mechanisms (i.e., movement) to maintain stable and likely optimal body temperatures when given the opportunity to select from a variety of temperatures. Environmental changes, such as temperature and food availability, induce local adaptations (or extinctions of) in ectotherms (Katz *et al*., 2017), though some species are able to avoid adaptation due to effective thermoregulation as reported for lizards by Muñoz and Losos (2018). To our knowledge there are limited studies on black soldier fly adult thermal preference. And while it is recognized that temperature is important when trying to mass produce black soldier fly larvae (Chia *et al*., 2018; Shumo *et al*., 2019), this study reveals that adult black soldier fly actively seek out different temperatures due to different life-history traits. Therefore, the ability to select an appropriate temperature range (e.g., heat lamps and cooling pads) across the lifetime of a black soldier fly ought to optimize and stabilize mass production. Continued research on the black soldier fly thermal preference (e.g., adult thermal preference based on diet) is needed to better understand which temperatures will help in standardizing optimal and stable breeding systems.

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CHAPTER 2

Different combinations of butchery and vegetable wastes on growth performance, chemical-nutritional characteristics and oxidative status of Black Soldier fly growing larvae

1. Introduction

Global average temperature has increased by about 0.7 °C in the last century (Cassandro, 2020). The Intergovernmental Panel on Climate Change (IPCC) reported that anthropogenic green-house gases (GHG), including carbon dioxide (CO2), methane (CH4), nitrous oxide (N2O) and halocarbons, have been responsible for most of the observed temperature increase. The effects of global warming are evident and led to an increasing attention of the world population towards the environment and biodiversity, thus, a greater sustainability of anthropogenic activities is required.

Among the anthropogenic activities contributing to global warming, the livestock sec-tor is under "special surveillance". Firstly, Gerber *et al*. (2013) estimated that the livestock sec-tor contributes for the 14.5% to global GHG emissions which are the mainly responsible of the climate change (IPCC, 2013). Secondly, the societal concern over the animal welfare has been in-creased according to the number of citizens in ask for farm animals treated as humanely as possible (Temple and Manteca, 2020). For these reasons, in the last years several authors studied the possibility to reduce the global impact of animal production.

Among the different solutions, the possibility to farm insects as food and feed source seems to be very promising due to the high sustainability of insect breeding. In fact, insect farming requires less land and water and produces lower GHG emissions in comparison to the traditional livestock productions (van Huis and Oonincx, 2017). In addition, insects have high feed conversion efficiencies and can transform low-value organic by-products into high-quality food or feed (van Huis and Oonincx, 2017). The industrial farming of insects, aiming to maximize the mass production, very often uses industrial products as growing substrate for larvae, such as poultry diet. Widening the possibilities of using alternative and more sustainable substrates will play a key role in enhancing the circularity of insect production, helping European insect farms to reach their full potential (IPIFF, 2020).

In recent years, some authors starting to investigate potential new substrates for in-sects, mainly for black soldier fly larvae (*Hermetia illucens*) (Meneguz *et al*., 2018; Scala *et al*., 2020; Giannetto *et al*., 2020; Fisher and Romano, 2021) obtaining interesting results in terms of growth performance and larval chemical quality. Black soldier fly is one of the most reared insects in the world, not only for its bioconversion ability (Lu *et al*., 2021) but also for the biological active molecules that can derive from it such as chitin (Triunfo *et al*., 2021), lipids (Franco *et al*., 2021) or antimicrobial peptides (Manniello *et al*., 2021). Due to the great potential of black soldier fly, a wide range of substrates merits to be explored for its larval growth, in particular wastes.

Butchery waste falls into category 3 of animal by-products defined by Regulation (EC) 1069/2009. They include mainly fat, bones and small amounts of meat which for only commercial reasons cannot

be used for human consumption. They must therefore be dis-posed of, and this operation has a cost. It could be interesting, with a view to a circular economy, to try to make the most out of this waste. The richness in fat of butchery waste could be "mitigate" by using vegetable wastes also readily available and notoriously rich-er in water and carbohydrates.

Since no indications are available concerning the welfare of larvae and the potential effect of stress on the quality of the final product, a first step could be to explore the metabolic conditions of larvae growing on different substrates. The evaluation of the used substrates on oxidation index responses could provide complementary physiological information for the assessment of health and well-being outcome of larvae. It is difficult to quantify the reactive oxygen species (ROS) in practice because of their very short half-life and it requires complex techniques over a long period of time (Sechi *et al*., 2017). Due to their high reactivity, ROS react with practically every organic molecule they meet, producing reactive oxygen metabolites (ROMs), which are more stable than the ROS and are therefore easier to quantify.

Conversely, the biological antioxidant potential (BAP) matches the total antioxidant capability of plasma and includes either exogenous (ascorbate, tocopherols, carotenoids) or endogenous (proteins, glutathione peroxidase, superoxide dismutase, catalase) components that can oppose the oxidant action of reactive species (Benzie and Strain, 1996). For these reasons, for the laboratory assessment of the oxidative status we used the pro-oxidizing component, through the d-ROMs for the determination of plasma hydroperoxides, and the antioxidant component, through the BAP test for the evaluation of the total plasma antioxidant barrier (Chiofalo *et al*., 2020).

The aim of the present research was to use wastes obtained from vegetable market and from butcheries in different combinations as substrate for black soldier fly larvae to produce high quality larvae for feed. Three different waste combination were compared to a standard diet with the purpose to test what combination give the best results in terms of larvae growth performance and chemical traits. In addition, the evaluation of the oxidative stress markers in hemolymph and the oxidative stability of larvae could supply interesting information about the animal health when farmed on different utilized substrates for their growth.

2. Materials and Methods

2.1 Larvae and substrates

Five days old black soldier fly larvae (*Hermetia illucens*) were purchased from the commercial insect rearing company Smart Bug's (Treviso, Italy) in February 2020 and used in a growing trial. Four different substrates were used in the trial: broiler feed as a control diet; a total vegetable mix diet (V100); a diet consisting of 75% of vegetable diet + 25% of butchery wastes (V75+B25); and a diet consisting of 50% of vegetable diet + 50% of butchery wastes (V50+B50). The vegetable diet consisted in a mix of vegetable wastes collected from fruit-vegetable shops in the province of Napoli (Italy), containing 75% of vegetables (broccoli 40%, celery 35%, cabbages 25%) and 25% fruits (50% oranges and 50% apples). The butchery wastes were obtained from butchers in the province of Napoli and mainly consisted in fat and meat resulting from the trimming of bovine carcasses and cuts of meat. The collected vegetable wastes were stored for two days at room temperature to reduce the water content. Vegetables and butchery wastes were cut into small pieces prior to use them. Diets were prepared mixing the ingredients accurately. (Fig 2.1).

Fig. 2.1 Vegetable and butchery wastes used for growing trials.

A batch feeding strategy was applied which means that the substrates were placed in plastic containers (60 cm × 40 cm × 15 cm) one day prior to placing the larvae, at the be-ginning of the experiment. This allows the substrates to heat up until the start of the experiment.

Each group (Control, V100, V50+B50 and V75+B25) consisted of 9 replicates, each placed in a plastic container for a total of 36 trays. In each replicate, 10 kg of substrate were placed. On the top of each substrate, 6,000, five days old larvae were transferred after weighing. To calculate the average weight 100 larvae/time were counted and weighed on an analytical balance (Adventure Pro balance, Ohaus, Pine Brook NJ, USA) for a total of 60 weighing. The trays were covered with a perforated cap with a black nylon grid and placed in a ventilated chamber (air flow around 2 m/s) under controlled environmental conditions (T: 27 ± 0.5 °C; RH: 70 ± 5%; L:D photoperiod: 16:8).

Moisture content of the substrates was measured at the beginning of the trial on 10 g of each substrate, using an electric oven for 24 h at 65 °C. The water contents of the vegetable and butchery wastes were 85.37 ± 1.12% and 38.92 ± 2.85%, respectively. Thus, the water percentage in the four diets was 70.05, 85.37, 70.13, and 63.65%, for Control, V100, V75+B25, and V50+B50 diets, respectively. The plastic containers were visually inspected daily to verify the adequate level of humidity. In addition, the temperature in each container was recorded every day to verify the optimal conditions for the larvae. (Fig 2.2).

Fig. 2.2 Plastic containers with moisture and H. illucens larvae.

2.2 Growing trial

One hundred larvae per replicate were randomly selected every two days, weighed, and measured for length and thickness (measured at the equator of each larva) and were then returned to their respective container.

Feeding of larvae was continued until more than 25% of the larvae in a tray had developed into prepupae. The evaluation of the prepupae percentage has been done by collecting exactly 100 g of substrate + larvae from each container (in three replicates) and counting the number of larvae and prepupae contained in each replicate.

The larvae were hand counted, washed, dried with a paper towel, and individually weighed and measured. The total final biomass (larvae + prepupae) and the residual rearing substrate were also weighed. The following parameters were then calculated according to Meneguz *et al*. (2018):

Larval mortality (LM), % = (ILN – (FLN + FPN))*100/ILN; Growth rate (GR), % = (LFW, g – LIW, g)/d; Substrate reduction (SR), % = (AS, g – RS, g)*100/AS, g ; Waste reduction index (WRI), % = (AS, g – RS, g)*100/AS, g/d; Efficiency of conversion of digested feed (ECD) = TFB, g/(TS, g – RS, g) where: ILN = initial larval number; FLN = final larval number; FPN = final prepupae number; LFW = larval final weight; LIW = larval initial weight; d = days of the trial; AS = administered substrate; RS = residual substrate; TFB = total final biomass; TS = total substrate; RS = residual substrate. All the weights are expressed on dry matter basis. Larvae yield (LY) was calculated as the ratio between larvae total biomass produced at the end of the trial and the total available substrate on dry matter basis. In addition, the protein conversion ratio (PR) was calculated considering the indications of Ewald et al. (2020) as follows:

PR = total protein in final larval biomass/total protein in the substrate.

2.3 Chemical-nutritional characteristics and oxidative stability of larvae

At the end of the trial, samples of substrate, larvae and frass from each tray were collected, freeze-dried using a Micromudulyo freeze drier (Thermo Electron Corporation, Thermo Fisher Scientific Inc., Whaltham, MA, USA) and analyzed for chemical composition. (Fig. 2.3). Dry matter (DM), ashes, and crude protein (CP) were analyzed according to AOAC, 2005. In brief, for DM and ashes around 2.5 g of sample were weighed into porcelain capsule and put in an electric oven at 103 °C until constant weight; then the capsule was transferred to an electric stove at 550 °C for the whole night. The crude protein was determined using the Kjeldahl method; only for larvae, the nitrogen to crude protein conversion ratio was 4.76 according to Jansen *et al*. (2017). Total lipids were extracted from each sample according to the Folch *et al*. (1957) method, and gravimetrically quantified. The amount of carbohydrates (CHO) in the diets was calculated as follows: CHO, % DM = 100 – Ash, % DM – CP, % DM – Lipids, % DM.

Oxidative stability of larvae lipids was analyzed following the primary and secondary oxidation products by means of conjugated dienes (CD) and 2-thiobarbithuric acid re-active substances (TBARS), determined according to the spectrophotometric methods previously proposed by Srinivasan *et al*. (1996) and Vyncke (1970), respectively. The analyses were performed in duplicate, and the results were expressed as mmol hydroperoxides (mmol Hp)/100 g larvae and malondialdehyde equivalents (MDA-eq.)/100 g larvae, respectively.

Fig. 2.3 Chemical composition analysis of the samples.

2.4 Oxidative status of larvae

At 20 days of age (15th day of the trial), hemolymph samples were collected from twenty larvae per replicate, according to Łoś *et al*. (2018). Briefly, the larvae were immobilized with tweezers, an incision of body layers was made with a scalpel, and the floating hemolymph was collected with a pipette and then frozen in a tube containing 150 µL of 0.6% physiological saline until analysis. d-ROMs and BAP tests were measured using re-agents from Diacron International s.r.l. (Grosseto, Italy). In the d-ROMs test, reactive oxy-gen metabolites (primarily hydroperoxides) in a biological sample, in the presence of iron released from plasma proteins by an acidic buffer, are able to generate alkoxyl and peroxyl radicals, according to the Fenton reaction. Such radicals can then oxidize an alkyl substituted aromatic amine (N, N-dietylparaphenylendiamine), thus producing a pink-colored derivative which is photometrically quantified at 505 nm (Alberti *et al*., 2000). The d-ROMs concentration is directly proportional to the color intensity and expressed as Carratelli Units (1 CARR U = 0.08 mg hydrogen peroxide/dL). In the BAP test, the addition of a sample to a colored solution, obtained by mixing ferric chloride solution with a thiocyanate derivative solution, causes a discoloration, whose intensity was measured photometrically at 505 nm and was proportioned to the ability of the plasma to reduce ferric ions **(**Benzie and Strain**,** 1996**)**. The results were expressed as µmol/L of reduced ferric ions.

2.5 Statistical analysis

Data were analyzed by a one-way ANOVA, using the GLM procedure of SAS, 2002 and considering the substrate as main effect.
The experimental unit was the replicate. To assess the differences among means, the Tukey's test was used SAS, 2002.

3. Results

All the groups reached the end of the experiment (25% of prepupae) after 17 days, when larvae were 22 days old. Considering the amount of administered substrate (10 kg), the number of larvae for replicate (6,000) and the length of the growing period (17 days), the feeding rate in the present trial was around 0.098 mg of substrate per larva/d.
Tab. 2.1 shows the chemical composition of the substrates used in the trial.

Tab. 2.1 Substrate chemical composition.

Substrate	Moisture, %	Ash, % DM	Lipids, % DM	Protein, % DM	Carbohydrates, % DM
Control	70.05	3.69	5.12	22.69	68.50
V100	85.37	10.41	3.98	19.11	66.50
V75+B25	70.13	3.64	36.97	16.15	43.24
V50+B50	63.65	1.84	49.76	14.88	33.52

V100: total vegetable diet; V50+B50: diet consisting in 50% of vegetables and 50% of butchery wastes; V75+B25: diet consisting in 75% of vegetables and 25% of butchery wastes.

The average live weight of BSF, measured on 100 larvae per replicate every 2 days along the trial, is reported in Tab. 2.2. In general, larvae of 75V+25B group showed a higher live weight (LW) compared to the other groups even if, at the end of the trial, had a similar weight of the control group. At 22 days of age, larvae from V100 group showed the lowest LW ($P < 0.001$).

Tab. 2.2 Live weight (g) of black soldier fly larvae from 5 to 22 days of age.

	Control	V100	V50+B50	V75+B25	RMSE	P-value
5 d	0.0582	0.0586	0.0579	0.0587	0.004	0.8598
7 d	0.0621	0.0627	0.0618	0.0629	0.009	0.9932
9 d	0.1262^{ab}	0.1092^{b}	0.0804^{c}	0.1280^{a}	0.014	<0.0001
11 d	0.1353^{b}	0.1413^{b}	0.1168^{c}	0.1533^{a}	0.0095	<0.0001
13 d	0.1365^{b}	0.1577^{a}	0.1353^{b}	0.1577^{a}	0.013	<0.0001
15 d	0.1549^{ab}	0.1584^{a}	0.1423^{b}	0.1636^{a}	0.013	0.0045
17 d	0.1653^{b}	0.1603^{b}	0.1798^{a}	0.1725^{ab}	0.012	0.0033
20 d	0.1913^{ab}	0.1656^{b}	0.1854^{b}	0.1971^{a}	0.0019	<0.0001
22 d	0.2162^{a}	0.1839^{c}	0.2047^{b}	0.2163^{a}	0.0078	<0.0001

V100: total vegetable diet; V50+B50: diet consisting in 50% of vegetables and 50% of butchery wastes; V75+B25: diet consisting in 75% of vegetables and 25% of butchery wastes. a, b, c: $P < 0.01$; RMSE: Root Mean Square Error.

Starting from 11 days of age, larvae from V75+B25 and V50+B50 groups showed a similar length and, at the end of the trial, were longer than the larvae of the control group, while larvae from the V100 group showed intermediate values (Tab. 2.3).

Tab. 2.3 Body length (cm) of black soldier fly larvae from 7 to 22 days of age.

	Control	V100	V50+B50	V75+B25	RMSE	P-value
7 d	1.14	1.12	1.16	1.18	0.067	0.1199
9 d	1.31^{b}	1.31^{b}	1.25^{b}	1.52^{a}	0.166	0.0046
11 d	1.31^{c}	1.39^{bc}	1.58^{ab}	1.67^{a}	0.161	<0.0001
13 d	1.34^{c}	1.48^{bc}	1.65^{ab}	1.71^{a}	0.151	<0.0001
15 d	1.43^{c}	1.57^{bc}	1.67^{ab}	1.77^{a}	0.121	<0.0001
17 d	1.44^{b}	1.63^{ab}	1.75^{a}	1.78^{a}	0.178	0.0003
20 d	1.47b	1.64^{ab}	1.77^{a}	1.84^{a}	0.210	0.0022
22 d	1.55^{b}	1.65^{ab}	1.80^{a}	1.87^{a}	0.182	0.0003

V100: total vegetable diet; V50+B50: diet consisting in 50% of vegetables and 50% of butchery wastes; V75+B25: diet consisting in75% of vegetables and25 % of butchery wastes. a, b, c: $P < 0.01$; RMSE: Root Mean Square Error.

The height of larvae, measured in the middle of the body, is pictured in Tab. 2.4. In general, the V75+B25 group showed the highest values up to 17 days of age. Then the Control group larvae overcame all the others. Starting from 11 days old, V100 groups showed the lowest height values.

Tab. 2.4. Body thickness (cm) of black soldier fly larvae from 7 to 22 days of age.

	Control	V100	V50+B50	V75+B25	RMSE	P-value
7 d	0.24^{b}	0.22^{b}	0.25^{ab}	0.29^{a}	0.034	0.0003
9 d	0.40^{a}	0.33^{b}	0.30^{b}	0.42^{a}	0.032	<0.0001
11 d	0.42^{ab}	0.37^{c}	0.40^{bc}	0.44^{a}	0.031	<0.0001
13 d	0.43^{b}	0.37^{c}	0.41^{b}	0.47^{a}	0.024	<0.0001
15 d	0.43^{b}	0.37^{c}	0.42^{b}	0.47^{a}	0.025	<0.0001
17 d	0.44^{b}	0.38^{c}	0.45^{ab}	0.48^{a}	0.032	<0.0001
20 d	0.55^{a}	0.38^{c}	0.47^{b}	0.50^{b}	0.028	<0.0001
22 d	0.55^{a}	0.38^{c}	0.49^{b}	0.50^{b}	0.025	<0.0001

V100: total vegetable diet; V50+B50: diet consisting in 50% of vegetables and 50% of butchery wastes; V75+B25: diet consisting in 75% of vegetables and 25% of butchery wastes. a, b, c: $P < 0.01$; RMSE: Root Mean Square Error.

The growth performance of the BSF larvae during the trial are summarized in Table 2.5. V50+B50 group showed the highest mortality rate ($P < 0.01$) followed by V75+B25 group and, together, Control and V100 groups. The total larval biomass in V100 group was the lowest ($P < 0.01$), while V75+B25 group has a higher total larval biomass than Control and V100 groups. The total larval frass showed the highest value in the Control, followed by V50+B50, V75+B25

and V100 groups ($P < 0.01$). The length to height ratio of V50+B50 group larvae was lower ($P < 0.01$) than that of V100 larvae and higher ($P < 0.01$) compared to the Control group larvae. The growth rate of V100 group was lower ($P < 0.01$) than the other groups; the opposite happened for the substrate reduction (SR) index. In addition, the Control group showed the lowest ($P < 0.01$) SR value. Larvae yield obtained in the V50+B50 group was lower ($P < 0.01$) than that of V100 and V75+B25 groups. The waste reduction index of V50+B50 group was lower ($P < 0.01$) than that of V75+B25 and higher than that of the Control group. The efficiency conversion of digested food was the highest ($P < 0.01$) in the Control group followed by both V100 and V75+B25 and then by V50+B50 groups. The protein conversion ratio was the highest ($P < 0.01$) in V100 and V75+B25 groups 1.

Tab 2.5. Growth performance of black soldier fly larvae calculated at the end of the trial.

	Control	V100	V75+B25	V50+B50	RMSE	P-value
Mortality, %	9.95^{c}	10.61^{c}	18.93^{b}	20.47^{a}	1.075	<0.0001
TLB, g DM	657.7^{b}	370.5^{c}	717.2^{a}	688.8^{ab}	18.49	<0.0001
TLF, g DM	1673.2^{a}	443.2^{d}	1013.5^{c}	1464.5^{b}	87.31	<0.0001
L/H	2.82^{c}	4.34^{a}	3.74^{ab}	3.67^{b}	0.23	<0.0022
GR	0.011^{a}	0.009^{b}	0.011^{a}	0.010^{a}	0.0009	<0.0001
SR	44.13^{c}	69.71^{a}	66.07^{b}	59.88^{b}	3.13	<0.0001
LY	0.22^{ab}	0.25^{a}	0.24^{a}	0.19^{b}	0.01	<0.0001
WRI	2.60^{c}	4.10^{a}	3.89^{ab}	3.52^{b}	0.25	<0.0001
ECD	0.48^{a}	0.36^{b}	0.36^{b}	0.32^{c}	0.015	<0.0001
PR	0.26^{b}	0.36^{a}	0.36^{a}	0.23^{b}	0.023	<0.0001

V100: total vegetable diet; V75+B25: diet consisting in 75% of vegetables and 25% of butchery wastes; V50+B50: diet consisting in 50% of vegetables and 50% of butchery wastes; TLB = total larval biomass; TLF = total larval frass; L/H: length to height ratio; GR = growth rate; SR = substrate reduction; LY = larvae yield; WRI = waste reduction index; ECD = efficiency conversion of digested food; PR: protein conversion ratio. Within rows: a, b, c: $P < 0.01$; RMSE: Root Mean Square Error.

The hydroperoxide levels and the antioxidant capacities found in hemolymph of larvae fed different diets are indicated in Table 2.6. The antioxidant barrier was not significantly different among groups, whereas the concentration of hydroperoxides was higher in the control group, thereby showing that the use, as well as the inclusion, of vegetables in the diet accumulated less oxidative damages.

Tab 2.6. Hemolymph oxidative stress profile of black soldier fly larvae at 20 days of age.

	Control	V100	V75+B25	V50+B50	RMSE	P-value
d-ROMs, U CARR	113.0^{a}	86.74^{b}	69.21^{b}	73.50^{b}	21.71	<0.0001
BAP, µmol/L	3860.5	3802.1	3967.3	3973.2	767.2	0.9016

V100: total vegetable diet; V75+B25: diet consisting in 75% of vegetables and 25% of butchery wastes; V50+B50: diet consisting in 50% of vegetables and 50% of butchery wastes; d-ROMs: Diacron Reactive Oxygen Metabolites; BAP: Biological Antioxidant Potential; within rows: a, b: $P < 0.01$; RMSE: Root Mean Square Error.

At the end of the trial, the larvae of V100 group showed the highest ($P < 0.01$) moisture, followed by Control and, together, V50+B50 and V75+B25 groups. V100 group showed the highest ($P < 0.01$) amount of ash followed by Control, V75+B25 and V50+B50 groups (Table 2.7). The highest percentage of lipids ($P < 0.01$) was found in V50+B50 group and the lowest in V100 one. V50+B50 group showed lower percentage of protein ($P < 0.01$) compared to

Control and V100 groups. The CD level in the Control and V50+B50 groups was higher ($P < 0.01$) than the other groups. The MDA of V75+B25 group was higher ($P < 0.01$) than the values found in the other groups.

Tab 2.7 Black soldier fly larvae chemical composition and oxidative stability.

	Control	V100	V75+B25	V50+B50	RMSE	P-value
Moisture, %	66.22^{b}	77.46^{a}	59.10^{c}	57.69^{c}	0.29	<0.0001
Ash, % DM	7.28^{b}	13.50^{a}	5.05^{c}	4.57^{d}	0.55	<0.0001
Lipids, % DM	22.12^{b}	6.01^{c}	27.08^{b}	35.56^{a}	4.10	<0.0001
Crude protein, % DM	44.24^{a}	44.87^{a}	40.67^{ab}	30.65^{b}	3.79	<0.0001
CD, mmol Hydroperoxide/100g	1.64^{a}	0.56^{b}	0.86^{b}	1.69^{a}	0.30	<0.0001
TBARS, mg MDA-eq/kg	0.18^{b}	0.22^{ab}	0.27^{a}	0.21^{ab}	0.045	0.0032

V100: total vegetable diet; V75+B25: diet consisting in 75% of vegetables and 25% of butchery wastes. DM: dry matter; V50+B50: diet consisting in 50% of vegetables and 50% of butchery wastes; CD: conjugated dienes; TBARS: thiobarbituric acid reactive substances; within rows: a, b, c: $P < 0.01$; RMSE: Root Mean Square Error.

Regarding the chemical traits of frass (Table 2.8), the moisture was the highest ($P < 0.01$) in the V50+B50 group followed by V100, Control and V75+B25 groups. The ash percentage was the highest ($P < 0.01$) in V100 group followed by Control, V75+B25 and V50+B50 groups. The highest lipid percentage ($P < 0.01$) was measured in V50+B50 group while the lowest was in Control and V100 groups. V100 group showed a higher protein content ($P < 0.01$) than Control and V75+B25 groups.

Tab 2.8 Frass chemical composition.

	Control	V100	V75+B25	V50+B50	RMSE	P-value
Moisture, %	33.18^{b}	41.63^{a}	21.28^{c}	42.39^{a}	0.31	<0.0001
Ash, % DM	12.57^{b}	15.13^{a}	8.67^{c}	3.11^{d}	0.89	<0.0001
Lipids, DM	1.77^{c}	2.47^{c}	19.98^{b}	39.32^{a}	2.41	<0.0001
Protein, % DM	16.60^{b}	21.72^{a}	15.65^{b}	18.98^{ab}	3.88	<0.0001

V100: total vegetable diet; V75+B25: diet consisting in 75% of vegetables and 25% of butchery wastes; V50+B50: diet consisting in 50% of vegetables and 50% of butchery wastes; DM: dry matter; within rows: a, b, c: $P < 0.01$; RMSE: Root Mean Square Error.

4. Discussion

Standing our knowledge, this is the first research in which butchery wastes have been tested as substrate for Black Soldier fly larvae. The inclusion of butchery wastes in the different proportions tested in our trial induced high total larval biomass production, expressed on dry matter basis, but also a higher mortality rate of larvae in comparison to the other groups. The best results were obtained when butchery wastes were "diluted" with high proportion of vegetable mix, but vegetable mix, alone, is not suitable for a good larval production. In fact, larvae on V100 diet showed, in general, the worst growing performance (live weight at 22 days, larvae length and thickness, growth rate) even if had a low mortality rate, high values of SRI, PR and a LY not different from the Control and V75+B25 groups. A lower growth rate of BSF larvae on V100 diet could be ascribed to both a lower protein availability and a high moisture content. Indeed, considering the protein and moisture percentages of each substrate, the total protein available for larvae growth were: 679.6, 279.8, 543.1 and 482.4 g, for Control, V100, V50+B50 and V75+B25 groups, respectively. Even if the moisture percentage in each

substrate were within the suitable range for BSF indicated by Cammack and Tomberlin (2017), Dzepe *et al*. (2021), testing five substrates with increasing moisture content from 40 to 80%, observed that increasing the substrate moisture content reduces the larval feed reduction, wet weight, development time, body size and body thickness. Lalander *et al*. (2020) also reported that high levels of moisture in the substrate reduced the biomass conversion ratio and survival rate of the larvae. However, in our trial, the ventilation applied in the larvae-growing chamber can alleviate the negative effects of high-moisture substrates, according to Pinotti and Ottoboni (2021).

Surprisingly, our results showed that the development time of larvae was not differ-ent among the groups. This result is in contrast with other researches, (Meneguz *et al*., 2018; Scala *et al*., 2020; Dzepe *et al*., 2021). It is not easy to explain this point, thus further insights need to clarify it, evaluating the metabolic profile of larvae in detail.

The V75+B25 and V100 diet (which larvae showed the second and the first lower amount of total protein) determined a high PR value. These results agree with the findings of Bonelli *et al*. (2020) who showed that the midgut of *H. illucens* larvae can adapt to diets with different nutrient contents, increasing proteolytic activity, and decreasing α-amylase and lipase activities when poor diets are available. The larvae obtained from substrates containing butchery wastes showed a higher percentage of lipids, but a lower percentage of proteins than the other groups and this was particularly true when butchery wastes were used at the highest level. In the larvae the body fat represents the tissue in which nutrients such as protein, carbohydrates and fats were stored (Arrese and Soulages, 2010) and used for growth and metamorphosis (Hahn *et al*., 2008). However, a high percent-age of fat does not indicate a satisfactory accumulation of nutrients reserves (Coppe Pimentel *et al*., 2017). In fact, the lipid tissue of insects is composed of trophocytes in which cytoplasm is possible to de-tect two types of roundish structures, associated to nutrient accumulation: lipid and pro-tein droplets differing in terms of size and coloring reactions (Coppe Pimentel *et al*., 2017). The diet containing only 25% of butchery wastes seemed to be more balanced for BSF larvae as their lipid and protein contents were not different from the Control group.

The V100 diet produced larvae with a protein content comparable to the Control but with a very low amount of lipids, despite a similar percentage of carbohydrates. This might surprise because larvae use free sugars, abundant in the vegetable mix, to produce triacylglycerol, accumulating it in the body fat (Carvalho *et al*., 2012). However, the V100 diet had 25.7% more moisture than the average of the other diets and this strongly diluted the nutrients available for larval growth. The average ash content in larvae was higher than in the rearing substrate and this suggests that larvae accumulate minerals in their body. Indeed, the rate of accumulation was different, according to the used substrate. When a high amount of vegetable mix was included in the diet, as happened in V75+B25 and V100 groups, the larvae showed 1.38 and 1.30 more ashes than the correspondent diets, respectively. On the contrary, with Control and V50+B50 diets, the rate of ash increase was 1.97 and 2.48, respectively. A possible explanation could be that, in the vegetable mix, part of minerals could be complexed with phytates that reduces the mineral availability for digestion. The effect of phytate on insect growth and development is still poor investigated (Callegari *et al*., 2020) but it can reduce the availability of essential minerals and proteins (Wodzinski and Ullah, 1996). In addition, phytate in plants plays a defensive role against phytophagous insects as showed by Green *et al*. (2001) who demonstrated a positive correlation between the presence of phytic acid in the diet and the mortality of three Lepidoptera species. The Control diet, consisting in a broiler standard feed, contained wheat and thus an amount of phytate. However, as a commercial diet, it contains a further supplementation of calcium, available phosphorous and other minerals, that may have been easily available for BSF

larvae. Also, the protein content in larvae was higher than in the correspondent substrates, ranging from 1.95 of the Control diet to 2.51 of the V75+B25 diet, according to Pinotti and Ottoboni (2021). In our trial, substrates containing the highest CP and moisture percentage (Control and V100) allowed to obtain BSF larvae with the highest CP level, according to Meneguz *et al*. (2018). The evaluation of lipid oxidation is a useful method to measure the integrity of BSF larvae (Bhattacharyya *et al*., 2014). MDA is one of the most important aldehydes produced during the secondary lipid oxidation of polyunsaturated fatty acids and is considered the major marker for lipid oxidation. Based on the standard values, the BSF larvae of all the tested groups can be considered not rancid (<1.5 mg MDA/kg) (Robles-Martinez *et al*., 1982). The increased production of CD, indicating a major lipid oxidation in the Control and V50+B50 groups (Min and Ahn, 2005). However, the measurement of CD could be interfered by com-pounds absorbing in the same region such as the presence of conjugated double bonds in the original fatty acids (Domínguez *et al*., 2019) or the presence of carotenoids (Yang *et al*., 2016). Concerning oxidative status of larvae, the lower level of d-ROMs in the hemolymph showed that the use of vegetable waste, at different levels, in the diet of *Hermetia illucens* larvae led to a significant reduction in ROS production. Conversely, the BAP did not show differences among groups, thus suggesting that the vegetable diets did not act increasing the antioxidant barrier, but some other mechanisms were involved.

In general, oxidative stress can be defined as a disturbance in the balance between the production of reactive oxygen species and antioxidant defenses (Betteridge, 2000). In insects, ROS are involved in the regulation of various mechanisms and intercellular signaling and act as bactericidal agents. They can also induce cellular senescence, apoptosis, cell growth regulatory pathways and are involved in immunity, also, in response to nutrient stress, cells enter autophagy that can lead to adaptation or death (Chaitanya *et al*., 2016). ROS activation is suspected to serve as a primary mechanism inhibiting development of the pathogen in situ (Huang *et al*., 2020). Since the ROS generation in the invertebrate systems may be due to many causes, further studies are needed to explore the mechanisms by which vegetable waste can act as a ROS limiting factor in *Hermetia illucens* diet. However, the higher level of ROS in the Control group is not accompanied to a high mortality rate that, on the contrary, was higher in V50+B50 and V75+B25 groups. The percentage of survived larvae was, in general, satisfactory considering that Nguyen *et al*. (2013) found a survival rate of 77% on BSF larvae growing on vegetable wastes. Some authors attributed the low survival rates to the intraspecific competition between individuals for the feed source (Tchuinkam *et al*., 2011; Mitchell-Foster *et al*., 2012) and to the type of substrate (Britis, 2017). Unfortunately, we cannot be able to evaluate at what stage of larval development the recorded mortalities occurred: this could be very interesting to determine for how long the larvae can be fed with a specific diet.

5. Conclusions

The use of butchery wastes as growing substrate for BSF larvae can be suitable, but they must be well combined with other ingredients to balance the high lipid a low protein contents. Vegetable wastes can be appropriate candidates to counteract the negative effects of butchery wastes. The use of vegetable wastes reduces the level of ROS in insect hemolymph, suggesting a positive effect of larvae welfare. However, the diet composed exclusively by vegetable wastes seems to be not indicated for black soldier fly growth as less larval biomass was obtained. Further analyses are in progress at our laboratories to assess the fatty acid, amino acid and mineral profile of substrates, larvae and frass.

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CHAPTER 3

Intestinal morphometry, enzymatic and microbial activity in laying hens fed different levels of a *Hermetia illucens* larvae meal and toxic/trace elements content of the insect meal and diets.

1. Introduction

According to the recent declaration of the chief commercial officer at insect producer Protix in the Netherlands and board member of the International Platform of Insects for Food and Feed (IPIFF), reported by All About Feed (September, 2018) the EU approval for the use of the insect meals in poultry nutrition is "not so distant in the future" and the approval by EU Member States could be possible during the first quarter of 2019 (IPIFF, 2018; All about feed, 2018). Thus, it is mandatory to try to understand all the possible aspects related to the chemical-nutritional characteristics, the effects on the animal health and welfare, the impact of feed and food safety of the different meals obtained from insects. This goal is not easy to reach as some characteristics can be modified according to the species and, within the species, in relation to the harvesting stage, the substrate for growth, etc. In the recent years, several studies pointed the attention on the use of insect meals in growing broiler (Bovera *et al*., 2015; Bovera *et al*., 2016; Loponte *et al*., 2018; Schiavone *et al*., 2018), quail (Cullere*et al*., 2018) and barbary partridge (Loponte *et al*., 2017; Secci *et al.*, 2018*)*. Also, for laying hens there are recent studies focused on laying performance, egg quality, metabolic and nutritional effects(Al-Qazzaz *et al*., 2016*;* Marono *et al*., 2017; Bovera *et al*., 2018; Secci *et al.*, 2018) of feeding insectmeals. However, the available literature on this topic is still limited and very often there are conflicting results due to the different kind of insects, the different strains and age in lay of the hens utilized in the trials.

An important aspect concerning the use of insect meal as feed, related to the human and animal health, is the possible accumulation of mineral elements in the insect body during the growing cycle. Some elements (Cu, Se, Cr, Fe, Mn, Ni) are essential for biological functions, but the heavy metals like Cd, Pb, Hg and As can induce adverse effects due to their potential toxicity and bioaccumulation in the food chain (Migliarini *et al*., 2005). To regulate the animal dietary exposure to toxic elements the EU Commission established maximum levels (MLs) for different undesirable substances in animal feeds (Directive (EC) No 2002/32). Data on transfer of chemical contaminants from different substrates to the insects are very limited (EFSA, 2015**)**. As a consequence, a monitoring of essential and non-essential elements concentrations, in the insect meal and diets is necessary from the point of view of both nutrition and contamination. The few studies in literature on the mineral profile of insect meals (Liland *et al*., 2017; Cullere *et al*., 2018; Irungu *et al*., 2018) often showed different minerals with a very wide range of variation according to the composition of the substrate used for the insect growing. This study represents the continuation and completion of a previous trial (Bovera *et al*., 2018) in which the laying performance, blood profiles and nutrient digestibility of hens fed *Hermetia illucens* (HI) larvae meal from 16 to 40 weeks of age have been investigated. Thus, the aim of the present research was to evaluate the effect of the inclusion of a defatted meal from HI larvae in the diet on the volatile fatty acids production in the caeca, intestinal morphometry and brush border enzymatic

activity of 40 weeks old layers. In addition, the research aims to contribute to the knowledge on the trace and toxic elements concentration in insect meals.

2. Materials and methods

All the animals were treated according to the principles of the animal welfare stated by the EC Directive 63/2010/EEC regarding the protection of the animals used for experimental and other scientific purposes. The experimental procedures were approved by the Ethical Animal Care and Use Committee of the Department of Veterinary Medicine and Animal Production of the University of Napoli Federico II, Italy (prot. N. 2017/0017676).

The trial was carried out in a private laying hens farm located in Sardinia (Italy) for 24 weeks, from February to July 2017.

A total of 162, sixteen weeks old Hy-line Brown hens (average live weight 1,41 kg ± 0.13 standard deviation) were equally divided into three experimental groups, differing for the dietary treatment. The control group was fed a corn-soybean meal based diet (SBM group) formulated to meet the hens requirements according to the Hy-line Brown commercial line management guide 2016 (Hy-line Brown Commercial Layers Management Guide, 2016**)**. In the other groups, the soybean meal was partially replaced by a partially defatted insect meal obtained from *Hermetia illucens* larvae (HI, *Hermetia* Deutschland GmbH & Co KG, Amtsgericht Potsdam, Germany) in order to formulate two isoproteic and isoenergetic diets. In the HI25 group, the 25 % of the dietary proteins was replaced by the HI protein (inclusion level of the HI in the diet 7.3 %); in the HI50 group the 50 % of the dietary proteins was replaced by the protein of the HI (inclusion level 14.6 %). The hens were housed in the same building in modified cages (800 cm^2/hen), under controlled temperature and humidity conditions. For each group, the hens were distributed into 3 cages (18 hens/cage) and each cage was divided by 2 internal transects in 3 equal areas, to obtain 9 replicates of 6 hens per group. Feed and water were manually distributed, and appropriate separations were placed along the trough and the line of the egg collection to control the feed intake and the egg production per each replicate. The dark:light cycle was 9:15 hours.

The main protein sources (soybean and insect meal) and the diets were analyzed for the chemical composition according to the AOAC official methods (AOAC, 2005). The metabolizable energy of the diets was calculated according to the NRC procedure of estimation (NRC, 1994), while the apparent metabolizable energy of the insect meal used in the present trial was obtained from the studies of (De Marco *et al*., 2005). The data on the amino acids, minerals and metabolizable energy values of all the ingredients were supplied by the respective producers and used to calculate the correspondent contents in the diets. The amount of protein linked to the acid detergent fibre (ADF) was determined (AOAC, 2005), and only for the insect meal it was used to estimate the amount of chitin, according to Marono *et al*. (2015) as follow: chitin (%) = ash free ADF (%) – ADF-linked protein (%). The chemical-nutritional characteristics of the two protein sources are reported in Tab. 3.1, while the ingredients and chemical-nutritional characteristics of the diets are indicated in Tab. 3.2.

Tab. 3.1. Proximate composition, mineral and essential amino acid composition (% as fed) of the Hermetia illucens larvae meal and soybean meal.

	***Hermetia illucens* larvae meal**	**Soybean meal**
Proximate composition		
Dry matter	92.7	90.0
Crude protein	55.6	43.4
Ether extract	8.34	1.1
ADF	11.5	5.9
ADF-linked protein	4.86	1.78
Ash	7.8	6.0
Mineral composition		
Ca[1]	6.47	2.83
Total P[1]	0.90	0.57
Na[1]	0.12	0.16
Essential Amino Acid composition		
Lysine[1]	4.12	2.92
Methionine[1]	1.09	0.61
Methionine+Cystine[1]	1.32	1.33
Isoleucine[1]	2.97	2.30
Tryptophan[1]	0.30	0.73
Valine[1]	5.02	2.11
Threonine[1]	2.32	1.74

[1] obtained by the producers

Tab. 3.2. Ingredients and chemical characteristics of the three diets: control (SBM), HI25 and HI50.

	SBM	**HI25**	**HI50**
Ingredients, g/kg			
Maize grain	605.5	597.5	630.5
Soybean meal	265	200	95
Insect meal	-	73	146
$CaCO_3$ grains	80	80	80
Vegetable oil	10	10	-
MinVit[*]	10	10	10
Methionine	2.5	2.5	2.5
Monocalcium phosphate	5	5	5
Celite	20	20	20
Salt	2	2	2

Chemical-nutritional characteristics			
Dry matter[1], %	91.53	91.39	91.62
Crude protein[1], %	16.45	16.32	17.03
Ether extract[1], %	3.17	3.61	4.06
NDF[1], %	10.38	11.29	12.49
ADF[1], %	5.85	5.90	5.67
ADL[1], %	2.67	2.94	2.29
Lysine[2], %	0.86	0.97	1.00
Methionine[2], %	0.53	0.58	0.61
Metabolizable Energy[2], kcal/kg	2832.3	2845.2	2842.2

SBM: soybean meal based diet; HI25: diet including *Hermetia illucens* as 25 % of replacement of the soybean meal protein; HI50: diet including *Hermetia illucens* as 50 % of replacement of the soybean meal protein; 1: determined according to AOAC (2004); 2: calculated according to NRC (1994); *Provided per kilogram: vitamin A (retinyl acetate) 20,000 IU, vitamin D3 (cholecalciferol) 6,000 IU, vitamin E (dl-α-tocopheryl acetate) 80 IU, vitamin B1(thiamine monophosphate) 3 mg, vitamin B2 (riboflavin) 12 mg, vitamin B6 (pyridoxine hydrochloride) 8 mg, vitamin B12 (cyanocobalamin) 0.04 mg, vitamin K3 (menadione) 4.8 mg; vitamin H (d biotin) 0.2 mg, vitamin PP (nicotinic acid) 48 mg, folic acid 2 mg, calcium pantothenate 20 mg, manganous oxide 200 mg, ferrous carbonate 80 mg, cupric sulphate pentahydrate 20 mg, zinc oxide 120 mg, basic carbonate monohydrate 0.4 mg, anhydrous calcium iodate 2 mg, sodium selenite 0.4 mg, choline cloride 800 mg, 4-6-phitase 1,800 FYT, D.L. methionine 2,600 mg, canthaxanthin 8 mg.

The trace elements contained in the insect meal and in the diets, were also determined. The samples were digested in ultrapure 65% HNO_3 and H_2O_2 in a microwave digestion system (Ariano *et al*., 2015). Concentrations of trace elements were determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) technique using a Perkin Elmer Optima 2100 DV instrument coupled with a CETAC U5000AT. The calibration curve and two blanks were run during each set of analyses, to check the purity of the chemicals. A reference material (CRM DORM-4, NRC, Canada) was also included for the quality control. All the values of the reference materials were within the certified limits. The instrumental detection limits are expressed as wet weight (w.w.) and they were determined following the protocol described by Perkin Elmer ICP application study number 57 (Barnard *et al*., 1993). The data are reported in the Tab. 3.3.

Tab. 3.3. Trace elements content (mg/kg) in the insect meal (HI meal) and in the three diets (SBM, HI25 and HI50).

	HI meal	SBM	HI25	HI50
As	0.23	0.95	0.86	0.81
Cr	0.21	1.87	1.83	1.47
Cd	0.06	0.001	-	0.007
Pb	0.03	-	-	-
Hg	0.01	-	-	-
Co	0.01	-	0.001	0.003

Cu	3.20	7.47	7.29	6.44
Ni	0.10	0.80	0.63	0.68
Se	0.05	0.12	0.14	0.13
Zn	453.6	479.4	657.6	510.8

At 40 weeks of age, two hens randomly chosen per replicate (18 per group) were slaughtered, the different digestive tracts were identified, each of them was excised and weighed.
The small intestine tracts were washed with an ice-cold isotonic saline buffer (pH 7) blotted with absorbent paper and divided into three segments, duodenum, jejunum and ileum and stored at -20°C until analysis.

2.1 Villus and crypt morphometry

The intestinal samples (0.5 cm) from duodenum, jejunum and ileum were fixed by immersion in 4% phosphate-buffered paraformaldehyde for 48 h. The samples were then washed in a phosphate-buffered saline solution, dehydrated in an ethanol series and embedded in paraffin according to Vargas *et al*. (2018). Cross sections were stained with Mayer's hematoxylin and eosin and examined under Zeiss Axio Imager.A2 microscope for the histopathological assay. For the analysis of the villus height and crypts depth ten microscopic fields of the duodenum, jejunum and ileum were acquired using a microscope combined color digital camera Axiocam 503 (Zeiss) and the measurements were performed using the Zen 2.3 lite software.

2.2 Brush Border Membrane enzymes activity

The Brush Border Membrane (BBM) enzymes were obtained according to Shirazy-Beechey *et al*. (1991) with some modifications as detailed in Messina *et al*. (2019). Briefly, 100 mg of the tissue were diluted 1:10 with a buffer (100 mM mannitol, 2 mM Hepes-tris, pH 7.1), added with MgCl2 at a final concentration of 10 mM, and crushed with a tissue lyser (Tissue Lyser II, Qiagen, Germany) at 30 Hz for 1 min. The samples were centrifuged at 2000 ×g at 4 °C for 10 min and the supernatant was transferred in a new vial and centrifuged at 15,000 x g at 4 °C for 10 min. All the steps were performed at 4 °C and the resulting supernatant was maintained at −20 °C until the analysis of the BBM enzyme activity.
The hydrolysis of sucrose and maltose by the mucosal maltase and sucrase, was determined according to Tibaldi *et al*. (2006). The intestinal alkaline phosphatase (IAP) and γ-glutamyl transpeptidase (γ-GT) activities were determined on the supernatant using two commercial kits (Paramedical, Pontecagnano Faiano, SA, Italy) as indicated by the manufacturer. The total protein concentration was determined according to Bradford, (1976) (Sigma-Aldrich cat. no. B6916) and bovine serum albumin (Sigma-Aldrich cat. no. 0834) was used as a standard. One unit (U) of enzyme activity corresponded to the amount of enzyme that transforms or hydrolyses 1 μmole of substrate ml−1 min−1. The specific enzymatic activity was calculated as U of enzyme activity per mg of protein.

2.3 Volatile fatty acids

The caeca were tied at both ends, separated by sterile instruments from the rest of the gastrointestinal tract, placed in tightly closed plastic bags and put in pre-warmed thermos. After the sampling, the material was transported as soon as possible (about 1 h) to the laboratory, where two quotes of the caecal content (about 5 ml each) were used for the volatile fatty acids (VFAs) determination. The samples were diluted with oxalic acid (1:1, v/v) and VFAs were analyzed by a gas chromatography method (Stanco *et al*., 2003) (Thermo-Electron mod. 8000top, FUSED SILICA Gaschromatograph, ThermoElectron Corporation, Rodano, Milan, Italy) equipped with OMEGAWAX 250 fused silica capillary column 30 m ×0.25 mm ×0.25 mm film thickness, flame ionisation detector (185 °C), carrier helium (1.7 ml/min) under isothermal condition (125 °C).

2.4 Statistical analysis

The data were processed by ANOVA using the PROC GLM of SAS, 2000. The differences among the groups regarding the volatile fatty acids in the caeca, the intestinal morphometry and enzymatic activity were analyzed by the one-way ANOVA according to the following model:

$$Yij = m + Di + eij$$

where Y is the single observation, m is the general mean, D is the effect of the diet (i = SBM, HI25 or HI50) and e is the error.
The comparison among the means was performed by the Tukey's test SAS, 2000; in addition, the orthogonal contrast analysis was performed to test the linear and quadratic effect among the means SAS, 2000.

3. Results

The data on the concentration of trace elements in the HI meal and in the experimental diets are reported in the Tab. 3.3.
The tab. 3.4 shows the morphometry traits (villi height, crypt depth and villi/crypt ratio) for each of the three tracts of the small intestine of the hens according to dietary treatments. In the duodenum, villi height and villi/crypt ratio were higher ($P < 0.01$) in the SBM than in both the HI groups. The contrast analysis shows a significant ($P < 0.01$) linear effect for villi height, while the quadratic effect was significant ($P < 0.01$) both for the villi height and villi/crypt. In the jejunum the villi height and villi/crypt ratio of the hens fed SBM was higher ($P < 0.01$) than that of both the levels of HI. The contrast analysis shows a significant effect for the linear component for the villi height, villi/crypt ($P < 0.01$) and for the crypt depth ($P < 0.05$). In the ileum the HI25 group had higher villi height than the HI50 ($P < 0.05$) and the only significant contrast was a linear effect ($P < 0.054$) for the villi height.

Tab. 3.4. Effect of the dietary treatments on the villi height and crypt depth of the different small-intestine tracts.

	SBM	**HI25**	**HI50**	**RMSE**	**P-values**		
					ANOVA	Contrast analysis	
	Duodenum					Linear	Quadratic
Villi height	1394^{A}	1031^{B}	1006^{B}	117.5	<0.0001	<0.0001	<0.0001
Crypt depth	350.8	381.3	395.8	90.56	0.4739	0.2345	0.8059
Villi/crypt	3.99^{A}	2.69^{B}	2.90^{B}	0.79	0.0006	0.0018	0.1023
	Jejunum						
Villi height	1149^{A}	825^{B}	790^{B}	114.2	<0.0001	<0.0001	<0.0001
Crypt depth	273.3^{b}	275.5^{b}	315.9^{a}	26.29	0.0461	0.0329	0.2684
Villi/crypt	4.27^{A}	3.09^{B}	2.68^{B}	0.84	<0.0001	<0.0001	0.1285
	Ileum						
Villi height	1006^{ab}	1013^{a}	843^{b}	161.2	0.0317	0.0162	0.1541
Crypt depth	321.3	335.9	328.7	32.11	0.8308	0.7601	0.6151
Villi/crypt	3.31	3.12	2.77	0.36	0.2351	0.0918	0.7821

SBM: soybean meal group; HI25 and HI50: Hermetia illucens groups in which the protein by insect meal replaced that of soybean meal by 25 and 50 %, respectively. A, B: $P < 0.01$; a, b: $P < 0.05$; RMSE: root mean square error.

The tab. 3.5 reports the activities of brush border enzymes in each of the three tracts of the hen's small intestine, according to dietary treatments. In the duodenum, the maltase was higher ($P < 0.05$) in the SBM and HI25 than the HI50 group and the contrast analysis indicates a significant linear effectfor the maltase and IAP ($P < 0.05$). In the jejunum, the IAP of the SBM was higher than that of boththe HI groups and the contrast analysis shows a significant linear effect ($P < 0.05$) for the IAP and γ-GT. In the ileum the γ-GT showed higher values in the SBM than both the insect meal groups ($P < 0.05$), with a quadratic significant effect ($P < 0.05$).

Tab. 3.5 Specific activity (UI) of the maltase, saccarase, intestinal alkaline phosphatase (IAP) ɤ-glutamyltransferase (ɤGT) measured in the different digestive tracts of the hens fed the test diets over 21 weeks.

	SBM	**HI25**	**HI50**	**RMSE**	**P-values**		
					ANOVA	Contrast analysis	
	Duodenum					Linear	Quadratic
Maltase	70.46^{a}	74.96^{a}	50.82^{b}	19.47	0.0498	0.0465	0.1044
Saccarase	12.12	13.02	11.54	3.49	0.7001	0.7422	0.4419

IAP	4.39	3.87	2.75	1.15	0.3165	0.0422	0.7479
γGT	129.4	159.2	119.9	59.9	0.2888	0.5582	0.1457
	Jejunum						
Maltase	71.39	69.94	76.77	16.97	0.7025	0.5325	0.5799
Saccarase	17.49	14.65	16.21	4.97	0.5308	0.6118	0.3194
IAP	5.48a	3.07b	2.87b	2.23	0.0436	0.0295	0.2680
γGT	206.4	199.1	154.9	70.42	0.3064	0.0435	0.5515
	Ileum						
Maltase	77.33	55.92	64.19	22.25	0.1780	0.2505	0.1385
Saccarase	17.34	14.51	17.20	3.90	0.2840	0.9445	0.1172
IAP	3.29	3.09	3.06	1.35	0.9351	0.7379	0.8899
γGT	145.0[a]	94.09[b]	132.73[ab]	37.0	0.0307	0.513	0.0108

SBM: soybean meal group; HI25 and HI50: Hermetia illucens groups in which the protein by insect meal replaced that of soybean meal by 25 and 50 %, respectively. a, b: $P < 0.05$; RMSE: root mean square error.

The tab. 3.6 reports the effect of the dietary treatments on the short chain fatty acids (SCFA) production in the hen's caeca. The production of acetate and total SCFA (mmo/l) was higher in the caecal content of the hens fed the HI50 diet than in that of the other groups ($P < 0.05$). The butyrate content was higher ($P < 0.05$) in the HI50 than in the SBM group, while the valerianic acid of the hens fed the SBM diet was higher ($P < 0.01$) than those fed both the HI diets. The contrast analysis showed a significant linear effect ($P < 0.05$) for the isobutyrate, butyrate, isovalerianic and valerianic acids.

Tab. 3.6. Volatile fatty acid production in the caecal content of the hens fed the test diets over 21 weeks.

	SBM	HI25	HI50	RMSE	P-values		
					ANOVA	Contrast analysis	
	Mmol/l					Linear	Quadratic
Acetate	58.24[b]	57.54[b]	65.99[a]	6.59	0.0242	0.1565	0.3406
Propionate	20.62	20.31	21.42	2.50	0.9169	0.7718	0.7735
Isobutyrate	1.78	2.11	2.34	0.17	0.1442	0.0107	0.8381
Butyrate	7.25[b]	7.72[ab]	8.81[a]	0.59	0.0125	0.0326	0.6576
Isovalerianic	2.78	3.00	3.42	0.25	0.1274	0.0453	0.7919
Valerianic	4.38[A]	2.99[B]	3.28[B]	0.31	0.0021	0.0176	0.3777
Total VFA	95.06[b]	93.67[b]	105.0[a]	6.69	0.0392	0.5826	0.4620
	% total VFA						

Acetate	61.27	61.43	62.85	5.38	0.8571	0.5743	0.9914
Propionate	21.69	21.68	20.40	2.54	0.8092	0.6036	0.7088
Isobutyrate	1.87^{b}	2.25^{a}	2.22^{a}	0.15	0.0212	0.1375	0.3203
Butyrate	7.62^{b}	8.24^{ab}	8.39^{a}	0.53	0.0456	0.0289	0.8072
Isovalerianic	2.92	3.20	3.26	0.12	0.0953	0.0423	0.8112
Valerianic	4.61^{A}	3.19^{B}	3.12^{B}	0.31	<0.0001	<0.0001	0.0014

SBM: soybean meal group; HI25 and HI50: Hermetia illucens groups in which the protein by insect meal replaced that of soybean meal by 25 and 50 %, respectively. A, B: $P < 0.01$; a, b: $P < 0.05$; RMSE: root mean square error.

In the Tab. 3.6 the SCFA content is also expressed as percentage of the total SCFA. In this case the isobutyrate has a higher production in the caeca of the hens fed the HI diets compared to the control ($P < 0.05$); the butyrate in the HI50 group was higher ($P < 0.05$) than the control and the valerianic acid in the control was higher ($P < 0.01$) than both the HI groups. The contrast analysis showed a significant linear effect for the butyrate and isolvalerianic acids ($P < 0.05$) and for the valerianic acid ($P < 0.01$). For the valerianic acid also the quadratic effect was significant ($P < 0.01$).

4. Discussion

The evaluation of essential and non-essential element concentrations, in insect meal and diets is necessary from the point of view of both nutrition and contamination. In fact, the trace mineral content of the insect meals is still poor investigated, but it is an important goal to formulate appropriate diets for poultry and avoid an excessive excretion in the environment. In addition, the knowledge on the potential accumulation of toxic elements is mandatory to guarantee the safety of human and animals. A recent study showed that the concentration of many minerals (magnesium, calcium, iodine, iron, sodium and potassium) in black soldier fly larvae linearly increases as the level of the correspondent mineral in the growing substrate increases, while manganese remained stable in the larvae, despite varying concentrations in the media (Liland *et al*., 2017). The data available in literature on the mineral concentration of the black soldier fly larvae showed a very high variability (Liland *et al*., 2017; Cullere *et al*., 2018; Irungu *et al*., 2018) and thus are difficult to compare to our results. The discrepancy of the results is tied to the different kind of substrates used for the insects growth but also, for some element as Zn, the technological process following the larvae harvesting. However, the concentrations of Co, Ni and Se found in edible insect of our trial were lower than those of the conventional food.

Concerning the toxic elements, the concentration of Cd, Pb, and Hg were negligible in all the analyzed samples. The mean value of the As was 0.23 mg/kg in the HI meal, approximatively comparable to the data reported in the literature and lower than the complete diets samples. Considering the trace elements requirements indicated by Hy-Line Brown commercial layers management guide (Hy-line Brown Commercial layers Management Guide, 2018), all the diets used in the present trial showed a higher amount of Zn (minimum required 80 mg/kg in a complete diet during laying period).

Compared to MLs of heavy metals set by EU Commission (Directive (EC) No 2002/32), Cd, Pb, Hg and As levels in the diets and insect meal resulted always lower than the maximum values established

for the feedingstuffs and feed materials. In fact, the EU regulation establishes the following MLs of heavy metals content in mg/kg (ppm) relative to a feed with a moisture content of 12 %: the Cadmium MLs in the feed materials and complete feed are 2.0 and 0.5 mg/kg respectively; the Lead MLs in the feed materials and complete feed are 10.0 and 5.0 mg/kg respectively; the Mercury MLs in the feed materials and complete feed are both 0.1 mg/kg; the Arsenic MLs in the feed materials and complete feed are both 2 mg/kg.

The inclusion of an insect meal from HI larvae as 25 or 50 % substitution of the proteins from the soybean meal had several effects on the small intestine morphometry and enzymatic activity as well as on the caecal microbial activity.

The morphometry changes mainly occurred in the duodenum and jejunum, but also in the ileum there were a few interesting modifications. In general, the villi height decreased as the inclusion level of the insect meal increased in the diet; the crypt depth was unchanged among groups or it tends to decrease (in the jejunum) as the level of the HI increased in the diet. The small intestine is involved in the digestion and absorption of almost all the nutrients of the diet Svihus, (2014): the duodenum digests around 95 % of the fats Sklan, (1975); jejunum digests and absorbs fats, starch and protein (Reisenfeld *et al*., 1980; Sklan and Hurwitz, 1980); the ileum is mainly involved in water and mineral absorption, but it also digests and absorbs fats, protein and starch Svihus, (2014). The morphological studies of the small intestine are often used to asses its functionality and, in general, an increased villi height is indicative of an improved intestinal function (Awad *et al*., 2011). Another important consideration is that the ileal villi in chickens, are smaller and lower than those of the previous tracts of the small intestine, as, feeding corn-soybean based diets, very little amount of nutrients are available beyond the jejunum (Imondi and Bird, 1965; Yamauchi *et al*., 1995; Yamauchi *et al*., 1996). In the present trial, the height of the ileal villi is lower than that of the duodenum but higher compared to the jejunum for hens fed both the insect diets. The effect of the diets on the intestinal villi height can be affected by the nutrient digestibility of the diets. As recorded in the first part of this trial (Bovera *et al.*, 2018) the dry matter digestibility of the hens fed the SBM and the HI25 diets (75.0 and 70.3 % respectively) was higher ($P < 0.01$) than that of the HI50 group (64.3) and the result was mainly attributable to the crude protein digestibility (86.2 vs. 81.1 vs. 76.1 %, respectively for SBM, HI25 and HI50, $P < 0.01$). The low nutrient digestibility in the hens fed the insect meal is tied to the chitin, present in the insect exoskeleton (Bovera *et al*., 2015). Thus, our hypothesis is that a higher availability of nutrients in the duodenum and jejunum of the hens fed the SBM, increased the intestine functionality, improving the villi height. In the hens fed the insect, the lower nutrient digestibility induced an increased amount of the potential digestible nutrients in the ileum. Yamauchi, (2007) stated that an increased load of nutrients deriving directly from the duodenum to the ileum (both for jejunum dissection or different diets) may stimulate the ileal absorptive function, resulting in a compensatory development of the villi. In general, longer villi are, the result of an activated cell mitosis in the crypts (Samanya and Yamauchi, 2001); thus, a larger crypt area indicates a more intensive cell production. In the present trial, the only crypt depth was recorded, and this was unchanged among the groups in the duodenum and jejunum tracts. However, the villi height to crypt depth ratio is strongly related to the epithelial cell turnover (Wang *et al*., 2007). In our trial, the cell turnover was higher in the SBM than in the insect meal groups for duodenum and jejunum, while no differences were observed among the groups in the ileum.

The presence of HI meals in the diet did not affect the activity of both the disaccharases except for the maltase in the duodenum of the hens fed the highest level of HI meal. Recently Khol *et al*. (2017)

showed that the activity of maltase in the small intestine of mallard, chicken and quail, varied depending on the species and, in the mallard, on the intestinal tract. Working with geese they also demonstrated an effect of the interaction with the protein and the fiber content of the diet, with the highest activity of maltase registered in the low protein-low fiber group. Likewise, in the present study a limiting action of the chitin in the HI meal on the availability of starch during the digestion process, that is a lower availability of the substrate to the activity of the maltase.

The linear decrease of IAP in the duodenum and jejunum of the hens as the dietary insect meal inclusion increased, shows that the SBM group presents the highest intestinal functionality. Similar results have also been observed by Cutrignelli *et al*. (2018) when the inclusion of HI larvae meal as 50 % substitution of soybean meal protein in laying hens, decreased the IAP levels in the jejunum and ileum. This enzyme is considered an excellent marker for the crypt–villus differentiation in chicken (Sabatakou *et al*., 2007) and in the present study the inclusion of HI meal resulted in a negative effect in the jejunum on both the villi/crypt ratio and both the IAP specific activity.

The γ-glutamil transpeptidase plays an essential role in the final digestion and absorption of dietary proteins being involved in the amino acid transport in the intestine (Smith *et al*., 1991; Cotgreave and Schuppe-Koistinen, 1994). Overall, the effect of the inclusion of HI meal on the activity of the γ-GT in the ileum seems to be in contrast with the increased ileal villi height, while is in agreement with the weight gain results reported by Bovera *et al*. (2018).

The inclusion of the insect meal in the hens diet induced several modifications in microbial activity in the caeca, as showed by the SCFA production, but the effects are particularly evident with the highest inclusion level (HI50 group). The increased total SCFA production is mainly due to a higher production of butyrate (+21.5 %) and acetate (+13.3 %) than in the SBM group, while the valerianic acid decreased in the two groups fed the insect meal. These results completely agree with the finding of Cutrignelli *et al*. (2018), in which the hens fed a meal from HI in total replacement of soybean proteins, showed an increased production of butyrate (+ 62.6 %) and acetate (+ 36.1 %) than the control. Similarly to our results, Loponte *et al*. (2018) found an increased amount of the total VFA (+45.6%), acetate (+40.3%) and butyrate (+64.6%) in broilers fed a *Tenebrio molitor* larvae meal as a complete replacement of the soybean meal. The increased activity of the microbial population in the caeca can be related to the chitin level of the HI diet, confirming the hypothesis of Loponte *et al*. (2018) and Cutrignelli *et al*. (2018). However, another important point emerging from our research is that the chitin needs to be at a sufficient level to act as "prebiotic", stimulating the intestinal microbial activity. Based on our analysis and taking into account the formula proposed by Marono *etal*. (2015) for the estimation of the insect chitin from the chemical composition, the amount of the chitin in the *H. illucens* larvae meal used in this trial was 6.64 % as feed. The feed intake of the hensinvolved in this trial and reported by Bovera *et al*. (2018), was 99.97, 97.69 and 101.9 g/d,respectively for the SBM, HI25 and HI50 groups. Thus, considering the inclusion level in the diets, the HI25 group ingested around 0.47 g/d of chitin, while the HI50 group ingested around 0.99 g/d. Our hypothesis is that the lowest level of chitin is not adequate to modulate the activity of microbialpopulation in the hens. The butyric acid is considered the main enterocytes energy source (Bovera *etal*., 2010) and it is also necessary for a proper development of the Gut-Associated Lymphoid TissueMroz, (2005). It is reported that the volatile fatty acids, in general, have a bacteriostatic effect againstsome enteric bacteria, including Salmonella typhimurium and in particular the butyrate is related to the decreased amounts of Enterobacteriaceae in chickens (van Der Wielen *et al*., 2000). Thus, both

the increases of the total SCFA and butyric acid can improve, through different mechanisms, the health of the of hens intestine.
Very interesting, the significant changes in the mutual proportions of the butyric, isobutyric and valerianic acid which were observed, this may indicate that not only the activity, but also the interactions among the different microbial species has been modified. This is in accordance with the findings of Borrelli *et al*., (2017), who observed changes in the gut microbiota of the hens fed HI larvae meal.

5. Conclusions

The inclusion of an insect meal from HI larvae as 25 or 50 % substitution of the proteins from the soybean meal influenced the small intestine morphometry and enzymatic activity as well as the caecal microbial activity. The SBM group showed the highest intestinal functionality, while some compensatory mechanism probably mediated by the chitin led to positive increase of SCFA and butyrate in the HM50 diet with potential positive effects on gut healthiness. However, considering the results of Bovera *et al*. (2018) to which these results are strongly linked, it is possible to conclude that protein replacement at 25% with insect meal from *Hermetia illucens* larvae in the diet of laying hens is more suitable and closer to the optimal level than replacement at 50%. Finally, the levels of Cd, Pb, Hg and As in the diets and insect meal resulted always lower compared to MLs of heavy metals set by EU Commission for the feedingstuffs and feed materials. This latter aspect provides important information on the safe use of these alternative ingredients in animal feeding.

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CHAPTER 4

In vivo performances, ileal digestibility, and physico-chemical characterisation of raw and boiled eggs as affected by *Tenebrio molitor* larvae meal at low inclusion rate in laying quail (*Coturnix japonica*) diet.

1. Introduction

The poultry sector grew from 9 to 122 Mt in the last five decades (1961-2017) and a further increment is expected (FAO, 2020) despite the overall stagnation of livestock productions. The success of poultry meat is mainly due to its high nutritional value, moderate costs, and because of its consumption not to be limited by religion. Among the "alternative" species, quail (*Coturnix coturnix*) represents approximately 12% of birds involved in meat and egg productions, the second largest one after chickens (Lukanov, 2019). Quail eggs are mainly produced in Asia, being China the largest producer with around 4.64 million tonnes laid in 2019, corresponding to more than 70% of the global production, followed by Thailand, Indonesia and Brazil. Europe, whose quail farming is mainly based on meat production, stands among the top 10 producers only with Russia that provides the 0.39% of the global production (FAOSTAT, 2021). Quail egg production increased its volume during the decade 2009-2019, passing from 5.2 to 6.04 million tonnes with a global export value of 702.96 USD/metric ton (FAOSTAT, 2021). As part of the poultry sector, even laying quails activities need to be fast orientated towards more sustainable horizons, in order to reduce its environmental impact: the livestock sector has been estimated to exceed 5.4 billion tons of CO_2-eq, equal to 14.5% of the total anthropogenic greenhouse gas emissions (Grossi *et al.*, 2019) and the poultry industry is responsible for the production of 0.1 giga ton of CO_2-eq (in comparison, dairy cattle provide 1 G ton). As recently underlined, feed production and processing are hot spots for the livestock sector, whose contribute in terms of greenhouse gas emissions has been esteemed at 45% of the overall impact (Grossi *et al*., 2019). In addition, the most common ingredients for poultry diets, as maize and soybean meal, are harshly criticised due to the water and broad spaces exploitations, as well as their competition with human consumption that is a product suggested to be integrated while evaluating the environmental impact (Costantini *et al*., 2021).

Despite the Food and Agriculture Organisation has endorsed edible insects in human diet, people from Western cultures still do not promote entomophagy but they can accept insect derived products as animal feeds. The increasing interest in insect meal as alternative protein source in poultry sector has been demonstrated both by the escalation of published works in the last decade and the expected approval of its use by the European Commission. The EU Regulation 2017/893 admitted the use of *Tenebrio molitor* (TM), however, to date, only the aquaculture sector can benefit of insects as ingredient for feed. However, very few studies specifically considered the use of insect meals in laying quails and they are divided into black soldier fly (Dalle Zotte *et al*., 2019), *Tenebrio molitor* (Shariat Zadeh *et al*., 2020) or other local insect species (Das and Mandal, 2014). The preliminary evaluation of Shariat Zadeh *et al*. (2020) found a positive correlation between the inclusion of TM larvae meal, the egg productive performance and the egg-related indices. Graded substitution level of fishmeal with TM (included at 15 and 22.5% as fed) significantly lowered the feed conversion ratio

(FCR) values, but egg quality traits such as yolk height and shell weight were significantly reduced. In general, Shariat Zadeh *et al*. (2020) highlighted the safety of using TM meal for quails' health, however additional data are necessary to evaluate the possibility to include TM in the diet of laying quails. Indeed, other studies aimed to substitute the conventional protein sources including the insect meal (TM larvae) in feeds for laying hens at 1, 2 and 3% (Ko *et al*., 2020) or 2.5 and 5% (Sedgh-Gooya *et al*., 2021) finding that the TM larva inclusions were effective in improving the FCR, but the authors suggest to limit the inclusion of TM at the 3% to avoid modification in blood parameters of laying hens and in egg quality characteristics.

To test if *T. molitor* larvae meal could represent a potential ingredient in laying quail diet and if its inclusion has similar effects than in laying hens, the present trial aimed to study the effect of low inclusion levels of a *T. molitor* defatted larvae meal on laying performance and egg physical and chemical characteristics of quails. Moreover, considering that in some countries, such as Japan, the market volume of boiled quail egg, generally preserved in cans, amounted to more than 1000 tons in 2019 (www.statista.com), a cooking trial was assessed to evaluate the boiled-egg quality.

2. Materials and Methods

2.1 Growing trial, egg collection, apparent ileal digestibility

All the animals were humanely treated according to the principles of the animal welfare stated by the Directive 63/2010/EEC regarding the protection of the animals used for experimental and other scientific purposes. The experimental procedures were approved by the Ethical Animal Care and Use Committee of the Department of Veterinary Medicine and Animal Production of the University of Napoli Federico II, Italy (prot. N. 2017/0017676). The trial was carried out in a commercial quail farm located in the province of Sassari (Sardinia, Italy).

A total of 120, 12-weeks-old female Japanese quails (*Coturnix japonica*, average weight 299.85 ± 15.87 g,) were equally divided into 4 groups (30 birds per groups, each containing 6 replicates of 5 birds). Each replicate was housed in galvanized metal cages (100 × 50 × 25 cm high) equipped with three feeding points and three nipples, providing feed and drinking water *ad libitum*, respectively. The cages were located in an environmental controlled room at 23.8 ± 0.7 °C temperature and 58.5 ± 5.7% relative humidity, under 16:8 h dark: light lighting regimen. The groups were submitted to different dietary treatments. The control group (CON) fed a corn-soybean diet formulated to meet or exceed the nutritional requirements of the birds, according to NRC (1994) and Arif *et al*., (2010). For the other 3 groups, indicated as T5, T10 and T20, an aliquot of soybean, respectively equal to 2.4, 4.1 and 9.6% (corresponding to 1.05, 1.80 and 4.20% of crude protein and around to 5, 10 and 20% of the protein of the control diet) was replaced with the protein from defatted *Tenebrio molitor* larvae meal (TML), respectively. The inclusion level of the TML was 1.4, 2.8 and 5.6% for T5, T10and T20 groups, respectively (corresponding to 0.96, 1.93 and 3.96% of crude protein). The defattedinsect meal was purchased at the ENTOMO Farm Company (Libourne, France). To measure nutrient digestibility, an indigestible marker (Celite®, Sigma-Aldrich, St. Louis, MO, USA) was added to the dosage of 5 g/kg during the last 10 days of the trial to each diet at the expense of corn.

Samples of the two main protein sources (soybean meal and TML) and of the diets were analysed for chemical-nutritional characteristics. The chemical composition (dry matter, ash, crude protein, ether extract and fibre fractions) was determined according to the AOAC (2005) methods. For the only

insect meal, the nitrogen-to-crude protein conversion factor was 4.97, according to Jansen et al. (2017). The acid detergent fibre (ADF) and the residual nitrogen in ADF (N-ADF; AOAC, 2005) were determined and used to estimate the amount of chitin according to Marono *et al.* (2015). The levels of calcium, phosphorous and amino acids as methionine, lysine and cysteine were determined according to Addeo *et al*. (2021). The metabolizable energy content of the diets was calculated from their chemical composition according to NRC (1994) equations; the apparent metabolizable energy for insect meals used in the present trial was calculated using the apparent digestibility coefficients of the total tract (CTTAD), as measured by De Marco *et al*. (2015). The chemical-nutritional characteristics of the protein sources and the ingredients and chemical-nutritional characteristics of the diets are reported in the Tab. 4.1 and 4.2, respectively. The fatty acid profile of the diets (Tab. 4.3) was determined according to the method described below for the eggs.

Tab 4. 1. Analysed nutrient composition (% as fed) of the soybean and Tenebrio molitor larvae meals used in the trial.

	Soybean meal	***Tenebrio molitor* larvae meal**
Dry matter	90.21	94.4
Ash	5.97	3.21
Crude protein	43.9	68.9
Ether extract	1.15	7.50
ADF[1]	5.47	6.97
N-ADF[2]	11.5	2.46
Chitin	-	4.51
Ca	0.30	1.97
P	0.63	1.25
Methionine, % CP[3]	0.59	1.35
Lysine, % CP	2.87	4.69

[1]ADF: acid detergent fibre. [2]N-ADF: nitrogen linked to ADF. [3]CP: crude protein.

Tab. 4.2. Ingredients and chemical-nutritional characteristics of the diets used in the trial.

Diet[1]	**CON**	**T5**	**T10**	**T20**
Ingredients, %				
Corn meal	52.1	53.7	55.0	57.0
Soybean meal 44%	34	31.6	29.2	24.4
Insect meal	-	1.4	2.8	5.6
Vegetable oil	6	5.5	5.3	5.3
Calcium carbonate	5.5	5.4	5.3	5.3
Dicalcium phosphate	1.5	1.5	1.5	1.5
Mineral Vitamin premix[2]	0.5	0.5	0.5	0.5
Methionine	0.1	0.1	0.1	0.1
Salt	0.3	0.3	0.3	0.3
Chemical composition				
Dry matter, %	90.48	90.77	90.55	90.42
Ash, % DM[3]	12.01	11.45	12.38	11.88
Crude protein, % DM	21.74	22.10	22.48	22.02
Ether extract, % DM	8.46	7.75	8.14	8.36
Ca, % DM	2.53	2.52	2.52	2.53
AvP[4], % DM	0.36	0.35	0.38	0.39
Crude fibre, % DM	9.94	8.16	9.12	7.23
NDF[5], % DM	9.68	11.16	10.45	9.73
ADF[6], % DM	6.26	6.58	6.07	6.91
ADL[7], % DM	2.51	2.77	2.88	3.01
Methionine+Cysteine, % DM	0.83	0.86	0.86	0.88
Lysine, % DM	1.16	1.22	1.24	1.28
ME, Kcal/kg[8]	2,890	2,890	2,890	2,891

[1] T5, T10, T20: diets in which 5, 10 and 20% of the protein of the control diet (CON) have been replaced with the protein from defatted *Tenebrio molitor* larvae meal. [2] Provides per kg of product: Fe, 50,000 mg; Co, 200 mg; Cu, 8,500 mg; Mn, 75,000 mg; Zn, 70,000 mg; Se, 250 mg; I, 1,500 mg; folic acid, 500 mg; pantothenic acid, 13.5 g; niacin, 30 g; vit. A, 10,000,000 IU; cholecalciferol 50,000 µg; vit. K3, 4,000 mg; vit. B2, 5,000 mg; vit. B6, 2,000 mg; vit., B12 10,000 µg; vit. E (dl-α-tocopheryl acetate) 21,978 IU. Celite® was added at the dosage of 5 g/kg during the last 10 days of the trial to each diet at the expense of corn meal. [3] DM: dry matter. [4] AvP: available phosphorous. [5] NDF: neutral detergent fibre. [6] ADF: acid detergent fibre. [7] ADL: acid detergent lignin. [8] ME: metabolizable energy, calculated value.

Tab. 4.3 Fatty acid profile of the administered diets (g/100 g total fatty acid methyl esters).

Diet[1]	**CON**	**T5**	**T10**	**T20**
C12:0	0.02	0.02	0.03	0.02
C14:0	0.09	0.09	0.13	0.14
C15:0	0.04	0.02	0.03	0.02
C16:0	22.38	15.56	16.01	14.39
C16:1n-9	0.09	0.08	0.11	0.08
C16:1n-7	0.21	0.17	0.32	0.20
C17:0	0.14	0.09	0.10	0.10
C18:0	3.82	2.73	3.05	2.60
C18:1n-9	40.61	34.19	33.28	31.00
C18:1n-7	0.98	0.79	0.80	0.73
C18:2n-6	27.63	43.10	43.27	46.87
C18:3n-3	0.68	1.03	1.02	1.12
C20:0	0.67	0.48	0.45	0.43
C20:1n-11	0.28	0.12	0.09	0.11
C20:1n-9	0.76	0.38	0.32	0.32
C20:4n-6	0.02	0.00	0.11	0.010
C20:5n-3	0.11	0.10	0.09	0.06
C22:0	0.42	0.21	0.21	0.23
C22:1n-11	0.30	0.30	0.17	0.20
C22:1n-7	0.32	0.26	0.16	0.20
C24:0	0.43	0.26	0.27	0.20
ΣSFA[2]	*27.60*	*19.24*	*20.06*	*17.89*
ΣMUFA[3]	*43.55*	*36.29*	*35.24*	*33.83*
ΣPUFA[4]*n-6*	*27.65*	*43.10*	*43.38*	*46.87*
ΣPUFAn-3	*0.79*	*1.16*	*1.11*	*1.18*

[1]T5, T10, T20: diets in which 5, 10 and 20% of the protein of the control diet (CON) have been replaced with the protein from defatted *Tenebrio molitor* larvae meal. [2]SFA: saturated fatty acids. [3]MUFA: mono-unsaturated fatty acids. [4]PUFA: poly-unsaturated fatty acids.

The trial was carried out for 54 days, from 5th May to 28th June 2019. The live weight and the feed intakes of the quails were recorded weekly per replicate. The amount of the administered feed and that of the leftover feed were measured daily to calculate the birds feed intake. The number of eggs produced, and the individual egg weights were recorded per replicate every week. Per each replicate of each group, the egg mass was calculated by multiplying the egg weight by the egg production

percentage and the feed conversion ratio (FCR) was calculated as the amount of feed intake per day divided by the amount of egg mass per day.
Overall, 288 eggs were collected during the 6 weeks of the trial (2 eggs per replicate, 12 eggs per group each week), they were weighed and stored at – 80 °C until analyses (see section *Physico-chemical analyses of raw and boiled eggs*).
At the end of the trial, the quails were slaughtered in a specialized slaughterhouse. After having measured the intestinal length, the ileum was separated from the Meckel's diverticulum to the ileocecal junction avoiding contamination of other intestinal contents and the digesta were pooled per replicate, immediately frozen and subsequently freeze-dried. The dried ileal digesta were ground to pass a 1-mm sieve and stored at –20 °C until chemical analysis (AOAC, 2005). The amount of acid insoluble ash (AIA) in the diets and in the ileal contents of the quails was measured according to Vogtmann *et al.* (1975). The apparent ileal digestibility of nutrients (dry matter, organic matter, crude protein, ether extract and calcium) was calculated as it follows: 100 – 100 × [(% AIA in the diet/% AIA in the ileal content) × (% nutrient in the ileal content/% nutrient in the diet)].

2.2 Physico-chemical analyses of raw and boiled eggs

The eggs of each group were randomly allotted to be analysed as raw or boiled. The physical characterization of raw eggs was conducted on 36 eggs per group which were thawed for 2 h at room temperature before being analysed. Firstly, the egg circumferences were evaluated with a measuring tape, then each egg was carefully broken to separate and weigh (PB503-S/fact, Mettler Toledo, Columbus, OH, USA) its components: eggshell, albumen and yolk. The eggshells thickness, including the testaceous membranes, was measured with a manual calliper (Salmoiraghi, Milan, Italy) in three points (equator, round and apex). The pH of both the albumens and yolks was measured (SevenGo pH meter, Mettler-Toledo, Columbus, OH, USA), while the yolk colour values expressed as lightness (L^*), redness index (a^*) and yellowness index (b^*) (CIE, 2004) were registered through a Chroma Meter CR-200 (Konica Minolta, Chiyoda, Japan). Once ended the physical analyses, the yolks were pooled within the collection week (n = 6) and lyophilized before the chemical characterisation.
The eggs allotted to the cooking trial (n = 36 per group) were peeled still frozen and inserted into silicone egg cooker cups (OmzgxGod, purchased on Amazon, Italy) prior to be immersed in boiling water for 9 minutes to obtain hard-boiled eggs. The colour values of both boiled albumens and yolks were evaluated using Chroma Meter CR-200 (Konica Minolta, Chiyoda, Japan). Then, the entire boiled eggs of each dietary group were pooled within the collection week (n = 6) and lyophilized for further analyses.
The water content of the pooled raw yolks was calculated by weighing the samples before and after lyophilization. The total lipids were extracted (Folch *et al*., 1957) from the experimental diets, the raw yolks and the whole boiled egg, then an aliquot of each extract, containing 400 mg of total lipids, was methylated according to Christie (1982). The fatty acid methyl esters (FAME) were chromatographically analysed for the fatty acid (FA) profile by a Varian GC 430 gas chromatograph (Varian Inc., Palo Alto, CA, USA) equipped with a capillary column Supelco Omegawax™ 320 (30 m × 0.32 mm × 0.25 μm; Supelco, Bellefonte, PA, USA). The injector was set at 220 °C, while the oven temperature was programmed to start at 100 °C, reaching 170 °C with 10 °C/min increment

(isotherm 6 min), then rising to 200 °C with 4 °C/min (isotherm 5 min). A Flame Ionisation Detector (FID) set at 300 °C was used. Helium was the carrier gas and flowed at 1.5 mL/min. The relative abundance of each single FA on the overall FAME content (g FA/100 g total FAME) was obtained by using tricosanoic acid (C23:0) (Supelco, Bellefonte, PA, USA) as internal standard and external calibration curves (standard Supelco 37 component FAME mix; Supelco, Bellefonte, PA, USA). The estimation of Δ9, Δ5 and Δ6 desaturase activity was obtained calculating the ratio between the product and the corresponding precursors, as reported by Mattioli *et al*. (2018). The following equations were used:

$$\Delta 9 \text{ desaturase } (16) = \frac{\text{C16: } 1}{\text{C16: } 1 + \text{C16}} \times 100$$

$$\Delta 9 \; desaturase \; (18) = \frac{C18{:}\,1}{C18{:}\,1 + C18} \times 100$$

$$\Delta 5 + \Delta 6 \; desaturase \; (n-6) = \frac{C20{:}\,2n6 + C20{:}\,4n6}{C18{:}\,2n6 + C20{:}\,2n6 + C20{:}\,4n6} \times 100$$

$$\Delta 5 + \Delta 6 \; desaturase \; (n-3) = \frac{C20{:}\,5n3 + C22{:}\,5n3 + C22{:}\,6n3}{C18{:}\,3n3 + C20{:}\,5n3 + C22{:}\,5n3 + C22{:}\,6n3} \times 100$$

The egg oxidative status was assessed quantifying spectrophotometrically conjugated dienes (Srinivasan *et al*., 1996) and thiobarbituric acid reactive substances (TBARS; Vyncke, 1970). The results are expressed as mmol hydroperoxides (mmol Hp) and malondialdehyde equivalents (MDA-eq.**)** on 100 g of fresh sample, respectively.

2.3 Statistical analysis

The data were processed by a one-way ANOVA, using the PROC GLM of SAS (2000) according to the following model:

$$Yij = m + PSi + eij$$

where Y is the single observation, m the general mean, PS the effect of the protein source (i = Control *vs*. *Tenebrio molitor*), e the error. The experimental unit was the replicate. The orthogonal contrast analysis was performed to test the linear, quadratic and cubic effects among the means (SAS, 2000).

3. Results

The tab. 4.4 shows the weights recorded at 12, 14, 16, 18 and 20 weeks of age together with weight changes and the *in vivo* performance of the quails according to the dietary treatments. No differences have been recorded for the weights; however, the contrast analysis shows a significant effect ($P < 0.05$) of the quadratic contrast indicating that low and intermediate inclusion levels of TML induced higher changes in the final weight compared to the other groups (+0.54 and +0.33 for T5 and T10, respectively). The feed intake was unaffected by dietary treatments. The laying rate and egg mass

linearly decreased (P < 0.01) as the TML inclusion level in the diet increased. The egg weight increased from the control to the T20 diet (linear contrast P < 0.01). The FCR linearly increased from the control to the T20 group (P < 0.01).

Tab. 4.4. Weight changes and in vivo performance of quails (n = 24), egg weight and egg mass (n = 72) of quails fed control or Tenebrio molitor diets from 12 to 20 weeks of age.

Contrast P values **Diet**[1]	**CON**	**T5**	**T10**	**T20**	**RMSE**[3]			
						Linear	**Quadratic**	**Cubic**
Initial weight (12 wk), g	302.0	298.5	290.8	308.1	10.48	0.423	0.082	0.992
Weight at 14 wk, g	301.9	297.5	287.3	307.2	9.87	0.429	0.165	0.875
Weight at 16 wk, g	297.2	293.2	292.4	304.7	8.67	0.365	0.141	0.668
Weight at 18 wk, g	307.6	307.6	306.5	312.7	10.11	0.372	0.831	0.348
Final weight (20 wk), g	302.3	328.7	309.2	314.3	9.88	0.333	0.044	0.152
Δ weight, g/d	+0.01	+0.54	+0.33	+0.10	0.31	0.940	0.348	0.388
Feed intake	35.06	35.86	35.28	35.83	0.56	0.273	0.726	0.111
FCR[2]	2.92	3.06	3.38	3.63	0.05	0.001	0.313	0.119
Laying, %	93.01	92.42	80.17	72.42	2.12	<0.001	0.219	0.318
Egg weight, g	12.89	12.68	12.98	13.63	0.24	0.004	0.014	0.083
Egg mass	11.99	11.73	10.42	9.87	0.36	<0.001	0.540	0.083

[1]T5, T10, T20: diets in which 5, 10 and 20% of the protein of the control diet (CON) have been replaced with the protein from defatted *Tenebrio molitor* larvae meal. [2] FCR: feed conversion ratio. [3] RMSE: root mean square error.

The coefficients of the apparent ileal digestibility for dry matter, organic matter, crude protein, ether extract and calcium are summarized in the Tab. 4.5. The digestibility of dry matter, organic matter and crude protein linearly decreased (P < 0.05) as the TML inclusion level in the diet increased. No effects of the dietary treatments were detected for the digestibility of ether extract and calcium.

Tab. 4.5. Coefficients of the apparent ileal digestibility of the nutrients (%) of quails (n = 24) fed control or Tenebrio molitor diets at 20 weeks of age.

Diet[1]	**CON**	**T5**	**T10**	**T20**	**RMSE**[2]	**Contrast P values**		
						Linear	**Quadratic**	**Cubic**
Dry matter	77.4	77.6	76.6	75.4	6.54	0.042	0.337	0.426
Organic matter	79.8	80.1	78.7	77.5	7.11	0.031	0.542	0.559
Crude protein	78.9	77.5	74.7	72.3	7.56	0.013	0.236	0.396
Ether extract	90.1	90.5	89.9	90.3	8.33	0.579	0.665	0.632
Ca	79.2	79.5	79.1	79.7	9.12	0.554	0.570	0.688

[1]T5, T10, T20: diets in which 5, 10 and 20% of the protein of the control diet (CON) have been replaced with the protein from defatted *Tenebrio molitor* larvae meal. [2]RMSE: root mean square error.

In relation to the physical and chemical traits of raw eggs (Tab. 4.6), the egg circumference tended to increase ($P < 0.01$) as the level of TML increased; however, the eggs from T5 group had lower circumference than CON (quadratic contrast, $P < 0.01$). The albumen weight showed a linear increasing trend when expressed both in absolute ($P < 0.01$) or relative value ($P < 0.05$). The yolk weight had a linear increase ($P < 0.01$) only when expressed in grams. Conversely, the eggshell weight linearly decreased ($P < 0.05$) when expressed as percentage of the whole egg. A significant effect of the cubic contrast ($P < 0.01$) has been detected for the yolk percentage and pH value. The yolk chemical characteristics (moisture and total lipid content) and oxidative status of the raw eggs were scarcely affected by the dietary treatments. Indeed, among the analysed parameters, only the conjugated dienes showed a significant cubic contrast ($P < 0.05$).

Tab. 4.6. Raw egg quality physical (n = 36) and chemical (n = 6 pools) traits according to quails fed control or Tenebrio molitor diets from 12 to 20 weeks of age.

Diet[1]	**CON**	**T5**	**T10**	**T20**	**RMSE**[4]	**Contrast P values**		
						Linear	**Quadratic**	**Cubic**
Egg circumference, cm	8.27	8.20	8.31	8.49	0.27	0.000	0.007	0.539
Shell thickness, µm	31.77	31.88	28.67	29.52	5.48	0.059	0.752	0.169
Albumen weight, g	5.48	5.34	6.05	6.30	0.97	<0.001	0.240	0.078
Albumen, %	45.47	45.80	47.64	47.89	5.17	0.02	0.961	0.428
Yolk weight, g	4.03	4.01	4.14	4.40	0.44	<0.001	0.071	0.942
Yolk, %	33.51	34.68	32.83	33.72	2.69	0.550	0.756	0.001
Eggshell weight, g	2.51	2.24	2.45	2.39	0.48	0.681	0.198	0.069
Eggshell, %	21.06	19.51	19.52	18.39	4.17	0.015	0.794	0.395
pH albumen	8.75	8.84	8.74	8.72	0.23	0.231	0.178	0.164
pH yolk	6.49	6.61	6.45	6.59	0.26	0.501	0.755	0.006
*L** yolk	50.67	50.71	50.63	49.79	15.17	0.815	0.864	0.955
*a** yolk	5.63	4.99	5.63	5.82	1.54	0.293	0.115	0.139
*b** yolk	23.15	22.62	23.52	23.64	3.78	0.416	0.610	0.431
Moisture, g/100 g yolk	49.33	50.01	50.60	49.74	2.86	0.741	0.492	0.835
Total lipids, g/100 g yolk	26.40	26.10	25.14	26.28	2.01	0.720	0.390	0.457
CD[2], mmol Hp/kg yolk	0.40	0.43	0.36	0.37	0.05	0.087	0.504	0.039
TBARS[3], mg MDA-eq/kgyolk	0.031	0.002	0.003	0.005	0.02	0.281	0.125	0.913

[1]T5, T10, T20: diets in which 5, 10 and 20% of the protein of the control diet (CON) have been replaced with the protein from defatted *Tenebrio molitor* larvae meal. [2]CD: conjugated dienes. [3]TBARS: thiobarbituric acid reactive substances. [4]RMSE: root mean square error.

The physical and chemical characteristics of the boiled eggs (Tab.4.7) highlighted that lightness (*L**) of the yolk showed higher values in T5 and T10 groups (quadratic contrast, $P < 0.01$). The green-red index (*a**) of the yolk showed lower values in T5 and T20 groups (cubic contrast, $P < 0.01$). The *L**, *a** and *b** indexes of the albumen revealed a significant effect of the quadratic contrast ($P < 0.05$). In addition, *b** index of the albumen also showed a significant effect ($P < 0.01$) of the cubic contrast. The total lipids tended to increase with the *Tenebrio molitor* larvae meal inclusion in the diets (cubic contrast, $P < 0.05$), having the T10 and T20 eggs the highest values.

Tab. 4.7. Boiled eggs quality physical (n = 36) and chemical (n = 6 pools) traits according to quails fed control or Tenebrio molitor diets from 12 to 20 weeks of age.

Diet[1]	**CON**	**T5**	**T10**	**T20**	**RMSE**[4]	**Contrast P values**		
						Linear	**Quadratic**	**Cubic**
Yolk colour parameters								
*L**	62.39	65.79	64.79	62.37	6.06	0.823	0.006	0.518
*a**	4.60	3.05	4.85	4.07	2.35	0.916	0.346	0.001
*b**	28.37	27.42	29.56	29.05	5.47	0.332	0.818	0.171
Albumen colour parameters								
*L**	84.05	86.69	85.66	82.22	6.78	0.222	0.011	0.807
*a**	-5.11	-5.76	-5.46	-5.18	1.17	0.916	0.022	0.285
*b**	7.14	9.89	6.82	7.30	2.77	0.237	0.019	<0.001
Total lipids, g/100 g	10.99	11.59	10.85	12.03	0.67	0.069	0.303	0.015
CD[2], mmol Hp/kg	0.16	0.16	0.15	0.17	0.01	0.136	0.245	0.068
TBARS[3], mg MDA-eq./kg	0.040	0.032	0.024	0.022	0.02	0.058	0.678	0.904

[1]T5, T10, T20: diets in which 5, 10 and 20 % of the protein of the control diet (CON) have been replaced with the protein from defatted *Tenebrio molitor* larvae meal. [2]CD: conjugated dienes. [3]TBARS: thiobarbituric acid reactive substances. [4]RMSE: root mean square error.

The fatty acid profile of the raw yolks is shown in the Table 8. Several fatty acids showed a linear increase (C14:0, C14:1n-5, C16:1n-7, C18:1n-7, C18:3n-6, C20:0, C20:4n-6, C22:4n-6, C22:5n-6) or a linear decrease (C15:0, C18:2n-6, C18:3n-3) according to the increase of TML percentage in the diets. For C20:2n-6, C20:3n-6, C22:4n-6 and C22:5n-6 a significant effect ($P < 0.05$) of the quadratic contrast has been detected, while a significant cubic effect was recorded for C16:0, C20:3n-6 and C22:1n-9 fatty acids. However, no effects of the dietary treatments were detected for the total saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA) and poly-unsaturated fatty acids (PUFA). A linear increase ($P < 0.05$) of the estimated activity of Δ9-desaturase (C16:0), Δ5+ Δ6-desaturase on both PUFAn-6 and PUFAn-3 was calculated as affected by TML inclusion in the quails' diet.

Tab. 4.8. Fatty acid profile (g/100 g of fatty acid methyl esters) of the raw eggs (n = 6 pools) according to quails fed control or Tenebrio molitor diets from 12 to 20 weeks of age.

Diet[1]	**CON**	**T5**	**T10**	**T20**	**RMSE**[5]	**Contrast P values**		
						Linear	**Quadratic**	**Cubic**
C12:0	0.06	0.01	0.07	0.01	0.0047	0.793	0.444	0.139
C14:0	0.38	0.39	0.41	0.42	0.025	0.013	0.861	0.906
C14:1n-9	0.05	0.05	0.06	0.06	0.008	0.003	0.555	0.103
C15:0	0.05	0.05	0.04	0.04	0.0045	0.000	0.060	0.533
C16:0	25.73	25.94	26.27	26.06	0.68	0.301	0.481	0.006
C16:1n-9	0.78	0.73	0.67	0.73	0.2059	0.158	0.122	0.623
C16:1n-7	3.20	3.40	3.81	3.64	0.23	0.001	0.075	0.082
C17:0	0.14	0.14	0.13	0.13	0.012	0.142	0.553	0.286
C18:0	8.87	9.23	8.99	8.81	0.23	0.336	0.011	0.136
C18:1n-9	36.65	36.24	36.69	36.56	0.52	0.858	0.527	0.140
C18:1n-7	1.56	1.55	1.69	1.67	0.107	0.026	0.880	0.010
C18:2n-6	18.18	17.81	16.58	17.20	0.87	0.016	0.182	0.105
C18:3n-6	0.28	0.29	0.31	0.30	0.02	0.050	0.226	0.167
C18:3n-3	0.23	0.21	0.19	0.20	0.24	0.037	0.281	0.424
C20:0	0.02	0.03	0.03	0.03	0.004	0.007	0.690	0.074
C20:1n-11	0.04	0.03	0.04	0.04	0.006	0.218	0.390	0.502
C20:1n-9	0.12	0.12	0.12	0.13	0.008	0.224	0.114	0.904
C20:2n-6	0.09	0.08	0.08	0.09	0.067	0.697	0.013	0.452
C20:3n-6	0.15	0.13	0.18	0.16	0.033	0.163	0.911	0.026
C20:4n-6	2.38	2.51	2.60	2.56	0.072	<0.001	0.010	0.538
C20:5n-3	0.01	0.01	0.01	0.01	0.005	0.119	0.143	0.454
C22:0	0.01	0.01	0.01	0.02	0.005	0.054	0.142	0.602
C22:1n-9	0.01	0.01	0.01	0.01	0.005	0.961	0.932	0.023
C22:4n-6	0.12	0.12	0.12	0.14	0.010	0.005	0.036	0.082
C22:5n-6	0.38	0.36	0.38	0.43	0.034	0.001	0.024	0.616
C22:5n-3	0.07	0.06	0.06	0.07	0.009	0.690	0.311	0.801
C22:6n-3	0.49	0.50	0.46	0.48	0.063	0.733	0.824	0.421
ΣSFA[2]	*35.21*	*35.77*	*35.88*	*35.50*	*0.7669*	*0.486*	*0.147*	*0.988*

ΣMUFA[3]	42.42	42.14	43.12	42.84	0.739	0.110	0.998	0.075
ΣPUFA[4]n-6	21.58	21.30	20.26	20.88	0.9137	0.074	0.241	0.161
ΣPUFAn-3	0.782	0.776	0.728	0.759	0.083	0.445	0.606	0.440
Δ9-desaturase (C16)	11.05	11.58	12.67	12.26	0.67	0.001	0.098	0.107
Δ9-desaturase (C18)	80.51	79.70	80.31	80.57	0.50	0.391	0.018	0.067
Δ5+Δ6-desaturase (n-6)	11.96	12.70	13.90	13.39	0.58	<0.001	0.015	0.052
Δ5+Δ6-desaturase (n-3)	71.00	72.81	73.72	73.67	2.13	0.033	0.295	0.989

[1]T5, T10, T20: diets in which 5, 10 and 20% of the protein of the control diet (CON) have been replaced with the protein from defatted *Tenebrio molitor* larvae meal. [2]SFA: saturated fatty acids. [3]MUFA: mono-unsaturated fatty acids. [4]PUFA: poly-unsaturated fatty acids. [5]RMSE: root mean square error.

The fatty acid profile of the boiled eggs according to the dietary treatments is reported in the Tab. 4.9. C12:0, C14:1n-5, C18:0 and C18:3n-3 linearly decreased as the TML percentage in the diets increased, whilst C18:1n-9, C18:1n-7, C20:4n-6 and C22:0 showed the opposite trend. A significant quadratic effect of the contrasts has been detected for C22:0 ($P < 0.5$), C22:4n-6 ($P < 0.01$) and C22:5n-6 ($P < 0.05$) and a significant effect ($P < 0.05$) of the cubic contrast for C18:1n-9 and C20:2n-6 fatty acids. The total MUFA linearly increased ($P < 0.05$) according to the increase of TML in the diets.

Tab. 4.9. Fatty acid profile (g/100 g of fatty acid methyl esters) of the boiled eggs (n = 6 pools) according to quails fed control or Tenebrio molitor diets from 12 to 20 weeks of age.

Diet[1]	**CON**	**T5**	**T10**	**T20**	**RMSE[5]**	**Contrast P values**		
						Linear	**Quadratic**	**Cubic**
C12:0	0.01	0.01	0.01	-	0.003	0.007	0.768	0.225
C14:0	0.40	0.40	0.40	0.42	0.031	0.369	0.474	0.788
C14:1n-9	0.06	0.05	0.06	0.06	0.0004	0.253	0.466	0.469
C15:0	0.05	0.04	0.04	0.04	0.005	0.008	0.858	0.856
C16:0	26.26	26.16	26.35	26.28	0.7366	0.857	0.978	0.682
C16:1n-9	0.76	0.76	0.76	0.72	0.1033	0.487	0.567	0.781
C16:1n-7	3.45	3.26	3.93	3.81	0.6001	0.126	0.878	0.150
C17:0	0.14	0.14	0.13	0.12	0.015	0.118	0.269	0.444
C18:0	9.02	9.25	8.80	8.72	0.274	0.012	0.176	0.048
C18:1n-9	36.61	36.23	36.94	37.12	0.580	0.046	0.261	0.140
C18:1n-7	1.58	1.54	1.69	1.75	0.193	0.008	0.534	0.388
C18:2n-6	17.24	17.78	16.36	16.44	1.58	0.201	0.724	0.242

C18:3n-6	0.28	0.28	0.30	0.30	0.030	0.254	0.818	0.421
C18:3n-3	0.23	0.22	0.18	0.19	0.037	0.025	0.402	0.226
C20:0	0.02	0.03	0.03	0.03	0.004	0.354	0.429	0.806
C20:1n-11	0.03	0.03	0.04	0.04	0.0084	0.101	0.828	0.427
C20:1n-9	0.11	0.11	0.12	0.12	0.010	0.167	0.331	0.879
C20:2n-6	0.08	0.08	0.07	0.08	0.010	0.589	0.653	0.027
C20:3n-6	0.15	0.16	0.17	0.16	0.0168	0.082	0.223	0.450
C20:4n-6	2.35	2.40	2.56	2.52	0.094	0.001	0.318	0.091
C20:5n-3	0.01	0.01	0.01	0.01	0.006	0.549	0.722	0.641
C22:0	0.02	0.02	0.01	0.02	0.004	0.008	0.016	0.217
C22:1n-9	0.01	0.01	0.01	0.01	0.004	0.987	0.587	0.362
C22:4n-6	0.13	0.11	0.12	0.14	0.0139	0.178	0.003	0.675
C22:5n-6	0.42	0.35	0.36	0.41	0.058	0.916	0.022	0.680
C22:5n-3	0.07	0.06	0.06	0.07	0.013	0.414	0.090	0.566
C22:6n-3	0.51	0.50	0.48	0.44	0.095	0.202	0.717	0.812
ΣSFA[2]	*35.90*	*36.03*	*35.76*	*35.60*	*0.644*	*0.335*	*0.578*	*0.660*
ΣMUFA[3]	*42.61*	*42.00*	*43.56*	*43.62*	*1.195*	*0.048*	*0.495*	*0.110*
ΣPUFA[4]*n-6*	*20.64*	*21.14*	*19.94*	*20.05*	*1.638*	*0.327*	*0.766*	*0.313*
ΣPUFAn-3	*0.83*	*0.78*	*0.73*	*0.70*	*0.138*	*0.114*	*0.904*	*0.930*

[1]T5, T10, T20: diets in which 5, 10 and 20% of the protein of the control diet (CON) have been replaced with the protein from defatted *Tenebrio molitor* larvae meal. [2]SFA: saturated fatty acids. [3]MUFA: mono-unsaturated fatty acids. [4]PUFA: poly-unsaturated fatty acids. [5]RMSE: root mean square error.

4. Discussion

To our knowledge, there are very few studies about the use of insect meals in laying quails and they are divided among local insect species (Das and Mandal, 2014), black soldier fly (Dalle Zotte *et al*., 2019) and *Tenebrio molitor* (Shariat Zadeh et al., 2020).

The absence of mortality and clinical signs of trouble (such as diarrhoea), as well as the absence of weight loss in all the groups, indicates that the inclusion of *Tenebrio molitor* in diets had no negative effects on laying quail's health status. In addition, the lack of differences in feed intake among the groups suggests an adequate palatability of the insect meal diets.

The most important difference among the groups regarding the productive performance was the laying rate which showed a progressive detriment due to the increasing inclusion levels of TML: the egg production of T20 and T10 groups was reduced by 22.14 and 13.80%, respectively, in comparison to the control group. This impairment could be attributed to the progressive reduction of dry matter (DM) and organic matter (OM) ileal apparent digestibility of the diets. On the other hand, the reduction of OM and DM digestibility is mainly due to the decrease of the crude protein digestibility when the insect diets were administered to the quails. This result was expected. It is well known that,

when insect meals are included in the diet, the reduced crude protein digestibility is registered due to their chitin content, which, because of its activity of binding proteins, makes them unavailable for digestion (Longvah *et al*., 2011). In the present study, considering the percentage of the crude protein of the diets, the feed intake of the birds and the coefficients of the apparent ileal digestibility, the amounts of crude protein available for digestion were: 6.01, 6.14, 5.92 and 5.70 g/d for CON, T5, T10 and T20 groups, respectively.

Previous studies on laying hens showed a strong correlation between dietary crude protein (CP) and laying performance of hens. Keshavarz and Nakajima (1995), Liu *et al*. (2005) and Gunawardana *et al*. (2008) showed that the egg production increased due to increasing dietary CP levels although the FI was not affected by these dietary changes. The effects of dietary CP on laying productive performance were also detected for egg mass and egg weight, but with different findings. Due to its close relation to the laying rate, the egg mass also increased or decreased as the dietary CP increased or decreased (Abd El-Maksoud *et al*., 2011; Alagawany *et al*., 2016); on the contrary some authors (Meluzzi *et al*., 2001) found that CP at 150 g/kg in the diet induced the higher egg size compared to the other higher or lower levels of protein (170 or 130 g/kg); completely different are the findings by Summers *et al*. (1991) and Lopez and Leeson (1995) who reported that the egg weight is strongly related to the content of CP in the diet. However, Summers and Leeson (1983) found that early egg size was not affected by the increase in dietary CP. This agrees with Khajali *et al*. (2008), Alagawany and Abou-Kassem (2014) and Alagawany *et al*. (2016), who indicated that low dietary crude protein has a negative effect on hens' performance, especially during the late stage of production.

In our trial, performed in an early phase of laying, the progressive decrease of laying rate was accompanied by a progressive increase in the weight of the eggs. This result should not be surprising. Unfortunately, in this trial we could not measure the amino-acid digestibility, but it is logical to assume that the reduction of proteins available for digestion also implies a reduction of the relative amino acid availability. Our hypothesis is that the strong reduction in eggs number laid by quails in T10 and T20 groups made the amount of amino acids available with the respective diets adequate to support a higher egg weight. These considerations are mainly for methionine and lysine which, as it is well known, are, respectively, the first and the second limiting AA in poultry and involve the regulation of egg weight (methionine) and egg mass (lysine; Fakhraei *et al*., 2010). However, as the laying rate strongly decreased from the control to T20 group, the egg mass linearly decreased accordingly. The FCR, which is tied to the egg mass, showed the same trend. According to our results, Shariat Zadeh *et al*. (2020) found a progressive decrease of egg mass of quails when *T. molitor* larvae meal increased from 25 to 100% in replacement of fishmeal and significant effects were already evident in the 25% group. In the study by Shariat Zadeh *et al*. (2020) the FCR values in the groups fed the insect meal diets were higher than in the control group.

According to Iqbal *et al*. (2017), increased egg weight resulted in the shell percentage significantly decreased, while the eggshell thickness was not influenced by the egg weight, corroborating the findings by Wolanski *et al*. (2007) and Crosara *et al*. (2019). The lack of differences among the groups for eggshell weights confirms the findings by Jonchère *et al*. (2012) who underlined that egg-laying birds have a limited amount of calcium available to produce the shell, approximately 2.0–2.5 g Ca^{2+}, irrespective of the egg size. However, the amount of Ca available for quails is sufficient in order not to cause significant changes in the shell thickness. The increased weight of eggs is accompanied by an increase in the circumference value.

The increase of the albumen and yolk weights from the control to T20 groups are in line with the increase of the egg weight according to Tebesi *et al*. (2012), Alkan *et al*. (2013) and Khawaja *et al*. (2013). However, when expressed as percentage of the egg weight, only the albumen showed a proportion progressive increase.

No difference in the yolk colour of the fresh eggs was detected among the groups. This is not in line with previous findings, in which the addition in the diet of insect meal from *Hermetia illucens* modified the yolk colour of hen (Secci *et al*., 2018) and quail eggs (Dalle Zotte *et al*., 2020). However, it must be considered the use of different insect species as ingredient in the diets, the lower level of insect meal inclusion in the diets of the present trial, as well as the overall feed formulation of the mentioned articles, which contained different amounts of carotenoid sources, as vegetable oils and maize. It is widely established that carotenoids are important not only for colouring but also for their protection against oxidative damages, even though we found these last slightly affected by the diet. Indeed, the overall content of the secondary oxidation products, that we observed, agreed with the values found by Ren *et al*. (2017) in raw yolk from laying hens.

The oleic (C18:1), linoleic (C18:2n-6) and palmitic (C16:0) fatty acids characterised the lipid fraction of *Tenebrio molitor* larvae with minor influence of their relative abundance in relation to the rearing substrates (Ruschioni *et al*., 2020). Despite the fatty acid profile of the control diet differed from the other ones containing the insect meal, it is of relevance that egg yolks showed only few modifications. Firstly, birds can synthesise ex novo C16 by the acetate/malonate way (Klasing, 2000), then can convert this fatty acid into C18:0 and desaturate into both in C16:1n-7 and C18:1n-9. This fact explains the highest values of these FAs found in the yolks despite their relative low amount in the feeds administered. However, the egg yolk from quails fed graded inclusions of TM meal showed significantly higher content of both C16:0 and C16:1n-7 than the control group, as a different enzymatic activity occurred, as supported by the significant linear increase in Δ9-desaturase activity on C16:0. In addition, some authors have previously observed that the overall fatty acid profile of yolk was only partially affected by the dietary intervention with insect meal (Secci *et al*., 2018; Dalle Zotte *et al*., 2020), probably due to the remarkable activity of desaturase and elongase on saturated fatty acids (as C14:0 and C16:0) for providing C14:1n-9, C17:1n-10, C16:1n-9, C18:1n-9 and C18:1n-11 fatty acids. In the present case, the retrieving in quail eggs of C14:1n-9 and C16:1n-9, not detected or scarcely contained in the feeds, might support this hypothesis. Interestingly, the groups fed the diets containing the insect meal showed a level of MUFA higher than the control group, thus possibly supporting the hypothesis that insect might affect the gene expression or the activities of these fundamental enzymes. In this regard, Nguyen *et al*. (2007) highlighted that the organ more affected by dietary TM was the liver, which is the main site for lipid metabolism and fatty acid synthesis and where the gene related to the elongation of fatty acids are expressed, especially after the sexual maturity of laying birds (Zhang *et al*., 2017).

Desaturase (Δ5 and Δ 6) and elongase enzymes are even able to catalyse the synthesis of long chain fatty acids, as PUFAn-3 and PUFAn-6, starting from C18:3n-3 and C18:2n-6, respectively (Güçlü *et al*., 2008). Our results highlighted this pathway in all the considered groups, since the FAs produced during the desaturation/elongation process have been detected in raw yolks (C22:6n-3, C20:3n-6, C20:4n-6, C22:4n-6, C22:5n-6), irrespective their scarce amount or the fully absence in the administered diets. As expected, since the content of the dietary precursors affects the enzymatic activity, the TM10 yolks were the richest in C20:3n-6 and C20:4n-6, while the TM20 yolks contained the highest amount of C22:4n-6 and C22:5n-6 together with a lower content of their precursor (*i.e.*,

the linoleic acid), abundantly contained in the feeds including TML. Noteworthy, the increase in C20 and C22 PUFAn-6 and the Δ5-Δ6 desaturase activity on PUFAn-6 was linear in the yolks of quails fed the diets including TML until the inclusion rate of 5.6%, while the enzymatic activity esteemed was contracted at the highest inclusion, despite the huge difference in dietary C18:2n-6 content (see Table 1). In addition, the predominance of PUFAn-6 on PUFAn-3 in the diet seemed to be responsible for the PUFAn-6 prevalence on the n-3 fraction, as previously noted by Dalle Zotte *et al*. (2020).
Quail eggs are commonly consumed as boiled, so the physico-chemical characteristics of the boiled eggs were evaluated. Yolk thermal processing, as pasteurization (de Souza *et al*., 2011) or water-bath treatment (Llave *et al*., 2018) increased yolk colour, especially the *b** value, due to the disruption of the fat-soluble carotenoids as lutein, zeaxanthin and beta-carotene in a minor extent and the subsequent formation of Maillard' products. Besides, Llave *et al*. (2018) showed a high correlation between colour changes and the non-denaturation ratio profile of high-density lipoproteins (α-HDL) and ovalbumin in yolk and albumen, respectively. Specifically, authors observed that increasing the thermal denaturation rate of these proteins augmented the colour difference. Hence, considering the significant quadratic and cubic contrasts emerged for the boiled eggs and the relation between colour and proteins, it could be presumed that a lower inclusion level of TM might affect in some extent at least the ovalbumin fraction of quail egg whites. Despite the studies on this field are still scarce and difficult comparisons can be done to debate the present data, we can mention that ovalbumin, whose content might slightly vary in egg white, contributes to the stability and the final volume of a batter in which the egg albumen is added (Lomakina and Míková, 2006). In this regard, Secci *et al*. (2020) found that the Angel cake made with the egg white from laying hens fed diet including 7.3% of *Hermetia illucens* larvae meal raised a significant lower final height than the control group, being 15.95 and 20.25 mm, respectively. Furthermore, Ko *et al*. (2020) noted that the concentration of yolk histidine linearly increased as the TM larvae meal inclusion increased in laying hens' diet (1, 2 and 3%). Despite the authors did not explain their findings, they supported the hypothesis that TML might in some extent interact with protein metabolism. This topic warrants further investigations.
From a nutritional point of view, the consumption of boiled eggs from quail fed TM meal included at 2.4, 2.8, or 5.6% in the diet brings the same quantity and quality of lipids than the eggs from quail fed the conventional protein sources. If we looked at the present results, as suggested by Sanders (2010), who highlighted the importance to consider food not only as a nutrient but also for its integrated role on environmental sustainability, it would seem reasonable to introduce TML meal in laying quails' diet.

5. Conclusions

Our results indicated that the inclusion of a defatted *Tenebrio molitor* larvae meal at 2.8 and 5.6% in laying quail diets negatively affected the laying performance and some physical characteristics of eggs, due to the impairment of nutrients digestibility, in particular of crude protein. In addition, TML inclusion in quail diets partly modified the fatty acid profile of the yolk, increasing the levels of C16:0 and C16:1n-7 fatty acids, possibly due to the increase in Δ9-desaturase activity. Thus, the best inclusion level of defatted TML meal for laying quails seemed to be 1.4% of diet. However, further efforts of research should be done to reduce the FCR, ameliorate digestibility and to increase PUFAn-3 deposition in egg yolks when the insect-based diets are utilised.

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CHAPTER 5

Potential use of a queen bee larvae meal (*Apis mellifera ligustica* Spin.) in animal nutrition: a nutritional and chemical-toxicological evaluation

1. Introduction

Nowadays, there is an increasing interest in the use of insects in animal and human nutrition, due to the lower impact of their production when compared to other animal species commonly used as protein sources such as bovine, swine, fish or poultry (Gasco *et al.*, 2020; Van Huis and Oonincx, 2017). However, one of the problems limiting the use of insect meals in intensive animal production is that the chemical-nutritional characteristics of the most common insect species farmed for feed production (*Tenebrio molitor, Hermetia illucens, Musca domestica*) are noticeably influenced by the rearing substrate (Meneguz *et al.*, 2018; Nguyen *et al.*, 2013; Spranghers *et al.*, 2016; Tomberlin *et al.*, 2002), so that it is actually impossible to have a standardised product, very important to guarantee a homogeneous production of animals under intensive farm conditions.

Previous research showed the possibility of including insect meals in fish (Caimi *et al.*, 2020; Chemello *et al.*, 2020; Nogales-Merida *et al.*, 2019), poultry (Bovera *et al.*, 2016; Dabbou *et al.*, 2019; Marono *et al.*, 2017; Secci *et al.*, 2018) and weaning pig (Biasato *et al.*, 2019; Di Giacomo andLeury, 2019; Spranghers *et al.*, 2018) nutrition as partial or total replacement of conventional protein/fat sources (soybean and fish meals and oils). High inclusion levels of the insect meals are responsible of a reduction of protein digestibility (Bovera *et al.*, 2018), but positive results have beenobserved in terms of animal health and performance, gut health aspects, and product quality (Borrelli*et al.*, 2017; Secci *et al.*, 2018). The utilisation of insects as novel feed additives to improve gut health,included at low levels in animal diets, has also attracted increasing interest, because they contain bioactive components, such as lauric acid, antimicrobial peptides, and chitin, which have immune- boosting properties (Gasco *et al.*, 2018).

Among the insect species, honeybees (*Apis mellifera* L.) are mainly considered for their role as pollinators, environmental indicators and for the honey production. However, honeybees can produce or collect from the environment several interesting products such as pollen, propolis, wax, royal jelly (RJ) and venom, towards which the attention of the producers and consumers for their properties grows more and more (Mustafa *et al.*, 2020). RJ is a milky substance secreted by hypopharyngeal and mandibular glands of young nurse (between 5 and 14 days) (Chauvin, 1968; Fujita *et al.*, 2013). Honeybees use RJ as exclusive nourishment for bee queen and as feed for all the larvae of the beehives within the first three days of life (Fratini *et al.*, 2016). The RJ is considered a ‘superfood’ due its nutritional characteristics and in particular its richness of minor components such as minerals (Fe, Na, Ca, K, Zn, Mg, Mn, and Cu), amino acids (eight essential amino acids: Val, Leu, Ile, Thr, Met, Phe, Lys, and Trp), vitamins (A, B complex, C, and E), enzymes, hormones, polyphenols, nucleotides, and minor heterocyclic compounds (Melliou and Chinou, 2014; Sabatini *et al.*, 2009; Xue *et al.*, 2017)

that confer it a lot of properties such as antimicrobial, antioxidant, anti-aging, immunomodulatory, and general tonic action (Ahmad *et al.*, 2020; Pavel *et al.*, 2011).
Around the world, China is the largest RJ producer with around 3,500 tons per year, that accounts for more than 90% of the world RJ market (Altaye *et al.*, 2019). In Italy, the consumption of RJ per year is estimated as 30-40 tons and only the 3% is produced in the same country (CO.PA.IT., 2020); the discrepancy between RJ production and consumption in Italy suggests the great potential for the expansion of this sector.
The intensive production of RJ is based on the fact that an orphaned honeybee colony rears queens from larvae of 1 to 3 days of age, and these larvae fed only RJ during their life; when the royal cells are near to closing by a wax operculum, it is possible to find the maximum amount of RJ in the cells (around 200 mg; Ali, 2017). The larvae grafted for the RJ production represent a waste of production, but could have a great potential in terms of animal and human nutrition, not only for their content of nutrients (as for other insect larvae) but also because little amounts of RJ can be stick to the larvae, given an added value to the product.
The aim of this research was to propose an additional income to RJ production, using the removed larvae as nutritional supplement for animal production. To the purpose, the first step was to collect and analyse queen bee larvae for their proximate composition, amino acid profile, fatty acid (FA) profile of the lipids, macro and micromineral content.

2. Materials and Methods

2.1 Insect rearing and collecting

The trial was carried out on an urban beekeeping of Napoli (Italy), from May to July 2019. A vertical method has been used for RJ production, in which the hives (5 frames each) with the queen bee were placed in the lower side, separated by the orphaned hive (in the upper side) by an exclude- queen grid. Honeybees larvae, 24-36 hours old, were moved using a scraping bar to the plastic queen-cell-base-bar (traslarvo), with a drop of RJ. The cell bars were inserted in the RJ production frame and moved to the upper hive. Each production frame contained 120 cells (=120 larvae) and each hive received 1 frame, placed in the middle of the hive. No nutrition was supplied to the honeybees. Three days after the graft, the RJ was collected in a specialised laboratory, uncapping the plastic cells, removing the larvae and thus collecting the RJ using a specific aspiration system; the age of scattered larvae ranged from 4 to 4.5 days.
Fifteen beehives (*A. mellifera ligustica*, Spin.) used for RJ production were monitored along the trial. At the collection of RJ, the scattered larvae were immediately placed in a glass jar, weighed and frozen at -80 °C. Along the trial, 2 collections of larvae per week have been made (8 collections per month, 3 months of collection); the frozen larvae obtained each week (2 collections) were freeze-dried using a Micromodulyo freeze dryer (Thermo Electron Corporation, Thermo Fisher Scientific Inc., Waltham, MA, USA). Thus, the obtained product per week was weighed, and the yield of meal production was calculated on a total of 12 samples (4 samples per month). The three-monthly aliquots (May, June and July) were ground, pooled and stored for the further analyses. So, all the chemical-nutritional evaluations were measured on 3 samples, each analysed in triplicate.

2.2 Chemical composition and amino acid profile

The chemical composition was determined using around 15 g of each sample, according to AOAC (2004); the nitrogen-to-crude protein conversion factor used in this trial was 4.76 according to Jansen *et al.* (2017). The acid detergent fibre (ADF) and the residual nitrogen in ADF were determined and used to estimate the amount of chitin according to Marono *et al.*, (2015).
The amino acid profile was determined on 20 g of each sample using a HPLC procedure after acid hydrolysis (6 N HCl for 24 hours at 110 °C) followed by ion exchange chromatography using an amino acid analyser (L-8800 Auto-analyser, Hitachi, Tokyo, Japan). For methionine and cystine analysis performic acid oxidation with acid hydrolysis-sodium metabisulfite method was applied (AOAC, 2005).

2.3 Fatty acid profile

The Fatty acids (FA) profile was assessed by gas-chromatography after a lipid extraction and methylation. Firstly, approximately 2 g of rehydrated sample (water content 75%) were subdued to the lipid extraction according to Folch *et al.* (1957). Then, around 400 mg of each extract were esterified to FA methyl esters (FAME) (Christie, 1982). The FA composition was determined by gas chromatography (GC) using a Varian GC 430 gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame ionisation detector and a Supelco Omegawax™ 320 capillary column (30 m × 0.32 mm i.d., 0.25-µm film and polyethylene glycol-bonded phase; Supelco, Bellefonte, PA, USA). The injector, oven, and detector settings were the same proposed by Secci *et al.*, (2020). FAs were identified by comparing the FAME retention time with the Supelco 37 component FAME mix (Supelco) and quantified towards the use of calibration curves using tricosanoic acid (C23:0, 0.4 mg/ml) (Supelco) as internal standard.

2.4 Mineral profile

For the mineral profile, before Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) analysis, about 10 g of each sample were homogenised by means of a laboratory mixer and 0.5±0.02 g tissue was digested in 5.0 ml of 65% HNO3 and 2.0 ml of 30% H2O2 in a microwave digestion system. Microwave assisted digestion was performed with a mineralisation program for 25 minutes at 190 °C. The vessel was then cooled at 32 °C, the digestion mixture was transferred into a 50.0 ml flask and the final volume was obtained by adding Milli-Q water (Ariano *et al.*, 2019). Concentrations of trace elements were determined by ICP-OES technique using a Perkin Elmer Optima 2100 DV instrument (Perkin Elmer, Inc., Wellesley, MA, USA) coupled with a CETAC U5000AT (ThermoFisher Scientific, Waltham, MA, USA). The calibration curve and two blanks were run during each set of analyses to check the purity of the chemicals. Reference material (BCR-422 cod muscle, IRMM Institute for Reference Materials and Measurements, DORM-2 fish protein, National Research Council, Canada) was also included for quality control. All the values of the reference materials were within certified limits. Instrumental detection limits expressed as wet weight and determined following the protocol described by Perkin Elmer ICP application study number 57 (Barnard *et al.*, 1993). The performance of the method was assessed through participation in

interlaboratory studies organised by Food Analysis Performance Assessment Scheme (Sand Hutton, UK).

2.5 Statistical analysis

The data of the amount of collected larvae, amount of freeze-dried meal and yield of meal production obtained per week were analysed by a one-way ANOVA, using the GLM procedure of SAS (SAS/STAT software, version 9, Cary, NC, USA), according to the model:

$$Yij = \mu + Mi + \varepsilon ij$$

where Y is the single observation, μ the general mean, M the month of collection (i = May, June, July), ε the error. Comparison between means was performed by Tukey's test (SAS/STAT). The data on chemical-nutritional characteristics were expressed as mean ± standard deviation.

3. Results

3.1 Insect rearing and collecting

Tab. 5.1 shows the weight of the collected larvae, their lyophilised weight, expressed as g/hive and the yield of meal production.

The amount of fresh and lyophilised larvae was the highest in June ($P<0.01$), as well as the yield of meal production was the highest in June, followed by July and May ($P<0.05$). The total amount of fresh larvae obtained for each month was 756.3 g in May, 1,124 g in June and 770.16 in July and the correspondent meals were 156.8, 290.5 and 175.4 g, respectively. Due to the little quantity of dried products obtained per month, it was not possible to perform a monthly evaluation of nutritional properties and the samples of each month were pooled for the final chemical-nutritional evaluations.

Tab. 5.1. Queen bee larvae weight and meal yield by month of collection (n=12)

	Wet weight/hive (g)	**Lyophilised** weight/hive (g)	**Yield (%)**
May	50.42^{B}	10.45^{B}	20.73^{c}
June	74.93^{A}	19.37^{A}	25.85^{a}
July	51.34^{B}	11.69^{B}	22.77^{b}
SEM	0.95	0.23	0.25

*Different superscript letters in the same column express significant differences.

3.2 Chemical composition and amino acid profile

Tab. 5.2 shows the chemical-nutritional characteristics of queen bee larvae (QBL) meal compared to that of the insect species mainly used in animal nutrition. The chitin amount estimated in our larvae was 12.99% of dry matter (DM), higher than that reported for other larvae meals. The percentage of ether extract was placed near the lowest values reported for *H. illucens* and *M. domestica*.

Tab. 5.3 displays the amino acid profile of queen bee larvae protein expressed as percentage of the crude protein. In comparison to the other larvae meal, queen bee showed lower amount of alanine and glycine and higher of cysteine.

Tab. 5.2. *Chemical-nutritional characteristics of the queen bee larvae meal (LM) compared to the most common full-fat and partially defatted insect meals used in animal nutrition (n=3) (Finke, 2007, 2013; Heuzé and Tran, 2015; Loponte et al., 2017; Pieterse and Pretorius, 2013; Rumpold and Schluter, 2013; Tran et al., 2015, 2019; Van Huis, 2013).* [1]

	Queen bee LM (mean±SD)	***Tenebrio molitor* LM (min-max)**	***Hermetia illucens* LM (min-max)**	***Musca domestica* LM (min-max)**
Dry matter (DM, %)	90.24±0.21	93.90	90-92.5	92.40
Ash (% DM)	7.61±0.10	1.9-4.5	14.6-28.4	5.16-10.7
Crude protein (% DM)	53.04±0.91	47.2-60.3	41.1-43.6	50.4-63.9
Ether extract (% DM)	16.74±0.04	31.1-43.1	15.0-34.8	14.08-24.31
Crude fibre (% DM)	2.72±0.13	5.00-14.96	7.0	5.7-8.59
ADF (% DM)	16.56±0.69	7.67	12.37	13.8
ADF-CP (% DM)	3.57±0.17	2.58	5.59	–
Chitin (% DM)	12.99±0.96	5.09	6.78	8.04

[1] ADF = acid detergent fibre; ADF-CP = crude protein linked to ADF; SD = standard deviation.

Tab. 5.3. Amino acid profile (% of crude protein) of the queen bee larvae meal (LM) compared to the most common full-fat and partially defatted insect meals used in animal nutrition (n=3) (Belluco et al., 2013; Finke, 2013; Heuzé and Tran, 2015; Józefiak et al., 2016; Loponte et al., 2017; Ramos-Elorduy et al., 1997; Rumpold and Schluter, 2013; Tran et al., 2015, 2019; Van Huis, 2013).[1]

	Queen bee LM (mean±SD)	***Tenebrio molitor* LM (min-max)**	***Hermetia illucens* LM (min-max)**	***Musca domestica* LM (min-max)**
Essential amino acids				
Leucine	7.34±0.59	7.4-10.6	7.1-8.4	4.5-6.4
Lysine	6.89±0.67	4.6-6.1	6.0-8.0	5.0-8.2
Valine	5.40±0.41	5.5-6.6	6.4-9.1	1.3-4.9
Isoleucine	4.57±0.39	4.1-5.0	4.7-5.6	2.3-3.7
Phenylanine	4.26±0.32	3.5-4.3	4.6-5.6	3.7-5.9
Threonine	4.05±0.33	3.5-4.4	1.3-4.8	2.0-4.1
Histidine	2.44±0.17	3.2-3.6	2.3-4.5	1.0-3.6
Methionine	2.13±0.01	1.3-2.0	1.7-2.4	1.3-3.7
Tryptophan	1.43±0.04	0-0.9	0.5	1.4-1.5
Non essential amino acids				
Glutamic acid	11.52±1.03	10.2-12.4	8.7-13.5	8.9-15.3
Aspartic acid	10.02±0.77	5.6-8.8	8.5-12.5	4.5-8.5
Arginine	5.25±0.34	3.8-5.6	5.3-6.1	3.7-5.8
Proline	4.90±0.38	6.6-7.0	5.5-7.7	2.5-4.0
Alanine	4.64±0.45	6.2-8.2	6.9-8.8	4.4-7.6
Serine	4.63±0.34	4.9-11.1	0.3-4.2	3.26-3.9
Tyrosine	4.38±0.32	7.1-7.4	6.0-7.7	2.9-7.1
Glycine	2.88±2.02	3.9-5.6	5.2-6.8	3.7-5.1
Cysteine	1.09±0.02	0.8-0.9	0.1	0.5-1.0

[1] SD = standard deviation.

The tryptophan was higher than that reported for *T. molitor* and *H. illucens* and similar to that of *M. domestica*.

In Fig. 5.1, the QBL essential amino acids (EAAs) contents, expressed as percentage of lysine, are compared to the correspondent levels of the same amino acids in the dietary ideal amino acid profile for laying hens (Schutte and De Jong, 1999). The first limiting amino acids seem to be methionine + cysteine, then in order methionine, isoleucine, threonine, and valine.

In Fig.5.2, the QBL EAAs contents, expressed as percentage of lysine, are compared to the correspondent levels of the same amino acids in the dietary ideal amino acid profile for broilers

(Schutte and de Jong, 1999). The first limiting amino acids seem to be methionine + cysteine then, in order, methionine, threonine and valine.
In Fig. 5.3, the percentage of each EAA in the QBL crude protein is compared to the correspondent level of the same amino acid in the dietary ideal amino acid profile for gilthead sea bream (*Sparus aurata*) (Tibaldi and Kaushik, 2005), rainbow trout (*Oncorhynchus mykiss*) (Webster and Lim, 2002), and common carp (*Cyprinus carpio*) (Shilo and Sarig, 1989). The first limiting amino acid appear to be arginine for gilthead sea bream and methionine + cysteine for rainbow trout and common carp.

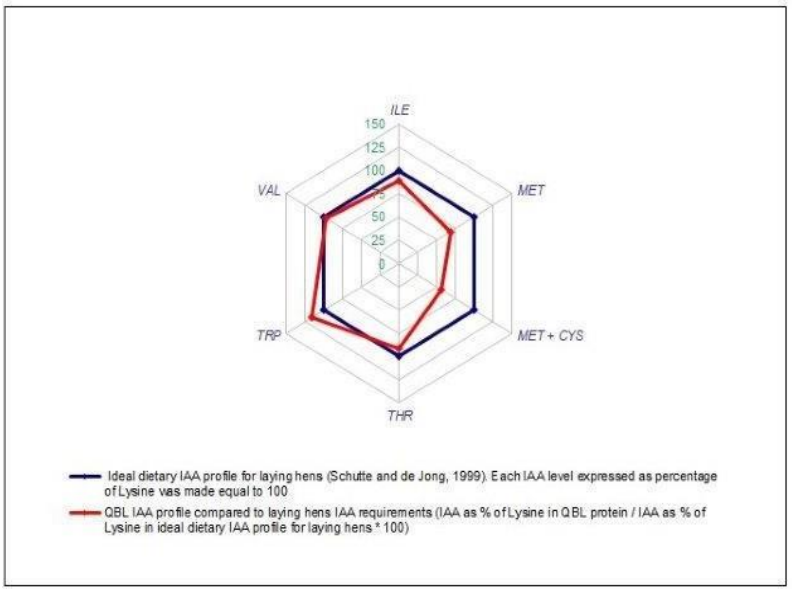

Fig. 5.1. Comparison between the queen bee larvae (QBL) essential amino acids (EAA) profile and the EAA requirements of laying hens (Schutte and De Jong, 1999).

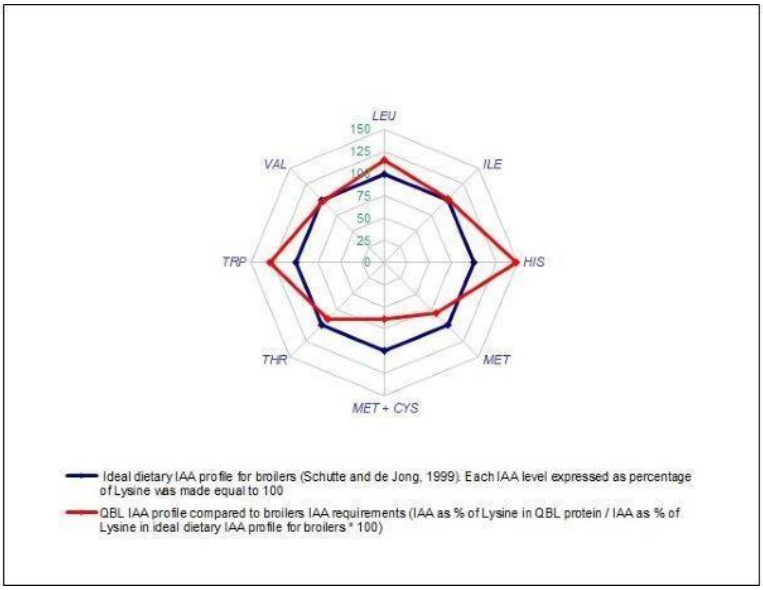

Fig. 5.2 Comparison between the queen bee larvae (QBL) essential amino acids (EAA) profile and the EAA requirements of broilers (Schutte and De Jong, 1999).

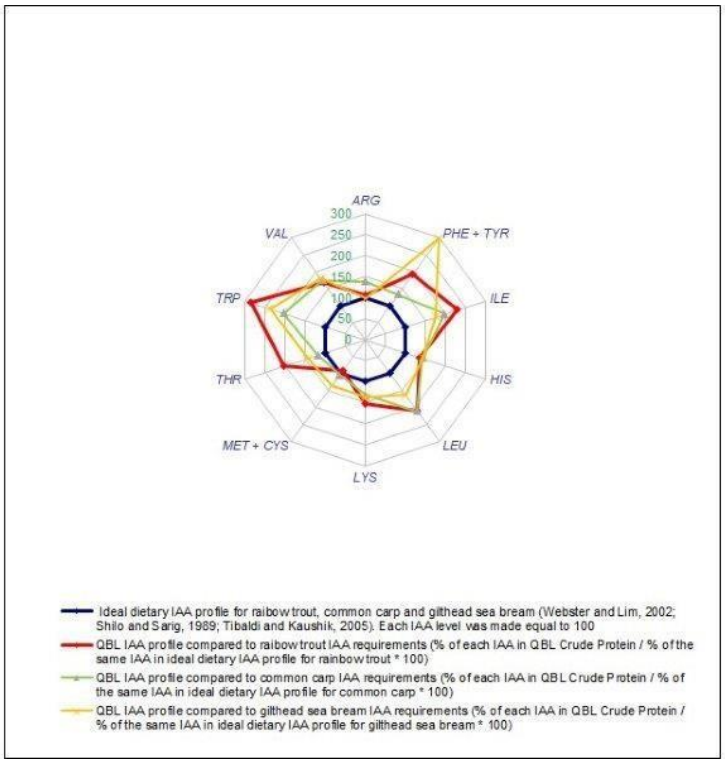

Fig. 5.3 Comparison between the queen bee larvae (QBL) essential amino acids (EAA) profile and the EAA requirements of three fish species.

3.3 Fatty acid profile

Tab. 5.4 shows the FA profile of the QBL meal expressed as percentage of total FA. in comparison to the most common insect species used as animal feed, QBL showed a higher amount of monounsaturated fatty acids (MUFA) and a lower of polyunsaturated fatty acids (PUFA). In comparison to the other insect species, the differences are very high because QBL are richer in saturated fatty acids (SFA) (particularly palmitic and stearic acids) and very poor in PUFA.

Tab. 5.4 Total lipid content (g/100 g) and fatty acid profile (% of total fatty acids) of the queen bee larvae meal (LM) compared to the most common full-fat and partially defatted insect meals used in animal nutrition (n=3) (Cullere et al., 2019; Dreassi et al., 2017; Ewald et al., 2020; Hashizume et al., 2019; Hussein et al., 2017; Makkar, 2014; Ruschioni et al., 2020; Starcevic et al., 2019; Stefanov et al., 2002; Tran et al., 2019).[1]

	Queen bee LM Mean±SD	***Tenebrio molitor* LM min-max**	***Hermetia illucens* LM min-max**	***Musca domestica* LM min-max**
Total lipids	14.88±3.90	34.42-48.31	11.0-58.0	6.8-33.0
C12:0	0.12±0.02	0.18-0.79	7.5-36.5	0.1
C14:0	1.42±0.09	2.15-7.21	2.3-8.08	3.10-6.8
C15:0	0.01±0.00	0.06-0.13	0.03-0.09	2.3-2.82
C16:0	34.95±2.04	14.93-21.1	12.69-19.2	25.28-38.0

C17:0	0.02±0.006	0.05-0.14	0.09-0.11	0-0.9
C17:0 anteiso	0.01±0.00	–	–	–
C18:0	5.39±0.50	2.51-3.46	1.74-6.9	2.3-5.0
C19:0	9.87±4.18	–	–	–
C20:0	0.23±0.04	0.09-0.19	0.04-0.23	0.2
C22:0	0.9±0.01		0.02	0.1
SFA	52.10±2.45	20.37-32.06	28.11-72.0	36.5-38.64
C14:1 cis 9	0.17±0.07	0.31-0.50	0.0-0.20	0.3-2.03
C16:1 trans7	0.10±0.01	–	–	–
C16:1 cis9	3.71±1.13	1.57-4.0	0.8-3.82	8.30-33.31
C17:1 cis10	0.00±0.00	0.05-0.08	0.13-0.16	0.5
C18:1 cis9	42.92±3.03	31.56-58.04	13.3-54.12	19.5-22.22
C18:1 cis11	0.43±0.13	0.56	0.52-1.1	–
C20:1 cis11	0.10±0.02	0.03-0.09	0.00-0.11	–
C20:1 cis n7	0.01±0.00	–	–	–
C22:1 cis13	0.00±0.00	–	0.03-0.08	–
MUFA	47.44±2.40	35.11-60.56	16.7-59.14	44.20-57.50
C18:2 cis9, cis12	0.11±0.03	16.63-36.42	4.28-31.4	2.82-16.4
C20:3 cis8, cis11, cis14-6	0.02±0.005	–	0.02-0.04	–
PUFAn6	0.12±0.03	17.33-36.71	4.28-31.4	2.82-16.40
C18:3 cis9, cis12, cis15	0.29±0.04	0.23-2.34	0.19-3.6	0.40-2.00
C20:4 cis8, cis11, cis14, cis17	0.01±0.00	–	–	–
C20:5 cis5, cis8, cis11, cis14, cis17	0.01±0.00	–	0.00-1.00	–
C22:5 cis7, cis10, cis13, cis16, cis19	0.03±0.01	–	–	–
PUFAn3	0.33±0.05	0.23-2.34	0.23-4.15	0.40-2.00

[1] MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; SD = standard deviation; SFA = saturated fatty acids.

3.4 Mineral profile

Tab. 5.5 shows the mineral and trace element content of the QBL. Almost all the minerals are lower than those reported in literature for the other larvae meal, except for phosphorous.

Tab. 5.5 Mineral profile (mg/kg) of the queen bee larvae meal (LM) compared to the most common full-fat and partially defatted insect meals used in animal nutrition (n=3) (Belluco et al., 2013; Finke, 2013; Gao et al., 2019; Józefiak et al., 2016; Moniello et al., 2019; Poma et al., 2017; Ramos-Elorduy et al., 1997; Rumpold and Schluter, 2013; Van Huis, 2013).[1,2]

	Queen bee LM	***Tenebrio molitor* LM**	***Hermetia illucens* LM**	***Musca domestica* LM**
	Mean±SD	**min-max**	**min-max**	**min-max**
Ca	360.5±4.6	300-6,200	9,340-86,300	3,100-20,100
P	7,840.3±41.9	440-14,200	3,560-15,000	9,700-24,000
Mg	293.3±8.62	2,000-2,800	1,740	700-11,500
K	519.5±11.7	8,500-9,300	4,530	1,000-12,700
Zn	23.15±2.14	58.6-136	36.2-453.6	43-325
Se	0.058±0.002	0.7	0.05-0.3	-
Fe	5.49±0.58	26-110	10.7-66.6	112.4-1,370
Cu	3.93±0.40	5-18	3.20-4.0	12.3-36
Mn	0.046±0.008	6-14	45.1-61.8	40-349
Cr	0.056±0.011	0.18	0.21	1.59-20.7
Ni	0.063±0.009	0.28	0.10	0.14-0.31
Pb	<LOQ	<0.03	0.03	–
Hg	<LOQ	–	0.01	–
As	0.158±0.019	<0.03	0.23	–
Cd	0.011±0.0001	0.06	0.06	0.66-0.98
Co	0.012±0.0001	0.05	0.01	–

[1] Mean values are shown if multiple different reports exist. 2 LOQ = limit of quantification; SD = standard deviation.

4. Discussion

4. 1 Rate of larvae collection and meal yield

To be considered for use as feed or food, an ingredient must show favourable chemical-nutritional characteristics and the interest increases if the same ingredient can act also as functional feed or food, that means to have an additional function (often related to health-promotion or disease prevention). The differences among yield percentages of QBL in meal by month of collection observed in the present trial could be ascribed both to the age of the larvae and the residual amount of RJ attached to the larvae. Regarding the first point, the larvae were collected by traslarvo at 24-36 hours old and removed after 3 days, so that, at RJ collection, the age of the scattered larvae ranged from 4 to 4.5

days. It is known that the amount of water is higher in younger larvae and also few hours of difference in terms of age can affect the dry matter content of the larvae, thus their yield in meal (Van der Fels-Klerx *et al.*, 2016). About the RJ attached to the larvae, actually, it is impossible to exactly quantify its amount that could be different from each larva due to several variables such as the total amount of RJ accumulated in each cell by the workers, the amount of RJ consumed by the larva, the efficiency of RJ removal. The RJ has an amount of water ranging from 60 to 70% (Kanelis *et al.*, 2015) and this difference as well as the amount of residual RJ can affect the total dry matter of the meal obtained from larvae lyophilisation.
In addition to the previous points, the differences in terms of grams of fresh larvae obtained per month also count the acceptance rate of grafted larvae by bee workers. This criterium can be strongly affected by several factors such as the larvae age, the number of grafted larvae, the environmental temperature and humidity (Okuyan and Akyol, 2018). In this trial, unfortunately, it was not possibleto measure the acceptance rate of the colonies.

4.2 Chemical characteristics

In general, the nutrients in the QBL are included in the range reported in the literature for the other insect meals. QBL meal showed interesting chemical nutritional characteristics for its potential use in animal but also in human nutrition. The high amount of crude protein, higher than that of *H. illucens*, and the lower percentage of ether extract suggest a potential use as alternative protein source. However, due to the lower mass production of the larvae, linked to the production of RJ, this kind of use, in the current state, seems impossible. The crude protein content of QBL is higher than that reported by Ghosh *et al.*, (2016) for worker larvae (35.3% DM), by Ramos-Elorduy *et al.* (1997) for bee larvae and pupae (42.0 and 49.0% DM, respectively) and that reported by Finke (2007) for bee brood (28.3% DM). This could be for the different kind of larvae but also for the different developmental stage as insects show a wide range of protein contents depending on the species and life stage (Bukkens, 1997; Finke, 2002). The amount of ether extract is in line with other findings (Finke, 2007; Ghosh *et al.*, 2016).
Few data are available in literature on the amount of ADF and of protein linked to ADF in honeybee larvae. The ADF analysis has been proposed by Van Soest *et al.* (1991) to measure the structural fibre in plants. Insects are from animal kingdom, thus the ADF analysis has a different meaning. ADF in insects represents both chitin (with a chemical structure similar to that of the cellulose) and protein bound to chitin in insect cuticle (Finke, 2007). The amount of ADF of QBL is very high in comparison to the finding of Finke (2007) who reported 13 g/kg DM of ADF in bee broad and is lower compared to the results of Pennino *et al.* (1991) who found 265 and 212 g/kg DM of ADF in adult bees. The interest for ADF analysis is that it can be used for an estimation of chitin (Marono *et al.*, 2015).
In our trial, the amount of the estimated chitin was higher than that estimated or measured in the other insect meals. The chitin could have a negative effect on protein digestibility, in particular when higher percentage of insect meals are included in animal diets (Bovera *et al.*, 2018) but can act as a potential prebiotic, positively modulating the short chain FA production of intestinal microbiota in laying hens (Borrelli *et al.*, 2017). Based on our knowledge, there are few studies available on the chitin content in honeybees: Ozimek *et al.* (1985) stated the amount of chitin in drones as 11.1%. Our results suggest a potential use of QBL as modulator of microbiota fermentations in the intestine. On this regard,

chitin also showed a great interest in human nutrition for its anticancer, antimicrobial, antioxidant and intestinal fermentation modulator activities (Tuyishime *et al.*, 2016).

4.3 Amino acid profile

Almost all the EAAs are present in honeybee protein, as indicated by Ozimek *et al.* (1985) and Ghosh *et al.* (2016). According to Ghosh *et al.* (2016), among the EAAs the leucine was dominating and this happens also in other edible insects such as *Brachytrupes orientalis* and *Chondacris rosea* (Orthoptera) (Chakravorty, 2014), *Bombyx mori* (Lepidoptera) (Tomotake *et al.*, 2010), *Oryctes monoceros* and rhinoceros larvae (Coleoptera) (Idolo and Henry, 2011; Okaraonye and Ikewuchi, 2009). The second EAA is lysine, followed by valine. Lysine is considered very important for animal and human nutrition due its poor presence in wheat, rice and maize which are largely used in the world for human and animal nutrition (Ozimek *et al.*, 1985). The relative abundance of EAAs is similar to that of *H. illucens*, *T. molitor* and *M. domestica*, even if for *M. domestica* the data available in literature indicates higher levels of lysine instead of leucine. Ghosh *et al.* (2016, 2020) and Tang *et al.* (2019) did not find two EAAs: methionine and tryptophan that, in our samples, have levels comparable or higher than other insect larvae meals. Ghosh *et al.* (2020) did not measure tryptophan while did not recover cysteine and methionine and this was attributed to the probably low values of both amino acids in honeybee larvae. The interesting amount of tryptophan, methionine and cysteine found in QBL could be probably attributed to the RJ attached to the larvae because, as well known, RJ is rich in amino acids, including the essential ones (Xue *et al.*, 2017). Compared to the other insect meals, QBL meal showed satisfactory amount of EAAs and a high level of tryptophan, similar to that recorded in *M. domestica*. Tryptophan and its metabolites (e.g. serotonin (5-hydroxytryptamine, 5-HT) and melatonin)) can regulate feed intake, reproduction, immunity, neurological function, and anti-stress responses; additionally, tryptophan may modulate gene expression and nutrient metabolism to impact whole-body homeostasis in organisms (Yao *et al.*, 2011). Thus, adequate intake of this amino acid from the diet is crucial for growth, development, and health of animals and humans. Among the non-EAAs, glutamic acid was the most represented in QBL protein and this is in accordance with the findings of Ghosh *et al.* (2016) and Tang *et al.* (2019) on honeybee larvae (workers) and with the data reported by Okaraonye and Ikewuchi (2009) and Idolo and Henry (2011) for other insect species. Comparing the amino acid profile of QBL to the ideal amino acid profile of protein for laying hens (Schutte and De Jong 1999), the QBL proteins appear lacking for valine, isoleucine, threonine, methionine and methionine + cysteine as the adequate values, expressed as percentage of lysine, are 81, 74, 64, 45 and 84%, respectively; instead the amounts of tryptophan (required: 18%) are adequate. Regarding broiler requirements, the amino acid profile of queen bee proteins falls in the limits indicated by Schutte and De Jong (1999) for leucine (92% lysine), isoleucine (66), histidine (24), tryptophan (16) while is lacking for valine (80), threonine (65), methionine (38) and methionine + tryptophan (73). For what concerns the correspondence of the amino acid profile of QBL to the amino acid requirements of reared fish species, it should be considered that, even if in general the amino acid patterns of insects are taxon-dependent, the amino acid profiles of most insects tested in fish diets show a good correlation with fish requirement values, and, in some cases, even exceed these requirements (Henry *et al.*, 2015; Gasco *et al.*, 2020). Honeybees (*A. mellifera*) meal inclusion has not been studied in fish diets, but it shows valuable EAA profile and, as other Hymenoptera, is particularly interesting due to its richness in methionine (Henry

et al., 2015). By comparing the QBL protein amino acid profile to the dietary ideal EAAs profile for gilthead sea bream (Tibaldi and Kaushik, 2005), one of the most commonly reared fish species in the Mediterranean basin, it can be noticed that it can ensure a well-balanced amino acid supply for a carnivorous fish such as gilthead sea bream (*S. aurata*): arginine 5.25 vs 5.4; phenylalanine + tyrosine 8.64 vs 2.9, isoleucine 4.57 vs 2.6, histidine 2.44 vs 1.7, leucine 7.34 vs 4.5, lysine 6.89 vs 5.0, methionine+ cysteine 3.22 vs 2.4, threonine 4.05 vs 2.8, tryptophan 1.43 vs 0.6, valine 5.4 vs 3.0 for QBL protein amino acid profile and dietary ideal EAAs profile for gilthead sea bream, respectively. The first limiting amino acid appears to be arginine, while it is worth noting excesses of phenylalanine and tyrosine. There is a considerable homogeneity of ideal profiles in the diets for most EAA when estimated from the EAA fish body composition, and they are not expected to vary significantly among fish species (Tibaldi and Kaushik, 2005). Thus, it is possible to affirm that the considerations made for sea bream can be extended to the most commonly reared marine carnivorous fish species, at least. Evaluating the suitability of the QBL to satisfy the requirements of the EAAs for rainbow trout (*O. mykiss*), it is possible to show that 4 amino acids considered limiting in feed for this freshwater species are well represented in the case of arginine (5.25 vs 5), lysine (6.89 vs 4.5) and tryptophan (1.43 vs 0.5), whereas methionine + cysteine (3.22 vs 3.5) is slightly below the needs for maintenance and growth. The other EAAs seem to cover the requirements of this salmonid: phenylalanine + tyrosine 8.64 vs 4.5; isoleucine 4.57 vs 2.0; histidine 2.44 vs 1.8; leucine 7.34 vs 3.5; threonine 4.05 vs 2.0; valine 5.40 vs 3.2 (Webster and Lim, 2002). Based on the EAA profile, the QBL can be accepted as candidate to supply the EAAs to rainbow trout. Considering the QBL to cover the needs of 10 EAAs in common carp (*C. carpio*), one of the most important warmwater fish species, it is possible to observe that in QBL all the EAAs are well balanced in relation to the requirements for growth of this cyprinid (Shilo and Sarig, 1989): arginine 5.25 vs 3.8; phenylalanine + tyrosine 8.64 vs 6.5; isoleucine 4.57 vs 2.3; histidine 2.44 vs 1.7; leucine 7.34 vs 3.5; lysine 6.89 vs 5.2; methionine + cysteine 3.22 vs 3.1; threonine 4.05 vs 3.4; tryptophan 1.43 vs 0.7; valine 5.40 vs 3.1.

4.4 Fatty acid profile

The FA profile of QBL is very different from that of the other larvae and is characterised by a negligible amount of PUFA. This is in line with previous research on workers (Ghosh *et al.*, 2016) and drones (Ghosh *et al.*, 2020) honeybees, with little differences. In comparison to honeybee workers and drones, queen larvae showed lower percentage of palmitic (35.35 vs 37.3 and 43.4%, respectively for worker and drone larvae) but mostly of stearic acid (5.43 vs 11.8 and 10.02%, respectively for worker and drone larvae). The differences in FA profiles compared to the other insect species could be attributed to the different genetic but mainly to the different larvae nutrition. It is well known that FA profile can be strongly affected by different feeding. Larvae from black soldier fly, mealworm and housefly were fed with different substrates to modulate and to optimise their FA profile. On the contrary, honeybee larvae were fed always with specific bee products, different according to the kind of larva. In fact, queen bees feed on RJ (a product of animal origin) for all their life, while both workers and drones feed on RJ for only three days, thus their diet is based on honey and bee bread (both vegetable products partly modified by honeybees). Probably, this is responsible for the differences detected among the different kinds of larvae. An assessment of fat quality is quite complicated. High levels of SFA, as that detected in our trial, are not desirable due to their linkage to cardiac diseases. However, not all the SFA have a negative impact on blood cholesterol. Lauric and

palmitic acids are hypercholesterolemic and lauric acid raises total cholesterol concentrations more than palmitic acid, which is partly due to a stronger rise in HDL cholesterol (Temme *et al.*, 1996). Both myristic acid and palmitic acid caused high LDL cholesterol and apoB levels and low HDL to LDL ratio (Zock *et al.*, 1994), and myristic acid resulted in increase in postprandial HDL TAG higher than stearic acid (Tholstrup *et al.*, 2003). In our trial, lauric, palmitic and myristic acids were in line with the finding of Ghosh *et al.* (2016), lower than those of conventional foods of animal origin like beef, veal, lamb and also of coconut oil (Ghosh *et al.*, 2016). The high proportion of MUFA (47.59%) is also interesting because MUFA lowers LDL cholesterol but does not lower high density lipoprotein (HDL) or causes increases of triglycerides (Grundy, 1986; Mensink and Katan, 1987). According to Ghosh *et al.* (2016), oleic acid levels were higher than those reported for almost all foods of animal origin and edible oils except for olive and canola oils.

4.5 Mineral profile and toxic elements

Also, for minerals, our samples presented values other than those reported in the literature (Ghosh *et al.*, 2016, 2020) for almost all the data reported in Table 6. The results can be ascribed to the different kind of larvae used in our trial as QBL had a feeding different than that of workers, used in the above-mentioned studies. As an example, RJ contains lower levels of elements (Wang *et al.*, 2016) than bee pollen (Somerville and Nicol, 2002).

The contents of micromineral and trace elements are, in general, lower than those measured in the other insect meals, except for P. This is due to the nutrition of the QBL, based only on RJ. The great range of variability of the other meals is also tied to the possible enrichment of some growth substrates with minerals. Some elements are essential for biological functions, but the heavy metals like Cd, Pb, Hg and As can induce adverse effects due to their potential toxicity and bioaccumulation in the food chain. An important aspect concerning the use of QBL meal in animal nutrition, related to the human and animal health, is the possible accumulation of toxic elements. To regulate the animal dietary exposure to heavy metals, the EU Commission established maximum levels for different undesirable substances in animal feeds (EC, 2002). Furthermore, the European Union legislation (EC, 2006 and its amendment EC, 2011) on food safety established the MRLs for some chemical contaminants in honey intended for human consumption. Concerning the toxic elements, the concentration of Cd, Pb, Hg and As in the QBL meal always resulted lower than the maximum values established for the feeding stuffs and feed materials. Thus, the results obtained in the current study showed negligible levels of Cd, Pb, As and Hg in all the analysed samples of QBL meal and were indicative of low risk for animal and human health. Although many studies reported data on metals concentrations in honey and other bee products (Giglio *et al.*, 2017; Bonsucesso *et al.*, 2018), data on concentration of heavy metals in QBL meal are very limited. As a consequence, a monitoring of toxic element concentrations is necessary from the point of view of contamination. In comparison to previous studies on honeybees larvae, queen bee appears very different from workers (Ghosh *et al.*, 2016) as the mineral contents are lower and more similar to that of drones (Ghosh *et al.*, 2020). Finke (2007) analysed the chemical characteristics of bee brood: in that trial the age of larvae was not well defined (the authors indicated most of them as pupae and less than 10% of mature larvae) as well as the kind of larva (worker or drone), however our results showed that QBL had higher amount of Ca, P and Mg, while the other minerals (K, Fe, Zn, Mn) were lower than those reported by Finke *et al.* (2007). Differences in the mineral contents are presumably due to the different resources available for the bees' nourishment in

the different geographical locations: the QBL nutrition is constant while that of workers and drones changes according to the natural available sources of pollen and nectar. Bee pollen mineral contents can vary according to the botanical origin but, in general, are higher than that of RJ (Machado De-Melo and de Almeida-Muradian, 2017; Wang *et al.*, 2016). QBL appears as a relatively poor source of Ca, but showed interesting amount of P and Mg. The mineral composition of insects can show a wide variability probably because it is the result of the minerals that the insect incorporates into its body from food as well as of the minerals in the food retained in the insect's gastrointestinal tract (Finke, 2007).

5. Conclusions

QBL meal showed very interesting characteristics under the nutritional point of view: it is rich in protein and EAAs, relatively poor in fat, rich in chitin and in some essential mineral such as phosphorous and magnesium and with negligible levels of toxic elements such as Cd, Pb, As and Hg. However, its FA profile is not completely favourable due to the low availability of PUFA. In addition, the meal is poor of Ca and other trace elements when compared to the most common insect meals used in animal nutrition. Another strength of queen bee meal is that larvae have a standard nutrition based on a specific product (RJ) with relatively constant chemical and nutritional characteristics. A weakness is that the collection of QBL does not allow to give high quantities of final product due both the low number of larvae collected and the relatively low yield in meal recorded. Thus, the insect meal obtained from QBL cannot be considered as an alternative protein source in animal production, but could be taken into account for the use as feed supplement, at low doses, mainly to exploit the possible activities as gut microbiota modulator due to the high levels of chitin. This could be of potential interest for animal but also for human nutrition. For example, its use in small scale farms, also in light of the fact that this product currently represents a production waste, could have a great potential in terms of improving a circular economy optimising the use of the natural resources.

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Considerations

Nowadays, industrial company scale is in the start-up phase, such as the mass breeding of black soldier flies, bred mainly for whole consumption or to be transformed into flour for feed. The critical elements for successful breeding include research on biology, control of breeding conditions and specific dietary formulations to maximize insect growth, product quality and sustainability of the production. Current manufacturing systems are expensive, with many patents pending. A major challenge of such industrial-scale farming is the development of automation processes to make plants economically competitive with the production of meat (or meat substitutes such as soy) from traditional farms or agricultural sources. The available evidence suggests that insect-based feeds are comparable to fishmeal and soybean-based feed.

Insects, as mentioned above, are often eaten whole, but they can also be transformed into granular or paste forms. It is also possible to extract proteins, fats, chitin, minerals and vitamins. At present, such extraction processes are too expensive and will need to be further developed to make them profitable and applicable for industrial use in the food and feed sectors. The processing and storage of insects and their products should follow the same health and sanitation standards as for any other traditional food or feed, in order to ensure food safety. Due to their biological composition, several problems should be considered, such as microbial safety, toxicity, palatability and the presence of inorganic compounds.

It is essential to raise public awareness, the political world and investors in the food and feed sectors by providing them with validated information on the potential of insects as food and feed sources.

The regulatory frameworks governing food and feed chains have expanded enormously over the past 20 years; however, regulations governing insects as food and feed sources are still largely absent. For developed countries, the absence of clear legislation and regulations guiding the use of insects as food and feed is one of the main limiting, hindering the industrial development of farmed insects for the supply of the food and feed sectors. The feed sector seems to take the lead by pushing for the development of more standards that embrace insects, while the concept of "novel food" seems to emerge as a leading tool for establishing rules and standards for the use of insects also in human food.

Over the past five years, scientific knowledge on insects as food and feed has grown exponentially. At the same time, the industrial sector is increasingly engaged in the breeding, processing and marketing of edible insects. Great attention is given to the black soldier fly as it can convert organic waste streams and transform them into different feed, food and industrial products. The profitability of industrial production of insects as feed is highly dependent on the availability and applicability of low-cost unused secondary streams. As insects have only recently been considered as food or feed, the legislation is careful to follow future developments. Therefore, policymakers need to be sure that farming and processing techniques are such as to ensure that insect products are free of chemical and microbial contaminants. Insects need to be processed into ingredients, which can be applied for safe and appetizing products. The insect industry is maturing rapidly, but still faces many challenges, which can only be addressed when all stakeholders cooperate closely.

Therefore, documentation on the nutritional values of insects is necessary, in order to promote insects as healthy food more efficiently. Secondly, the environmental impacts of the collection and breeding

of insects must be studied to allow comparison with traditional agricultural and breeding practices that can be more harmful to the environment. Furthermore, the increase in the socio-economic benefits that insects reap, and that agriculture can offer is necessary, in particular to improve the food security of the poorest in society. Finally, a clear and comprehensive legal framework at the international level is needed to pave the way for greater investment, leading to the full development (from domestic to industrial scale) of the production and international trade of insect-based products as food and feed sources. In recent years, more and more conferences and workshops have been organized. Public interest is high considering the numerous newspaper articles that are published on this topic. The number of scientific articles, books and book chapters on this topic is increasing almost exponentially. With all this interest it is believed that insects will be increasingly commercialized. The production of sustainable food and feed is increasingly seen as a necessity. So, most likely under these circumstances, hopefully, there will be a bright future for insects as food and feed.