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CICLO: XXXIII

Insect Protein Meal: an alternative raw material for freshwater fishes

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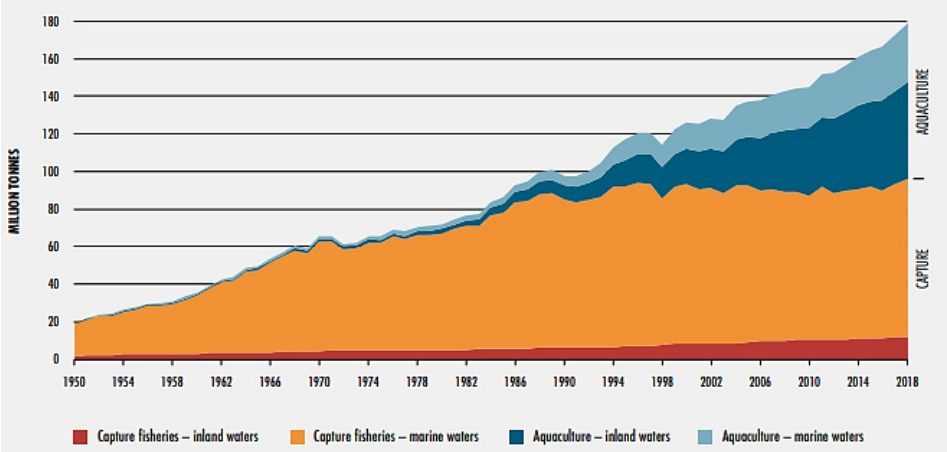
1. Global Status of Fish and Aquafeed Production

Aquaculture is the world fasted growing food production sector (FAO, 2020; Garlock et al., 2020; Tacon, 2020), able to support the growth of the global demand of animal product correlated with the increase in world population (Engle et al., 2017; Astuti et al., 2020).

In 2018, the global aquaculture production (fish, crustaceans, molluscs and other aquatic animals) has reached about 82 million tonnes (Fig. 1), of which 53.4 million tonnes (FAO, 2020) of fish production. Inland freshwater aquaculture currently dominates the world fish production (Tacon, 2020). In 2018, over 86% of the 53.4 million tonnes of the global aquaculture fish production was obtained from freshwater species (FAO, 2020) with carps and cyprinids as the most farmed species, followed by tilapia and other cichlids (FAO, 2020).

According to FAO, global aquaculture production showed an annual growing rate of 9.8% over the period 1980 – 2000 (FAO, 2018), and 5.3% per year in the period 2001 – 2018 (FAO, 2020), and in 2018 the global aquaculture production exceeding the global capture fisheries production by over 18.32 million tonnes (Tacon, 2020) reaching the 46% of the total production (FAO, 2020). Future prospects estimate that almost two-thirds of the total aquaculture consumption will be farm-raised in 2030 (Kobayashi et al., 2015).

Fig. 1: World capture fish and aquaculture production (FAO, 2020)



The European Union (EU) production regards three main sectors: marine fish, freshwater fish and shellfish. As regards the marine sector, production of seabass (*Dichentrarchus labrax*) and seabream (*Sparus aurata*) is the most extended in EU whit Greece as main producer, while Atlantic salmon (*Salmo salar*) is the most farmed fish in United Kingdom and Ireland. In the freshwater fish sector (trout, carp, European eel, sturgeon), the main producer countries are Italy, Denmark, France and Easter Europe, while shellfish are most produced in France, Spain and Italy (Gutierrez et al., 2020).

In order to contribute to the human nutrition, in the last few decades we have witnessed at the fast development of aquaculture called "Blue Revolution" (Ahmed & Thompson, 2019; Garlock et al 2020). Asia, African continent and Americas, showed a sector growing of 5.89%, 9.81% and 5.45% per year since 2000, respectively. Compared to the other producers, the EU aquaculture have shown a lesser growing trend. During the period 1980 – 2000, the EU production have registered an increasing annual growth rate of 3.5% per year (Gutierrez et al., 2020), decreased at 2.27% per year from 2001 to 2018 (Tacon, 2020).

In April 2013, the European Commission to the European Parliament launched the "Strategic Guidelines for the Sustainable Development of EU Aquaculture" (European Commission, 2013) with the objective to promote consumption and production of seafood in a way that is environmentally, socially and economically sustainable.

After two years, the 2030 Agenda for Sustainable Development whit the 17 Sustainable Development Goals (SDGs), were launched from the United Nations (UN) (United Nations, 2015). One objective of the 14th SDG (Conserve and sustainably use the oceans, seas and marine resources) is the necessity to increase the sustainability of the management of aquaculture. However, if on the one hand aquaculture provided up to 50% of the average per capita intake of animal proteins (FAO, 2020), on the other hand, is also related to unsustainable production practices, especially concerning the use of feed for fish (Osmundsen et al., 2020). The aquafeed usage was almost 59 million tonnes in 2020, and this number is expected to rise about 73 million tonnes by 2025 (Tacon, 2020) due to the increasing number of fish facility.

Traditionally, the aquafeed manufactory is related to the fishmeal (FM) and fish oil production. Due to the high nutritional value and digestibility, for many decades, FM was considered the main dietary protein sources for farmed fish (Caimi et al., 2020a). FM can be obtained from whole fish (small pelagic wild stocks), fish trimmings or fish by-product. In the last few years, the inclusion rate of FM has shown a decreasing trend due to the restricted availability and the price variation (FAO, 2020). As a result, it is now estimated that the inclusion rate of FM in aquafeed is between 10 and 20% (Chemello et al., 2020; FAO, 2020) and many country use fish by-product to produce up to 50% of the total volume of FM (FAO, 2020). From an economic point of view, the global increase of the aquafeed price

due to the increase of the fish demands, has made the feeds' cost a primary problem for the aquaculture sector (Arru et al., 2019). From an environmental viewpoint, the currently existing oceanic resources are not enough to satisfy the huge quantity of FM necessary to produce fish feed (Gasco et al., 2018). The constant increasing of the aquafeed price and the restricted supply of FM, shown that a total dependence on FM is not possible in future years (Chakraborty et al., 2019).

Against this background, researchers and aquafeed producers, acknowledging the importance to improve the environmental and economic sustainability of feed production, have increased the interest to search alternative protein source (Sogari et al., 2019; Hua, 2021).

2. Insect as Feed

2.1 European Regulation on the Use of Insect Meal

In the last few years, insects have been identified as an important protein source of sustainable raw materials for aquafeeds, to reduce or replace the use of FM in aquafeeds. Therefore, after the European Food Safety Authority scientific opinion (EFSA, 2015), in May 2017, the European Commission launched the Commission Regulation (EU) 2017/893 (European Commission, 2017) that amending the previous Regulations on the field of processed animal proteins (PAPs). The use of insect PAP in aquafeeds is allowed since July 2017. Seven species of insect are currently allowed to produce meal: black soldier fly (BSF) (*Hermetia illucens*, HI), common housefly (*Musca domestica*, MD), yellow mealworm (*Tenebrio molitor*, TM), lesser mealworm (*Alphitobius diaperinus*, AD), house cricket (*Acheta domesticus*), silent cricket (*Gryllus assimilis*) and banded cricket (*Gryllodes sigillatus*).

Insects are the natural prey of several farmed animals, such as fish and poultry (Pinotti et al., 2019; Gasco et al., 2020a) and the use of insect to produce meal as feed ingredient shown several strenght: nutrient composition, low space and water requirement, low emission gasses and the capacity to grow on different substrates.

The 12th Sustainable Development Goal expressed by the UN 2030 Agenda is: Ensure sustainable consumption and production patterns (United Nation, 2015). One objective concerns the reduction of waste generation through “prevention, reduction, recycling, and reuse”. Along the food supply chain, one third of the produced food are lost or wasted. Food waste could be used to produce biogas and biofertilizer but are mainly burned or landfilled. As a direct consequence of the rapid expansion of human population, the amount of food waste has been

projected to increase (Paritosh et al., 2017), therefore a better solution to manage food waste is necessary to reduce the environmental impact. The technologies currently used do not recovery macro- and micro-nutrients. The commercial use of insect meal (IM) for aquaculture is a good approach to transform low-nutritional material (i.e., organic waste) in forms of a high amount of protein-rich insect larvae to produce feed (Henry et al., 2015; Gasco et al., 2020b). From an environmental point of view, insect biomass production requires significantly fewer inputs in the form of feed, water and land, compared with other livestock system (Smetana et al., 2019; Ojha et al., 2020). Furthermore, the ability to bioconvert organic waste into high-nutritional value material constitute an example of sustainable circular economy (Sogari et al., 2019; Cadinu 2020) that could help to reduce the food waste accumulation expected in the next years (Ites et al., 2020). Nevertheless, to preserve the food safety policy, in Europe, materials such as animal and catering waste, are forbidden (Gasco et al., 2020a, b). The list of organic substrate included in the EU Regulation, are FM, blood products from non-ruminants, di and tricalcium phosphate of animal origin, hydrolysed proteins from non-ruminants, hydrolysed proteins from hides and skins of ruminants, gelatine and collagen from non-ruminants, eggs and egg products, milk, milk based-products, milk-derived products and colostrum, honey and rendered fats.

2.2 Nutritional Composition of Insect Meal

IM is an interesting protein source for aquafeed, but the nutritional value depends on several factors. The chemical composition and the nutritional value of the meal largely depends on insect species, physiological status, rearing substrate (Meneguz et al., 2018; Ewald et al., 2020) and manufacturing process (i.e., drying procedures, defatting method, addition of diets enzyme) (Gasco et al., 2018; Ravi et al., 2019; Mikołajczak et al., 2020). An overview of the composition of different type of insect meal (defatted, partially defatted and whole meal) from *Hermetia illucens*, *Musca domestica*, *Tenebrio molitor*, *Alphytobius diaperinu*s and crickets (*A. domesticus*, *G. bimaculatus*, *G. sigillatus*) is reported in Table 1.

On average, insect meals are generally rich in protein (30 – 70% on dry matter, DM). The crude protein (CP) content is not particularly affected by the rearing substrate, while is affected by the insect species and the fat content of the meal (Gasco et al., 2018). Typically, lower CP content are reported for full-fat meal, while highest values are reported in defatted and partially defatted meal. The CP content of larva meal, ranging between 39.00 and 60.84% with a balanced essential amino acids (EAA) profile. High content of methionine, phenylalanine and tyrosine was recorded for MD (Hussein et al., 2017), and the CP varying from 28.63 to 58.60%, while in TM larvae meal ranged from 37.87 to 71,28% with a lack of lysine and/or methionine (Henry et al., 2015; Jeong et al., 2020). Concerning the AAE profile, MD and HI are similar to FM (Hussein et al., 2017; Koustos et al., 2019; Reyes et al., 2020) while TM are close to soybean meal (Gasco et al., 2018).

Table 1: Proximate composition and amino acidic content of *H. illucens*, *M. domestica*, *T. molitor*, *A. diaperinus* and cricket meal (*A. domesticus*, *G. bimaculatus*, *G. sigillatus*)

	HI [a]	MD [b]	TM [c]	AD [d]	Crickets [e]
DM (g/100g DM)	91.70	74.03	82.66	93.81	93.72
CP (g/100g DM)	49.61	43.50	56.51	61.76	54.27
EE (g/100g DM)	20.99	19.70	26.52	18.24	17.95
Ash (g/100g DM)	9.85	17.24	4.25	4.34	4.87
Lysine (% protein)	6.4	6.1	4.5	n.d.	5.1
Methionine (%protein)	1.8	2.3	1.5	n.d.	2.0
Methionine + Cystine (% protrein)	2.2	3.0	2.3	n.d.	4.0
Tryptophan (% protein)	0.8	1.8	0.9	n.d.	0.8
Threonine (% protein)	3.6	3.8	3.6	n.d.	3.9
Leucine (% protein)	7.3	5.7	7.6	n.d.	6.7
Isoleucine (% protein)	4.7	2.9	4.1	n.d.	3.7
Valine (% protein)	6.9	3.3	5.5	n.d.	4.3
Histidine (% protein)	3.1	3.0	3.0	n.d.	2.2
Arginine (% protein)	5.4	4.9	4.5	n.d.	6.2
Phenylalanine + Tyrosine (% protein)	11.2	9.8	10.7	n.d.	7.5

Abbreviation: HI, *H. illucens*; MD, *M. domestica*; TM, *T. molitor*; AD, *A. diaperinus*; DM, dry matter; CP, crude protein; EE, eater extract; n.d., not detected. [a] Data from: Devic et al. (2017), Renna et al. (2017), Dietz & Liebert (2018), Dumas et al. (2018), Gasco et al. (2018), Xiao et al. (2018), Cardinaletti et al. (2019), Rimoldi et al. (2019), Fawole et al. (2020), Stejskal et al. (2020), personal data. [b] Data from: Ogunji et al. (2007, 2008, 2009), Aniebo et al. (2009), Gasco et al. (2018), Saelh (2020a), personal data. [c] Data from: Belforti et al. (2015), Sanchez-Muros et al. (2015), Gasco et al. (2018), Iaconisi et al. (2018), Rema et al. (2019), Jeong et al. (2020), personal data. [d] Data from: Adámková et al. (2016), personal data. [e] Data from: Taufek et al. (2016, 2018a, 2018b), personal data.

As far as the lipid content is concerned, insects are rich in lipid (about 10 to 30% on DM) (Gasco et al., 2019). The fat quantity is mainly affected by the physiology. In holometabolous insects (i.e., Diptera and Coleoptera, such as HI, MD, TM and AD), the larval fat content increased with the age and drops in adults, while the moisture content follows an opposite trend. On the contrary, in hemimetabolous insect (i.e., Orthoptera, such as cricket and locusts) the fat content also increased whit the age but in adults is similar to that of late-stage nymphs (Koutsos et al., 2019).

As regard the fatty acids (FA) profile, the composition of the meal depends on the insect species and the rearing substrate. The lipid fraction of HI meal is lacking in n3 and mainly composed form saturated FA (SFA), of which the most represented is lauric acid (C12:0) (Liland et al., 2017; Belghit et al., 2019). Contrary to the other FA, BSF larvae contains high level of lauric acid regardless of the dietary treatment, as larvae neo-synthesized and accumulate this FA (Koutsos et al., 2019). Compared to HI, the FA profile of the common housefly larvae is predominant in monounsaturated FA (MUFA) (57.5%), followed by SFA (38.64%) and polyunsaturated FA (PUFA) (3.86%). The quantity of n3 is lesser than 1% on the total FA (Hussein et al., 2017). In TM the lipid content is characterized by high level of linoleic (C18:2 n6), oleic (C18:1 n9) and palmitic (C16:0) acids (Gasco et al, 2018), the absence of n3 FA (Jeong et al., 2020) and consequently a high n6/n3 ratio (Paul et al., 2017). Crickets are generally lower in long chain PUFA (Grapes et al., 1989) and rich in linoleic, oleic and palmitic acids (Starčević et al., 2017). Overall, terrestrial insects are characterized by a high ratio n6/n3 and a lack of in n3 PUFA. Eicosapentaenoic (EPA, C20:5 n3) and docosahexaenoic (DHA, C22:6 n3) acids are known to exert beneficial effects on human health (Calder, 2018). The FA composition of fish reflects that of the administered diet

(Renna et al., 2017), therefore, the absence of EPA and DHA in insect is the main limitation on the use of insect meal in fish nutrition. Nevertheless, the insects' FA profile can be modified manipulating the rearing substrate. In particular, studies conducted on black soldier fly (St-Hilaire et al., 2007; Ewald et al., 2020; Oonincx et al., 2020), housefly (Hussein et al., 2017), lesser mealworm (Oonincx et al., 2020), and crickets (Starčević et al., 2017; Oonincx et al., 2017) show that the enrichment of the rearing substrate with EPA and DHA sources (i.e., fish by-product, fish oil, flaxseed oil) can be increase the content of these FA in the insect and consequently on the obtained meal. As for the FA profile also the mineral content depends on the insect species and could be manipulated with the rearing substrate (Anderson, 2000; Klasing et al. 2000; Spranghers et al., 2017).

Besides protein and lipid, other valuable compound of insects are chitin and antimicrobial peptides (AMPs) (Mikołajczak et al., 2020). Chitin is an indigestible polysaccharide (Lindsay et al., 1984) and is the primary component of the exoskeleton of insects. The amount of chitin in insect varies according to the species and the development status (Finke, 2007) and its effect on fish is controversial. Several studies shown that at low quantity, chitin is a component whit beneficial effect, able to improve the innate immune response, stimulating the production of immune mediators and cytokine (Gopalakannan & Arul, 2006; Henry et al., 2018a; Stenberg 2019). On the other hand, high chitin negatively affects the diets palatability and the nutrient digestibility, particularly proteins (Kroeckel et al., 2012; Gasco et al., 2016; Piccolo et al., 2017; Caimi et al., 2020a). To overcome these problems, the IM producers can extract the chitin (Belluco et al., 2013; Sánchez-Muros et al. 2014; Gasco et al., 2018) or include dietary enzyme to reduce the amount of chitin and improve the digestibility

(Henry et al., 2015; Mikołajczak et al., 2020). AMPs play an important role on the microbiological homeostasis, stabilizing the activity between animal and host gut microbiota, due to their activity against potentially pathogenic bacteria, viruses, parasites and fungi (Yi et al., 2014; Mikołajczak et al., 2020). Antimicrobial peptides can be used as alternative to antibiotics, improve the fish health and promote the animal welfare (Yi et al., 2014; Henry et al., 2018b; Kvalsvik Stenberg et al., 2019; Gasco et al., 2020c).

3. Use of Insect Meal in Aquaculture Feed

As regards the use of insect meal in aquaculture, a high number of research are focused on freshwater fish due to the high interest for the aquaculture. The use of insect meal in aquaculture feeds it is not a recent topic. The first studies on the inclusion of whole insect larvae or adult and insect meal were performed on bluegill (*Lepomis macrochirus*) (Heidinger, 1971), common carp (*Cyprinus carpio*) (Kim, 1974; Jeyachandran & Raj, 1976), channel catfish (*Ictalurus punctatus*) and blue tilapia (*Tilapia aurea*) (Bondari & Shepard, 1981).

In the last two decades, the number of publications on the use of insect meal in aquaculture is rapidly increased and the research area was expanded with several studies testing different insect species on a high variety of fish species. Although the high number of published papers, some works shown contradictory results. Many factors, such as insect species, rearing substrate of the larvae, and insect meal processing method dramatically affect the results. Generally, high inclusion level negatively affects the growth performances, but results are influenced by species-specific factors. For all these reasons, more studies are necessary to increase the knowledge about the use of insect meal on aquaculture feed.

3.1 Black Soldier Fly in Freshwater Fish

Black soldier fly is one of the most promising insect species for aquaculture feeds production (Caimi et al., 2020a).

The first trial on the use of BSF in freshwater fish was conducted by Bondari & Sheppard (1981) in channel catfish and blue tilapia. In this study, they evaluated the growth response and the consumer acceptance of the fish fed with six experimental diet: two commercial diet with high protein and low protein contend used combined, alone and combined with chopped larvae of BSF. After 10 weeks of trial no significant differences among treatment were recorded for body weight, total length and consumer acceptance. A second trial was performed by the same authors in same fish species (Bondari & Sheppard, 1987). In this case the trial lasted for more weeks - from 10 to 20 weeks – and fish were fed with: control diet (commercial), control diet plus a 10% of FM or 10% of BSF ground larvae, and two diets where fish were fed with only whole or chopped BSF larvae. Compared to the first trial (Bondari & Sheppard, 1981), results show that the use of 100% whole or chopped larvae depressed fish growth on the long period (more than 10 weeks) probably due to a deficiency of nutrients, especially for dry matter and protein.

The evaluation of the inclusion of BSF larvae meal as FM replacement has been widely studied in rainbow trout (*Oncorhynchus mykiss*). The first study on the evaluation of the growth performance was conducted by St-Hilaire et al. (2007) using 14.9% and 29.8% of inclusion of grinded prepupae meal. Results showed that increasing level upper than 14.9% negatively affect the growth performance, moreover, a reduction on n3 was observed. On the contrary, more recent study shown that inclusion level up to 50% of BSF larva meal did not affect growth performance and feed utilization (Renna et al., 2017; Cardinaletti al., 2019; Jozefiak et al.,

2019). This difference is probably due to several factors, such as the processing method and the fat and chitin content of the HI meal. In the last decade, the use of BSF larvae meal as feed ingredient was more explored, including investigation about the influence on gut microbiota (Huyben et al., 2019; Jozefiak et al., 2019; Rimoldi et al., 2019; Terova et al., 2019), nutritional trait of the fillet (Mancini et al., 2017; Secci et al., 2019), sensory quality (Sealey et al., 2011; Borgogno et al., 2017) and the stress response of the fish (Dumas et al., 2018; Elia et al., 2018; Cardinaletti et al., 2019). Overall, all the main effect on the inclusion of BSF meal in rainbow trout was detected, showing that an inclusion level lower than 25% is recommended to prevent unfavourable effects.

Many other studies on the effect of BSF on the performance of freshwater fish were conducted in Eurasian perch (*Perca fluviatilis*) (Stejskal et al., 2020), Jian carp (*C. carpio* var. Jian) (Li et al., 2017; Zhou et al., 2018), grass carp (*Ctenopharingodon idellus*) (Lu et al., 2020), Nile tilapia (Devic et al., 2018; Liebert & Dietz, 2018; Toriz-Roldan et al., 2019) African catfish (*Clarias gariepinus*) (Fawole et al., 2020), yellow catfish (*Plateobagrus fulvidraco*) (Xiao et al., 2018) and Siberian sturgeon (*Acipenser baerii*) (Jozefiak et al., 2019; Caimi et al., 2020a, 2020b; Rawski et al., 2020). Overall, results show that inclusion level up to 14, 22 and 30% of BSF meal can be used to produce aquafeeds for Cyprinids, catfish and Siberian sturgeon, respectively, while an inclusion level lower than 8% is recommended for Nile tilapia.

3.2 Common Housefly in Freshwater Fish

The potential of MD as an alternative protein source has been tested in different freshwater species with promising results. MD were extensively tested in the form of maggot meal, whole larvae or maggot and live maggot.

Oyelese (2007), indicated that juveniles of African catfish feed with 50% live larvae and 50% commercial feed had the best growth performance and benefits in terms of reducing feed cost. Also, Ipinmoroti et al. (2019), reported that African catfish juveniles can be feed with up to 75% of wet maggot reporting best growth, feed efficiency and feed conversion ratio. Other studies showed that up to 75% of maggot meal can be include in substitution of FM in diets for African catfish juveniles (Aniebo et al., 2009; Okore, 2016). A study conducted by Fasakin et al. (2003), showed that defatted maggot meal can be included up to 32% as the only animal protein source in African catfish fingerlings. In other research, results showed that the growth performance were significantly higher in fry catfish feed with 50% of artificial diets mixed with 50% of fresh maggot (Saleh, 2020a).

According to Ogunji et al. (2007), housefly maggot meal could be included up to 68% as total replacer of FM without any adverse effect on the oxidative stress response and growth performance in Nile tilapia fingerlings, while a reduction of digestibility of CP and gross energy was observed in diet with a 30% of maggot meal (Ogunji et al., 2008). On the contrary, Wang et al. (2016), did not reported digestibility reduction using up to 43% of maggot meal while, lower growth performances were recorded. As suggested by the authors, contradictory results could be due to different fish size, feed ingredients and rearing condition (Wang et al., 2016). In addition, the higher ash content of the maggot meal used by

Ogunji et al. (2007) might be the cause of a lower digestibility. The use of live housefly maggot was also tested. Ebenso & Udo (2003) showed that a blend of wheat offal powder with 20% of live maggot could be successfully used to feed Nile tilapia fry. Similar results were obtained by Alofa et al. (2020) in tilapia fingerlings feed with 25% of live maggot, pre-cooked, grinded and blended with the other feed ingredients.

Other study with housefly maggot meal were also performed in common carp (Ogunji et al., 2009, 2011; Herawati et al., 2019) and rainbow trout (St-Hilaire et al., 2007). The use of housefly meal as alternative protein sources had positive results and could reduce the feed cost, particularly in developing countries where the high price of the FM is the main problem (Saleh, 2020b).

3.3 Yellow Mealworm in Freshwater Fish

As far as TM is concerned, the use of both whole larvae and meal was evaluated.

Inclusion level up to 18% of TM meal could be included in diet for different species of catfish without any adverse effect in growth performance and improved immune response and bacterial resistance (Ng et al., 2001; Roncarati et al., 2015; Su et al., 2017). It was also reported that fingerlings of African catfish fed with sliced larvae in combination with commercial pellets grow similarly or better than fish fed with only commercial pellets (Ng et al., 2001).

No negative effects on growth performance were reported in rainbow trout with inclusion levels up to 50% of TM meal (Belforti et al., 2015; Rema et al., 2019; Chemello et al., 2020; Jeong et al., 2020). Substitution of FM with yellow mealworm meal did not affect fillet's physical quality traits (Iaconisi et al., 2018), nutrient retention (Rema et al., 2019), plasma and immune biochemical parameters (Jeong et al., 2020) and metabolic response (Chemello et al., 2020) in rainbow trout. Furthermore, Henry et al. (2018b) reported an increase of the anti-oxidative activity and a reduction in lipid peroxidation in proximal and distal intestine, confirming the improve in the health response of fish. Instead, a worsening on the fillet's fatty acids composition (Belforti et al., 2015; Iaconisi et al., 2018) and a reduction in CP digestibility (Belforti et al., 2015; Chemello et al., 2020) were reported.

In Nile tilapia, inclusion higher than 21% did not affect biometric indexes and fillet's amino acids composition, while negatively affect growth performance (Sanchez-Muros et al., 2015). In a digestibility trial with Nile tilapia fingerlings, TM meal showed the highest apparent digestibility coefficient compared to other insect meals (Fontes et al., 2019). Overall,

in this specie, inclusion level up to 10% of TM meal are recommended to not worsen growth performance, carcass composition, somatic and haematological indexes (Tubin et al., 2020).

3.4 Cricket Meal in Freshwater Fish

The use of different species of cricket has been tested in freshwater fishes. In fingerlings of red hybrid tilapia (*Oreochromis* sp.), inclusion level greater or equal to 60% of *A. domesticus* meal negatively affect the growth performance (Lee et al., 2017). In contrast, Alfaro et al. (2019) reported that *G. assimilis* meal blended with corn meal and commercial feed can support the growth in tilapia in the nursery stage. Fontes et al. (2019), reported lower apparent digestibility coefficient in tilapia fingerlings fed with *G. assimilis*. The authors suggested that low results could be due to the protein's profile, fatty acids quality and chitin content.

Inclusion up to 30% of *G. bimaculatus* improve growth performance and diets digestibility in African catfish (Taufek et al., 2016; 2018a), while inclusion of 35% enhance the disease resistance and the innate immune system (Taufek et al., 2018b).

4. Aim of the PhD project

The focus of the PhD project was to evaluate the use of insect meal, as fishmeal replacement, on growth performance, biometric and morphometric indices, apparent digestibility of diets, whole body and fillet proximate and fatty acid compositions, gut health, stress and metabolic response, in freshwater fishes.

The insect meal used at this purpose were provided by different producers, obtained using various processing methods and different insect species (*Hermetia illucens* and *Tenebrio molitor*).

During the PhD, four papers were published; all the papers presented in the following pages represent the final version of the manuscript currently published or submitted to the Editors.

Over the three years of the PhD, I also had the opportunity to follow several studies on the evaluation of the use of live larvae and insect meal in laying hens, broiler and Muscovy duck.

From this research, has been published: Gariglio M., Dabbou S., Biasato I., Capucchio M. T., Colombino E., Hernández F., Madrid J., Martinez S., Gai F., **Caimi C.**, Oddon, S. B., Meneguz M., Trocino A., Vincenzi R., Gasco L., Schiavone A. (2019). Nutritional effects of the dietary inclusion of partially defatted *Hermetia illucens* larva meal in Muscovy duck. Journal of Animal Science and Biotechnology, 10(1), 37. https://doi.org/10.1186/s40104-019-0344-7

5. Literature Contribution

5.1 First insights on Black Soldier Fly (*Hermetia illucens* L.) larvae meal dietary administration in Siberian sturgeon (*Acipenser baerii* Brandt) juveniles

Published in:

Caimi C., Renna M., Lussiana C., Bonaldo A., Gariglio M., Meneguz M., Dabbou S., Schiavone A., Gai F., Elia A. C., Prearo M., Gasco L. (2020). First insights on Black Soldier Fly (*Hermetia illucens* L.) larvae meal dietary administration in Siberian sturgeon (*Acipenser baerii* Brandt) juveniles. Aquaculture, 515, 734539. https://doi.org/10.1016/j.aquaculture.2019.734539

Abstract

The *Hermetia illucens* (L.) larvae meal (HIM) has been tested on different fish species but its use on Siberian sturgeon has not been investigated yet. The aim of this study was to evaluate the effects of fish meal (FM) substitution with a highly defatted HIM on growth performance, biometric and morphometric indices, apparent digestibility of diets, whole body proximate and fatty acid compositions of *Acipenser baerii* (Brandt) juveniles. Five experimental groups were fed with a FM-based diet without HIM (HIM0), three diets with 25% (HIM25), 50% (HIM50) and 100% (HIM100) of FM substitution with HIM, or a vegetable protein based diet (VEG) without HIM. The feeding trial lasted 118 days and 4 replicates per diet were used. The HIM100 diet was refused by the fish and therefore this experimental group was excluded from statistical evaluation. Moreover, a decrease in feed consumption was recorded with the increase of HIM inclusion. The HIM50 diet negatively affected the growth performance of the fish. The somatic indices were not affected by treatment. Increasing levels of HIM showed increases of dry matter and ether extract contents in the whole body. Compared to HIM0, HIM diets caused modification in lauric acid (up to 65-fold increase) and total saturated fatty acids (up to 1.4-fold increase) contents in the fish whole body. The apparent digestibility coefficients of dry matter, crude protein and gross energy were the highest for the VEG diet (77.0%, 90.4% and 85.8%, respectively). HIM25 and HIM50 showed lower apparent digestibility coefficients of crude protein (86.5% and 86.6%, respectively) when compared to HIM0 (88.5%). Overall, this study showed that it is possible to replace up to 25% of FM with HIM in the diet of Siberian sturgeons (equal to 18.5% HIM inclusion level) without affecting the growth performance, condition factor, biometric and morphometric indices, and whole body proximate

composition of the fish. The fatty acid composition of the sturgeons' whole body was significantly modified already at 18.5% dietary HIM inclusion level.

Keywords: Insect meal; Fishmeal substitution; Animal performance; Fatty acid profile; Apparent digestibility coefficient.

Abbreviations: AA, amino acid; ADC, apparent digestibility coefficient; ADF, acid detergent fibre; ADL, acid detergent lignin; ANFs, antinutritional factors; BCFA, branched chain fatty acids; *c*, *cis*; CP, crude protein; DM, dry matter; EE, ether extract; FA, fatty acid; FAME, fatty acid methyl ester; FBW, final body weight; FC, feed consumption; FCR, feed conversion ratio; FM, fish meal; FR, feeding rate; GE, gross energy; HI, *Hermetia illucens*; HIM, *Hermetia illucens* larvae meal; HSI, hepatosomatic index; IBW, initial body weight; K, Fulton's condition factor; MCFA, medium chain fatty acids; MUFA, monounsaturated fatty acids; nd, not detected; NFE, Nitrogen free extracts; PAPs, processed animal proteins; PER, protein efficiency ratio; PUFA, polyunsaturated fatty acids; SEM, standard error of the mean; SFA, saturated fatty acids; SGR, specific growth rate; *t*, *trans*; TFA, total fatty acids; VEG, vegetable protein based diet; VSI, viscerosomatic index; WG, weight gain.

1. Introduction

By 2050, the demographic growth will result in an increase of 60% in the consumption of products of animal origin and aquaculture is considered as one of the livestock sectors able to support the global demand of animal products (Engle et al., 2017). Between 2001 and 2016, aquaculture production grew about 5.8% per year and such trend is set to grow (FAO, 2018). The intensification of production has led to an increase in the demand for raw materials, especially protein sources, to produce aquafeeds. For many years, fishmeal (FM) represented the main protein source in the production of feed for carnivorous farmed fish. However, in the next few years FM production will not be able to support aquaculture growth anymore (Gasco et al., 2018). To reduce both ecological impact and feed costs, at commercial level FM is partially replaced by vegetable protein meals (Soliman et al., 2017). However, plant feedstuffs raised some nutritional issues that reduce their potential in fish feed formulation (Soliman et al., 2017). The technological advances in plant ingredient processing and the feed extrusion process have solved most of these problems (Merrifield et al., 2011; Gai et al., 2016), but the formulation of diets containing 100% vegetable proteins still causes performance and health issues in carnivorous species where FM can hardly be totally replaced by vegetable proteins (Gai et al., 2012). Globally, terrestrial processed animal proteins (PAPs) are largely used in aquaculture. However, under European regulations only PAPs from non-ruminant animals (poultry and pigs - category 3) are allowed in fish feed formulation (EC No 56/2013). Alternative protein ingredients of animal origins, such as insect meals, may be an alternative solution to overcome this problem and replace, at least partly, the amount of FM in aquafeeds (Henry et al., 2015). Insect meals are rich in essential aminoacids (in particular lysine,

methionine and leucine), have a fatty acid (FA) profile that can be manipulated choosing appropriate rearing substrates (Liland et al., 2017; Meneguz et al., 2018) for insects, and do not have any anti-nutritional factors (Spranghers et al., 2017). One of the most promising insect species to partially replace FM is the Black Soldier Fly (*Hermetia illucens* L. - HI; Stratiomyidae family). HI is able to convert low value by-products and wastes into protein and fat sources (Spranghers et al., 2017; Meneguz et al., 2018) representing a sustainable way to produce edible proteins for livestock feeding. The partial substitution of FM with HI larvae meal (HIM) has been successfully tested in various fish species including rainbow trout (*Oncorhynchus mykiss* Walbaum) (Renna et al., 2017; Elia et al., 2018; Huyben et al., 2018), Atlantic salmon (*Salmo salar* L.) (Lock et al., 2016; Belghit et al., 2018a), European seabass (*Dicentrarchus labrax* L.) (Magalhães et al., 2017), yellow catfish (*Pelreobagrus fulvidraco* Richardson) fry (Xiao et al., 2018), Jian carp (*Cyprinus carpio* var. Jian) (Li et al., 2017; Zhou et al., 2018), and Nile tilapia (*Oreochromis niloticus* L.) (Devic et al., 2018).

The production of caviar and sturgeon's meat is a financially relevant aquaculture sector. Compared to other sturgeon's species, Siberian sturgeon (*Acipenser baerii* Brandt) has a short reproductive cycle (7 to 8 years) and can be reared in fresh water (Bronzi et al., 2011). For these reasons, Siberian sturgeon is one of the most commonly farmed sturgeons (Şener et al., 2006; Bronzi et al., 2011). Compared to other species (e.g., Atlantic salmon, rainbow trout, and European sea bass), less attention has been paid to the nutrition requirements of sturgeons. However, the research of FM substitutes is a crucial point for the future of sturgeon rearing and production (Sicuro et al., 2015). In the last ten years, several authors have evaluated the use of vegetable ingredients other than soybean

meal (Liu et al., 2009), like spirulina microalgae (Palmegiano et al., 2008), rice concentrate (Sicuro et al., 2015) and sesame oil cake or corn gluten (Jahanbakhshi et al., 2013) or animal proteins such as meat and bone, poultry by-products or feather meals (Liu et al., 2009; Zhu et al., 2011) as FM substitutes in sturgeon's diets concluding that a partial FM replacement with these alternative protein sources is feasible without adverse effects. So far, the use of HIM in sturgeon's feeds has not been investigated. Therefore, the purpose of the present study is to assess the effects of the dietary inclusion of *H. illucens* larvae meal as FM replacer on growth performance, biometric and morphometric indices, whole body composition (proximate constituents and FA profile) and apparent digestibility of diets of *A. baerii* juveniles.

2. Materials and methods

A growth trial and a digestibility trial were conducted at the experimental facility of the Department of Agricultural, Forest and Food Sciences (DISAFA) of the University of Torino (Italy). The experiment was designed according to the guidelines of the current European Directive (2010/63/EU) on the protection of animals used for scientific purposes. The experimental protocol was approved by the Ethical Committee of the University of Turin (Italy) (protocol N° 143811).

2.1 Experimental diets

A highly defatted HIM (ether extract – EE: 4.03 g $100g^{-1}$, as fed) purchased from Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, Germany) was used in the trials. The HIM was defatted with a mechanical method; no solvent was used for this purpose. Five experimental diets were formulated to be isonitrogenous (crude protein – CP: on average 50.5 g

$100g^{-1}$, on as fed basis), isolipidic (EE: on average 12.4 g $100g^{-1}$) and isoenergetic (gross energy – GE: on average 20.84 MJ kg^{-1}). The five experimental diets were the following: (i) a FM based diet (HIM0), without HIM inclusion; (ii, iii, iv) three diets with an inclusion, on as fed basis, of 18.5% (HIM25), 37.5% (HIM50) and 75.0% (HIM100) of HIM, with the purpose to replace the 25%, 50% and 100% of FM in the HIM0 diet, respectively; and (v) a vegetable protein based diet (VEG), without HIM inclusion, formulated to replicate currently available commercial feeds. Due to the different chemical composition of HIM compared to FM, and in order to maintain diets isonitrogenous, isolipidic and isoenergetic, the amounts of some other dietary ingredients (i.e. wheat meal and fish oil) were modified with the increase of HIM inclusion in the diets. The experimental diets were prepared at the experimental facility of DISAFA. The ground ingredients were individually weighed and subsequently mixed with fish oil. From 250 to 500 mL kg^{-1} of water was added to promote greater malleability and to obtain a suitable compound for pellet preparation. The pellets were obtained using a 3.0 mm die meat grinder and were dried at 50°C for 48 h. The diets were stored in dark bags at -20°C until utilization. The ingredients of the experimental diets are listed in Table 1.

2.2 Fish feeding and management

A 118 day growth trial was carried out using 20 square fibreglass tanks of 400 L supplied by artesian well water (constant temperature of 13 ± 1°C) in an open system (flow-through) with each tank having a water inflow of 8 L min^{-1}. Dissolved oxygen was measured every week and ranged between 8.5 and 9.3 mg L^{-1} while water pH was equal to 7.6. The fish were exposed to natural photoperiod (April to July 2016). *A. baerii* juveniles

were purchased from a private sturgeon farm (Cisliano (MI), Italy), transported to the experimental facility of DISAFA and acclimated to the rearing conditions for 2 weeks. After the acclimatization period, 440 fish were lightly anesthetised (MS-222 100 mg L^{-1}; PHARMAQ Ltd, Fordingbridge, UK), individually weighed (mean individual initial body weight - iIBW: 24.2 ± 7.59 g) and randomly distributed to each tank (22 fish per tank). The experimental diets were randomly assigned to the tanks (four replicate tanks per diet). The fish were fed by hand to apparent visual satiation, three times a day (8:00, 14:00 and 20:00 h), six days per week. Not ingested feed was siphoned, dried and weighed to record the actual feed consumption (FC) per tank. The fish were weighed individually at the end of the trial (individual final body weight – iFBW). Mortality was checked every day.

2.3 Sampling

At the end of the growth trial, after 24 h of fasting, 6 fish per tank (24 fish per treatment) were sacrificed by over anaesthesia (MS-222, 300 mg L^{-1}), individually weighed (KERN PLE-N v. 2.2; KERN & Sohn GmbH, Balingen-Frommern, Germany; d: 0.001), photographed orthogonally (Lumix G1; Panasonic Corp., Kadoma, Osaka, Japan) with a metric scale (mm) and measured to record fish length (i.e. from mouthpart to the bottom of the caudal fin) with 1 mm accuracy. Data were elaborated using ImageJ software (ImageJ 1.50b, Wayne Rasband, Public domain, National Institute of Health, USA) for the calculation of the Fulton's condition factor (K). Subsequently, 3 of the 6 fish sampled (12 fish per treatment) were frozen, finely ground with a knife mill (Grindomix GM200; Retsch GmbH, Haan, Germany), freeze-dried and stored (-80°C) for final whole body proximate composition and FA profile analyses. The remaining 3 fish

(12 fish per treatment) were eviscerated; the liver and viscera were weighed to calculate the hepatosomatic (HSI) and viscerosomatic (VSI) indices, respectively.

2.4 Data calculation

The growth performance indices were calculated as follows:

- Survival rate (SR, %) = (number of final fish / number of fish at start) × 100;
- Weight gain (WG, g) = iFBW (g) – iIBW (g);
- Specific growth rate (SGR, % day^{-1}) = [(lnFBW - lnIBW) / number of days] × 100;
- Feed conversion ratio (FCR) = total feed supplied (g, dry matter (DM)) / tank WG (g);
- Protein efficiency ratio (PER) = tank WG (g) / total protein fed (g, DM).

Individual initial (iIBW) and final body (iFBW) weight were used to calculate the WG while SGR, FCR and PER were calculated per tank.

The Fulton's condition factor and somatic indices were calculated as follows:

- K = [fish weight (g) / (body length)³ (cm)] × 100;
- HSI (%) = [liver weight (g) / fish weight (g)] × 100;
- VSI (%) = [viscera weight (g) / fish weight (g)] × 100.

2.5 Diets digestibility

At the end of the growth trial, the remaining fish (on average 16 per tank) were used for the digestibility trial. The fish were maintained in their respective tanks and fed the same experimental diets added with 1% celite® (Fluka, St. Gallen, Switzerland) as inert marker. The diets were

prepared as described in section 2.1, and the inert marker was added at the expense of 1% of starch gelatinized. Feed was administered by hand to apparent visual satiation three times per day (8:00, 14:00 and 20:00 h), six days per week. After the first feed administration, all the fish were placed in a metal cage that was perforated on the bottom side, and placed on a rigid plastic tray. In this way, the faeces were settled in the plastic tray, allowing their sampling. As described in Guo et al. (2012), four hours after the meal, the faeces were collected using a suction pipe, placed on blotting paper to remove the excess water and immediately frozen (-80°C) until analysed. After the first faeces collection, the fish were released in their tanks and the same procedure was performed after the second feed administration. To avoid digestibility overestimation, only intact faeces were collected, and faeces collection lasted 20 days. The apparent digestibility coefficients (ADC) of DM, CP and GE were calculated as reported by Renna et al. (2017) and expressed as a percentage.

2.6 Chemical analyses of dietary ingredients, diets and fish whole body

The chemical analyses of the dietary ingredients were performed before diet formulation. Feed samples were ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland) and analysed for DM (#934.01), CP (#984.13) and ash (#942.05) contents according to AOAC International (2000); EE (#2003.05) was analysed according to AOAC International (2003). The GE content was determined using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany). Chitin was estimated according to Finke (2007) by correction considering the amino acid (AA) content of the acid detergent fibre (ADF) fraction and assuming the remainder of the ADF fraction is chitin. The FA composition of HIM and of the experimental diets was assessed as described by Renna et al. (2017). Fatty

acid methyl esters (FAME) were separated, identified and quantified on the basis of the chromatographic conditions reported by Renna et al. (2014). The results were expressed as mg 100 g^{-1} DM (Table 2). The proximate composition of the fish whole body was determined according to the same procedures implemented for feed analyses (AOAC International, 2000; 2003). The freeze-dried and ground samples of the fish whole bodies were also used to assess their FA composition, as reported by Renna et al. (2017). FAME were separated using the same analytical instruments and temperature program previously described for the FA analysis of feeds. Peaks were identified by injecting pure FAME standards as detailed by Renna et al. (2012). The results were expressed as mg 100 g^{-1} ww. All chemical analyses were performed in duplicate.

2.7 Statistical analyses

Data were analysed by one-way ANOVA using IBM SPSS Statistics v. 25.0 for Windows. The following model was used: $Y_{ij} = \mu + D_i + \varepsilon_{ij}$

where Y_{ij} = observation; μ = overall mean; D_i = effect of diet (HIM0, HIM25, HIM50, VEG); ε_{ij} = residual error.

The Kolmogorov–Smirnov test was used to check the assumption of normality. The assumption of equal variances was assessed by Levene's homogeneity of variance test. If such an assumption did not hold, the Brown-Forsythe statistic was applied to test the equality of group means instead of the F one. Pairwise multiple comparisons were performed to test the difference between each pair of means (Tukey's test and Tamhane's T2 in the cases of equal variances assumed or not assumed, respectively). The results were expressed as mean and pooled standard error of the mean (SEM). Significance was declared at $p \leq 0.05$.

3. Results

3.1 Chemical composition of insect meal and experimental diets

The proximate composition of HIM and of the experimental diets are reported in Table 1. The DM, CP and EE contents were comparable among the diets. The ash content showed a reduction at the increase of HIM inclusion in the diet (13.15 to 7.90 g $100g^{-1}$ in HIM0 and HIM100, respectively), while the chitin content increased at increasing inclusion levels of HIM. The FA composition of HIM and of the experimental diets is shown in Table 2. The most represented FA in HIM was lauric acid (C12:0), followed by palmitic (C16:0), myristic (C14:0) and oleic (C18:1 *c*9) acids (2020.65, 435.36, 325.51 and 303.20 mg $100g^{-1}$ DM, respectively). In the experimental diets containing HIM, lauric acid increased proportionally at the inclusion of insect meal. The concentrations of C12:0 in the diets containing HIM were noticeably higher than those observed in HIM0 and VEG. When compared to the other diets, the HIM0 diet showed a higher amount of total n3 FA (1348.15 mg $100g^{-1}$ DM), with C22:6 n3 as the most represented individual FA in this group (666.59 mg $100g^{-1}$ DM). The total n3 polyunsaturated fatty acids (PUFA) decreased proportionally at increasing levels of HIM. The VEG diet showed a lower concentration of total saturated fatty acids (SFA) and was richer in total n6 PUFA (in particular linoleic acid, C18:2 n6) when compared to the other diets. HIM0 showed the highest Σ n3 / Σ n6 PUFA ratio among the considered experimental diets.

3.2 Growth trial

The fish fed HIM100 showed a low diet acceptance, rapidly decreased the voluntary ingestion of feed and, few weeks after the beginning of the trial, they refused the diet. Therefore, to avoid stress or suffering in the animals,

we decided to stop this dietary treatment. These fish were fed again with a commercial diet and excluded from further investigations. The survival and growth performance of the fish are reported in Table 3. Siberian sturgeons fed the HIM0, HIM25, HIM50, and VEG diets easily accepted the experimental diets. Nevertheless, a decrease in feed consumption (FC) was recorded in diets added with HIM (-5.3% for HIM25 and -6.0% for HIM50). This led to a worsening of FBW (-10.9%), WG (-12.9%) and SGR (-6.9%) in the HIM50 group compared to HIM0. Nevertheless, FCR and PER were similar among the treatments. The fish fed the VEG diet reported values comparable to the fish fed the control diet for all the considered dependent variables.

3.3 Biometric and morphometric indices

The Fulton's condition factor, HSI and VSI are reported in Table 4. All the biometric and morphometric indices were not significantly affected by treatment.

3.4 Proximate composition and fatty acid profile of fish whole body

The proximate composition and FA profile of the fish whole body are reported in Table 5. The substitution of FM by HIM progressively increased the DM and EE contents of the fish. Particularly, the fish fed with HIM50 showed a significantly higher DM (+10.0%) content when compared to those fed with HIM0 and VEG, and a significantly higher EE (+38.4%) content when compared to those fed with HIM0. The DM and EE contents were comparable among HIM0, HIM25 and VEG. The CP and ash contents were unaffected by the treatment. As far as the FA composition is concerned, the concentration of C12:0 significantly increased (from 2.70 to 61.23 and 151.61 mg 100 g^{-1} ww in HIM0, HIM25

and HIM50, respectively) as the level of HIM increased in the diet, while VEG and HIM0 showed comparable values. The fish fed with HIM50 showed a higher concentration of C14:0 when compared to the fish fed with HIM0 and VEG (+47.9% in both cases), while the fish fed with HIM25 showed intermediate values. Various branched chain fatty acids (BCFA) were detected, but only a few of them (C17 *anteiso* and C18 *iso*) were affected by the diet. The concentration of total BCFA in the fish whole body did not significantly differ among the treatments. The concentration of C18:1 *c*9 was significantly higher in the fish fed with HIM50 than in those fed with HIM0 (869.27 and 600.58 mg $100g^{-1}$ww, respectively), while the fish fed with HIM25 and VEG showed intermediate values. The concentrations of C18:2 n6, C18:3 n6, and total n6 PUFA were the highest in the fish fed with VEG, followed by the fish fed with HIM50 and HIM25, and finally by those fed with HIM0. As regard to individual long-chain n3 PUFA, the absolute lowest values were recorded in the fish fed with VEG (C20:5 n3) and HIM0 (C22:5 n3 and C22:6 n3), while the absolute highest values were observed in the fish fed with HIM25. The Σ n3 / Σ n6 FA ratio in fish whole body ranked in the order HIM0 = HIM25 > HIM50 > VEG.

3.5 Digestibility trial

The ADCs of the experimental diets are reported in Table 6. The VEG diet showed significantly higher values than all the other diets for all the considered ADCs. ADC_{DM} and ADC_{GE} did not differ significantly among HIM0, HIM25 and HIM50, while ADC_{CP} was significantly lower in HIM25 (- 2.3%) and HIM50 (-2.1%) when compared to HIM0.

4. Discussion

Insects, such as Diptera and Coleoptera, are part of the natural diet of Siberian sturgeons (Pyka and Kolman, 2003). To our knowledge no studies using HIM as protein source have been carried out with sturgeons yet, and our results indicate that up to 18.5% of highly defatted HIM can be included without impairing survival rate and growth performance in this species. Nevertheless, WG and SGR decreased with the increase of HIM in the diets as a consequence of a decrease in feed consumption. At 75% of HIM inclusion (total FM substitution – HI100), we observed a noticeable decrease in feed ingestion by the sturgeons. The decrease in feed consumption recorded with the increase of HIM could be attributable to different causes. One factor could be the chitin content of the diets. It has been reported how even low levels of chitin could decrease feed consumption in fish (Gopalakannan and Arul, 2006; Olsen et al., 2006; Kroeckel et al., 2012). In particular, when feeding turbot juveniles (*Psetta maxima*) with diets containing insect meals, Kroeckel et al. (2012) faced a decrease of feed acceptance at increasing levels of insect meal, and the diet with 75.6% of inclusion of HI prepupae meal showed the lowest daily feed intake and performances. These authors argued that the dietary level of chitin could have been one of the reasons due to the fact that turbot is a fish without gut chitinase activity. Chitin, an indigestible polysaccharide, is a primary component of the exoskeleton of arthropods (such as insects and shrimps). In insects, the amount of chitin varies according to the species and the development stage (Finke, 2007). In the current trial, following the method proposed by Finke (2007), the chitin contents of the experimental diets were equal to 0 (HIM0 and VEG), 0.72 (HIM25), 1.92 (HIM50) and 3.75 (HIM100) g $100g^{-1}$ as fed. Not all studies performed using insect meals reported palatability problems (Lock et al., 2016;

Magalhães et al., 2017; Renna et al., 2017; Belghit et al., 2018a; Devic et al., 2018). It could be argued that these inconsistent results are due to differences in meal composition and processing (as already reported for other PAPs) or as a consequence of the presence/absence of endogenous chitinases in the investigated fish species such as Atlantic salmon, European seabass, rainbow trout and Nile tilapia. Another cause could be the excessive hardness of the pellet observed in the production phase, particularly in the experimental diet where HIM completely substituted FM (HIM100). In white sturgeons (*A. transmontanus* Richardson) larvae, Gawlica et al. (2002) reported that the physical texture of the diet significantly affected feed intake, with negative effects being associated with dry and hard diets. The absolute lowest FC recorded in the fish fed with HIM50 (37.5% of HIM inclusion) reduced the nutrients intake and thus the final fish weight and SGR (1.48). The fish fed HIM25 (18.5% of HIM inclusion) showed comparable FC values than those fed HIM50. They also suffered a reduction of iFBW and SGR when compared to the fish fed HIM0, but such difference was not statistically significant, as it was instead for the fish fed HIM50. On the contrary, Renna et al. (2017) showed that an inclusion of up to 40% of a partially defatted HIM did not affect the FBW and SGR of rainbow trout. Moreover, Xiao et al. (2018) reported better weight gain rate and SGR than a control diet (containing fish and soybean meals as primary protein sources) when yellow catfish were fed with diets containing up to 22.3% of HIM inclusion while, when the inclusion level was higher than 34.3%, a decrease of growth performance was observed. The inconsistency among available literature may be also due to the different fish species considered in the trials. The overall reduction of the growth performance observed in this study can also be related to the reduction of ADC_{CP}, which occurred already at 18.5%

level of HIM inclusion in the diet. The observed decrease of protein digestibility could be determined by the presence of chitin in the insect meal (Renna et al., 2017; Zhou et al., 2018). In European seabass, the inclusion up to 19.5% of HI prepupae meal did not negatively affect the performances or the ADCs of the diets (Magalhães et al., 2017). Renna et al. (2017) reported significant differences for ADC_{DM} and ADC_{CP} between diets with 25 and 50% inclusion of HIM, with the lowest values found in the diet with 50% of HIM inclusion. Chitin interferes with the digestibility of proteins (Marono et al., 2015), but it has also been observed that several fish species are able to synthesize endogenous chitinases, probably due to differences in their gut microbiota. This may explain the apparently contrasting results obtained by different authors while working with different fish species (Henry et al., 2015). To our knowledge, no chitinase activity has been reported in sturgeons. The fish fed with the VEG diet reported similar values than those fed with HIM0 in terms of performance while, as far as digestibility is concerned, the fish fed with the VEG diet showed the highest ADC values. Our results indicate that Siberian sturgeons have good capability to use raw plant materials. A comparative study between rainbow trout and *Acipenser naccarii* Bonaparte showed that sturgeons digest proteins and lipids like carnivorous fish and carbohydrates like omnivorous fish (Furnè et al., 2009). Such a good capability to digest raw plant materials by sturgeons has already been observed by other authors (Liu et al., 2009). Kaushik et al. (1989) showed how Siberian sturgeon juveniles are not able to use complex carbohydrates (such as crude starch) fully, but these authors suggested that the inclusion of pre-treated starch or cereals in diets for sturgeons improve the growth rate and nutrient utilization. It is well known that extrusion can strongly improve the availability of complex carbohydrates. In our trial, the diets

were not extruded and, to balance diets, the level of wheat meal inclusion was reduced from 140 (HIM0) to 100 (HIM50) g kg^{-1} of wet feed. Considering average starch values found in literature for the individual feed ingredients used in our experimental diets (ARRAINA 2015; Xie et al., 2017), we were able to estimate the starch content of the diets that were equal to 111.6, 95.6, 79.7 and 30.8 (g kg^{-1}, as fed) for HIM0, HIM25, HIM50 and VEG respectively. Compared to the VEG diet, the other diets contained a considerable amount of complex carbohydrates that could have contributed to the decrease of ADCs. Even if the VEG diet contained high levels of vegetable meals, no wheat meal was used, obtaining a diet with an overall lower content of complex carbohydrates and then likely more digestible by the sturgeons. Moreover, about 30% of the vegetable feed ingredients used to produce the VEG diet was soybean meal, and it has been reported how sturgeons are able to digest soybean meal efficiently (Degani, 2002; Liu et al., 2009). In particular, Liu et al. (2009) demonstrated that, in sturgeons, the ADC_{DM} and ADC_{CP} of soybean meal are higher than those of FM and PAPs, such as meat and bone meal or poultry by-product meal. Considering the Fulton's condition factor, rainbow trout and yellow catfish fed with HIM showed K ranging from 1.18 to 1.21 (Renna et al., 2017) and from 1.00 to 1.09 (Xiao et al., 2018), respectively. K is an index of the health status of fish and, in some species, values less than 1 are considered indicators of bad health status. In the present trial, K values averaged 0.25 - 0.26, being lower than those found in other fish species. In agreement with our results, in other studies conducted on sturgeons, K values were always less than 1. Indeed, in a growth trial performed by Zhu et al. (2011) on Siberian sturgeons fed with a blend of rendered animal protein, K values equal to 0.7-0.8 were reported. In a study conducted on lake sturgeons (*Acipenser fulvescens*

Rafinesque), K measured at fork length or total length was equal to 0.58 and 0.73 respectively (Jackson et al., 2002). In sturgeons, K values lower than 1 seem then to be a direct consequence of fish morphology (narrow and elongated body) and not a symptom of bad health status. This assumption is confirmed by the high survival rate and overall good performance observed in all the considered treatments. The lack of significant differences in the morphometric indices among dietary treatments confirms the findings of other authors in other fish species fed with insect meals (Renna al., 2017; Xiao et al., 2018). Results on the effects of the dietary inclusion of insect meals on the proximate composition of fish lack consistency in the available literature. Similarly to what previously found by Renna et al. (2017) in rainbow trout fillets, in the current trial the increase of HIM in the diets caused a progressive increase of the DM and EE contents of the sturgeons whole body. In European sea bass juveniles, no significant differences were observed in DM and EE contents of whole body at different inclusion levels of a full-fat *Tenebrio molitor* larvae meal (Gasco et al., 2016). Kroeckel et al. (2012) found decreasing DM and EE contents in the whole body of turbot juveniles while increasing the inclusion level of HI prepupae meal in the experimental diets. As far as HIM0 and diets containing HIM are concerned, the results obtained in our trial are somehow ambiguous. Even if not statistically significant, from Table 4 we can observe an increase in HSI and VSI values from HIM0 to HIM50. Contemporarily, a general decrease of the growth performance of the fish was observed (Table 3). An increase in the TFA content of the whole body was observed in the sturgeons fed with insect meal when compared to the sturgeons fed with the control diet, supporting the recent findings of Belghit et al. (2018b) on freshwater Atlantic salmon. The observed sharp increase (from 0.11% to

4.56% of TFA) in the content of lauric acid at the increase of HIM inclusion in the diet was expected. In fact, as outlined in Table 2, the HIM used in this trial was very rich in lauric acid. This confirms the recent findings of other authors who investigated the chemical composition of HI larvae and prepupae when fed different rearing substrates (Liland et al., 2017; Spranghers et al., 2017; Meneguz et al., 2018). Concerning SFA, a significant increase in the fish whole body was also found for the contents of myristic and arachidic (C20:0) acids, even if to a lesser extent. Particularly because of the substantial increase in C12:0, the total SFA concentration of the sturgeon whole body was found to increase while increasing the level of dietary HIM inclusion. Similar findings were reported by other authors on rainbow trout (Mancini et al., 2017; Renna et al., 2017), Jian carp (Zhou et al., 2018) and Atlantic salmon (Belghit et al., 2018b, 2019) fed *H. illucens* larvae or prepupae meal. The chromatographic conditions applied in our trail allowed us detecting various BCFA with a number of carbon atoms ranging between 15 and 18 (both *iso* and *anteiso* forms) in the sturgeons whole body. Such findings are in line with the individual BCFA found by Wang et al. (2016) in other common freshwater fish species. Other authors previously reported the FA composition of different sturgeon species, such as white sturgeon (Xu et al., 1993; 1996; Gawlicka et al., 2002), Russian sturgeon (*A. gueldenstaedtii* Brandt) (Şener et al., 2005; Zhu et al., 2017), Gulf sturgeon (*A. oxyrinchus desotoi* Vladykov) (Chen et al., 1995), and the hybrid *A. naccarii* × *A. baerii* (Vaccaro et al., 2005). To the best of our knowledge, only two studies previously investigated the FA composition of Siberian sturgeons (Nieminen et al., 2014; Aidos et al., 2019). Despite the available above-mentioned literature on the FA composition of sturgeons, information on BCFA is very scant. Until now, only Chen et al. (1995)

reported BCFA in the muscle samples of cultured and wild Gulf sturgeons. The overall amount of BCFA found in the Siberian sturgeons in our trial (up to 2.30% of TFA) was comparable to that found in ruminant-derived food products (usually considered the most important source of BCFA in the human diet), most probably because we analysed the fish whole body. Indeed Wang et al. (2016) found that fish skin contains on average higher amounts of BCFA compared to different muscle types. Some BCFA were also detected in the insect meal (Table 2). Also Spranghers et al. (2017) found variable amounts of total BCFA in *H. illucens* prepupae reared on different organic waste substrates. However, these authors reported no details regarding the detected individual *iso* and *anteiso* forms, and therefore no comparisons at this regard can be made with our findings. The dietary treatment did not affect the total amount of BCFA, nor the majority of the detected individual *iso* and *anteiso* forms, in the Siberian sturgeons in our trial. In the current trial, the amount of C17 *anteiso* slightly differed in the experimental diets, ranking in the order HIM50 > VEG = HIM25 > HIM0 (Table 2), thus mostly mirroring the concentration of this FA found in the whole body of the fish (Table 5). Regarding MUFA, from a quantitative point of view the most interesting result found in the sturgeon whole body was the proportional increase in the concentration of oleic acid at the increase of HIM inclusion in the diet. Such increase reflected the oleic acid concentration in the experimental diets (Table 2). Oleic acid is by far the most abundant individual MUFA in *H. illucens* larvae and prepupae, and the amount detected in the HIM used in our trial (about 8% of TFA) was very similar to that found in the available literature (Spranghers et al., 2017; Meneguz et al., 2018). As far as PUFA are concerned, the highest total n6 PUFA concentration found in the whole body of the sturgeons fed with the VEG diet was expected. The VEG diet

used in this trial contained corn and soybean as plant materials, linoleic acid being the most abundant FA in both corn and soybean. It is also interesting to notice that the concentrations of C18:2 n6 and total n6 PUFA were higher in the whole body of the sturgeons fed HIM25 and HIM50 when compared to HIM0, most probably as the consequence of the C18:2 n6 content (195.96 mg $100g^{-1}$ DM) found in HIM. Regarding the n3 PUFA, in HIM C18:3 n3 was detected in very low amounts, while EPA, DPA and DHA were not detected (Table 2). The total n3 PUFA in HIM only reached the 0.7% of TFA and this has to be considered a big issue from the point of view of both fish dietary requirements of essential fatty acids and human health related outcomes, when trying to replace fishmeal with insect meal in aquafeed. In the whole body of the sturgeons used in our trial, the total n3 PUFA concentration, as well as the concentrations of EPA, DPA, and DHA did not decrease while increasing HIM in the diet because, with the aim of maintaining the diets isolipidic and isoenergetic, we contemporarily increased the amount of fish oil (Table 2). Adding even low amounts of fish oil while replacing fishmeal with insect meal in commercial aquafeeds may be a strategy to meet the lipid nutritional requirements of the fish and to prevent the lowering of the quality of the lipid fraction of the fish destined to human consumption. Another very promising strategy may be the modulation of the n3 FA concentration in the insects using appropriate growth media (Liland et al., 2017).

5. Conclusion

In this first study performed on Siberian sturgeons, results showed that it is possible to replace up to 25% of FM with a highly defatted HIM without impairing the growth performance, condition factor, biometric and morphometric indices, and whole body proximate composition of the fish.

Only few variations were observed in the fatty acid composition of the sturgeon whole body already at 25% of fishmeal substitution with the insect meal. For future practical applications, it will be necessary to evaluate different insect species and lower levels of insect meal inclusion in the diets for Siberian sturgeons.

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Table 1. Ingredients and proximate composition of *H. illucens* larvae meal and experimental diets.

	HIM	HIM0	HIM25	HIM50	HIM100	VEG
Ingredients (g kg^{-1})						
Fish meal (Chile, super prime)[a]	-	700.0	525.0	350.0	0	320.0
HI larvae meal[b]	-	0	185.0	375.0	750.0	0
Wheat meal	-	140.0	120.0	100.0	55.0	0
Corn gluten meal	-	0	0	0	0	150.0
Soybean protein concentrate	-	0	0	0	0	200.0
Soybean meal	-	0	0	0	0	140.0
Starch gelatinized,D500	-	80.0	80.0	80.0	80.0	80.0
Fish oil	-	60.0	70.0	75.0	95.0	90.0
Vitamine mixture[c]	-	10.0	10.0	10.0	10.0	10.0
Mineral mixture[d]	-	10.0	10.0	10.0	10.0	10.0
Proximate composition[e]						
DM (g 100g^{-1})	94.94	96.41	96.39	96.29	96.83	97.37
CP (g 100g^{-1}, as fed)	62.51	50.29	50.65	50.20	50.27	50.87
EE (g 100g^{-1}, as fed)	4.03	12.68	12.62	12.10	11.73	12.81
Ash (g 100g^{-1}, as fed)	8.20	13.15	11.71	10.24	7.90	9.91
CF (g 100g^{-1}, as fed) [f]	7.0	0.32	1.61	2.89	5.25	1.77
Chitin (g 100g^{-1}, as fed)[g]	4.97	nd	0.72	1.92	3.75	nd
NFE (g 100g^{-1}, as fed)[h]	18.26	23.56	23.41	24.57	24.85	24.65
GE (MJ kg^{-1}, as fed)	20.76	19.77	19.65	20.64	20.38	20.44

Abbreviations: HI, *Hermetia illucens*; HIM, *Hermetia illucens* larvae meal; VEG, vegetable protein based diet; DM, dry matter; CP, crude protein; EE, ether extract; NFE, Nitrogen free extracts; GE, gross energy.

[a] Purchased from Corpesca S.A. (Santiago, Chile). Proximate composition (g $100g^{-1}$, as fed basis): 88.7 DM; 63.8 CP; 8.4 EE; 14.9 ash.
[b] Purchased from Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, Germany).
[c] Vitamin mixture (IU or mg kg^{-1} diet): DL-α tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B_{12}, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg (purchased from Granda Zootecnici S.r.l., Cuneo, Italy).
[d] Mineral mixture (g or mg kg^{-1} diet): dicalcium phosphate, 500 g; calcium carbonate, 215 g; sodium salt 40, g; potassium chloride, 90 g; magnesium chloride, 124 g; magnesium carbonate, 124 g; iron sulphate, 20 g; zinc sulphate, 4 g; copper sulphate, 3 g; potassium iodide, 4 mg; cobalt sulphate, 20 mg; manganese sulphate, 3 g; sodium fluoride, 1 g (purchased from Granda Zootecnici S.r.l., Cuneo, Italy).
[e] Values are reported as mean of duplicate analyses
[f] Crude Fiber estimated according to Feedipedia and Arraina booklet ingredients database
[g] Estimated as ADF – ADFN (Finke, 2007)
[h] Nitrogen Free Extracts; Calculated as 100 − (CP + EE + Ash + Crude Fiber)

Table 2. Fatty acid profile (mg 100g^{-1} DM) of *H. illucens* larvae meal and experimental diets.

	HIM	HIM0	HIM25	HIM50	HIM100	VEG
C12:0	2020.65	22.17	388.43	673.58	1402.94	16.65
C14:0	325.51	452.72	488.41	506.04	471.53	375.93
C15 *iso*	nd	16.61	17.65	15.51	11.18	14.71
C15 *anteiso*	nd	5.40	2.55	3.33	1.27	3.34
C14:1 *c*9 +						
C15:0	15.24	46.20	44.70	45.20	34.13	36.39
C16 *iso*	9.14	16.99	14.42	12.65	14.98	22.64
C16:0	435.36	1102.78	1113.22	1148.65	800.06	942.69
C17 *iso*	nd	29.35	31.33	27.57	13.90	25.57
C17 *anteiso*	3.32	17.07	23.34	29.11	27.27	24.47
C16:1 *c*9	124.05	514.11	555.83	580.22	502.10	527.68
C17:0	3.33	38.92	43.13	44.51	26.63	31.82
C17:1 *c*9	nd	18.05	16.48	18.87	12.12	19.02
C18:0	64.87	271.14	260.78	265.69	165.27	227.11
C18:1 *t*	nd	85.72	103.68	123.01	108.05	124.49
C18:1 *c*9	303.20	930.75	1045.14	1203.62	984.07	1100.78
C18:1 *c*11	9.34	329.89	313.57	325.14	228.54	313.62
C18:1 *c*12	nd	nd	16.70	33.83	20.07	Nd
C18:1 *c*14 +						
*t*16	nd	nd	139.64	275.37	161.70	Nd
C18:2 n6	195.96	195.35	263.29	274.97	253.95	383.26
C18:3 n3	25.08	64.47	81.94	99.46	76.93	79.69
C18:3 n6	nd	10.77	6.89	7.79	3.67	14.07
C20:0	15.84	16.69	36.05	67.64	33.27	17.09
C20:1 *c*9	nd	52.96	68.62	89.99	68.43	80.15
C20:1 *c*11	nd	370.41	437.66	545.61	432.73	512.71
C20:2 n6	7.33	154.85	172.02	148.84	101.83	147.51
C20:4 n6	nd	31.44	9.24	7.68	7.40	27.97

C20:5 n3	nd	580.27	573.06	481.13	301.52	469.54
C22:1 n9	nd	197.72	256.58	339.27	245.21	295.80
C22:5 n3	nd	36.82	43.17	41.52	17.64	40.86
C22:6 n3	nd	666.59	584.14	590.03	248.17	512.89
∑ SFA	2925.97	1989.84	2419.33	2794.26	2968.29	1702.01
∑ MUFA	451.82	2545.82	2998.59	3580.15	2797.14	3010.64
∑ PUFA	228.36	1740.57	1733.76	1651.42	1011.10	1675.79
∑ PUFA / ∑ SFA	0.08	0.87	0.72	0.59	0.34	0.98
∑ n3	25.08	1348.15	1282.31	1212.14	644.26	1102.99
∑ n6	203.29	392.42	534.63	439.29	366.84	572.80
∑ n3 / ∑ n6	0.12	3.44	2.40	2.76	1.76	1.93
TFA	3606.16	6276.23	7151.68	8025.84	6776.54	6388.44

Abbreviations: DM, dry matter; HIM, *Hermetia illucens* larvae meal; VEG, vegetable protein based diet; *c*, *cis*; *t*, *trans*; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; nd, not detected.Values are reported as mean of duplicate analyses.

Table 3. Survival and growth performance of Siberian sturgeon juveniles fed the experimental diets (n = 4).

	HIM0	HIM25	HIM50	VEG	SEM	*p*-value
Survival rate (%)	98.81	97.50	97.62	100.00	0.739	0.528
iIBW (g)	24.20	24.26	24.21	24.19	0.017	0.494
iFBW (g)	159.32^{a}	148.12^{ab}	141.94^{b}	153.32^{ab}	2.198	0.015
WG	135.12^{a}	123.86^{ab}	117.73^{b}	129.13^{ab}	2.203	0.015
FC (g DM)	3003.04^{a}	2844.81^{b}	2823.15^{b}	3052.78^{a}	27.879	0.000
SGR (% d^{-1})	1.59^{a}	1.51^{ab}	1.48^{b}	1.58^{a}	0.015	0.008
FCR	1.03	1.08	1.12	1.05	0.016	0.213
PER	1.94	1.84	1.78	1.88	0.028	0.236

Abbreviations: HIM, *Hermetia illucens* larvae meal; VEG, vegetable protein based diet; SEM, standard error of the mean; *p*, probability; iIBW, individual initial body weight; iFBW, individual final body weight; FC, feed consumption; WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio. Different superscripts within a row indicate significant differences ($p \leq 0.05$).

Table 4. Biometric (n = 24) and morphometric indices (n = 12) of Siberian sturgeon juveniles fed the experimental diets.

	HIM0	HIM25	HIM50	VEG	SEM	*p*-value
K	0.26	0.25	0.26	0.26	0.002	0.051
HSI	2.69	3.02	3.39	3.41	0.123	0.117
VSI	8.04	8.50	8.91	8.76	0.158	0.233

Abbreviations: HIM, *Hermetia illucens* larvae meal; VEG, vegetable protein based diet; SEM, standard error of the mean; *p*, probability; K, Fulton's condition factor; HSI, hepatosomatic index; VSI, viscerosomatic index.
Different superscripts within a row indicate significant differences ($p \leq 0.05$).

Table 5. Whole body proximate (g 100 g^{-1} ww) and fatty acid (mg 100g^{-1} ww) compositions of Siberian sturgeon juveniles fed the experimental diets (n = 12).

	HIM0	HIM25	HIM50	VEG	SEM	*p*-value
Proximate composition						
DM	21.25^{b}	22.37^{ab}	23.37^{a}	21.30^{b}	0.239	0.002
CP	13.66	14.10	13.96	13.45	0.106	0.116
EE	4.50^{b}	5.13^{ab}	6.23^{a}	5.19^{ab}	0.198	0.016
Ash	2.33	2.39	2.40	2.14	0.041	0.095
Fatty acid composition						
C12:0	2.70^{c}	61.23^{b}	151.61^{a}	2.42^{c}	9.364	0.000
C14:0	140.46^{b}	178.88^{ab}	207.80^{a}	140.53^{b}	7.216	0.001
C15 *iso*	6.43	7.13	6.82	6.30	0.225	0.566
C15 *anteiso*	1.74	1.89	1.83	1.59	0.071	0.475
C14:1 *c*9 + C15:0	20.13^{ab}	23.75^{ab}	24.10^{a}	19.36^{b}	0.705	0.024
C16 *iso*	2.82	3.52	3.60	3.67	0.133	0.080
C16:0	531.70	640.78	642.28	551.19	22.924	0.183
C17 *iso*	15.20	16.63	14.20	13.09	0.534	0.110
C17 *anteiso*	16.95^{b}	20.76^{ab}	23.68^{a}	21.18^{ab}	0.791	0.021
C16:1 *c*9	209.56^{b}	264.07^{ab}	289.54^{a}	230.04^{ab}	10.732	0.036
C17:0	15.86	17.11	16.20	15.67	0.515	0.777
C18 *iso*	5.22^{ab}	5.47^{a}	4.08^{b}	4.12^{ab}	0.196	0.012
C17:1 *c*9	12.16	14.81	14.41	12.14	0.521	0.127
C18:0	87.63	104.56	94.80	93.03	2.988	0.245
C18:1 *t*9-11	4.45^{b}	6.10^{ab}	5.84^{ab}	7.15^{a}	0.258	0.001
C18:1 *c*9	600.58^{b}	828.26^{ab}	869.27^{a}	747.45^{ab}	34.283	0.024
C18:1 *c*11	142.82	180.69	180.24	168.59	5.705	0.059
C18:1 *c*12	7.04^{c}	13.68^{b}	16.34^{ab}	18.50^{a}	0.840	0.000
C18:2 n6	71.37^{c}	130.18^{b}	138.15^{b}	180.38^{a}	7.438	0.000

C18:3 n6	3.16^{c}	7.71^{b}	8.64^{b}	11.90^{a}	0.557	0.000
C18:3 n3	19.10^{b}	30.46^{a}	32.04^{a}	30.39^{a}	1.392	0.001
C20:0	12.21^{c}	19.97^{b}	22.26^{ab}	27.04^{a}	1.172	0.000
C20:1 *c*9	41.57^{b}	61.05^{a}	58.10^{a}	58.89^{a}	2.021	0.001
C20:1 *c*11	151.74^{b}	221.46^{a}	216.53^{a}	222.42^{a}	7.966	0.001
C20:2 n6	8.25^{b}	12.79^{a}	13.17^{a}	13.69^{a}	0.532	0.000
C20:3 n6	2.79^{b}	4.80^{a}	4.92^{a}	5.96^{a}	0.240	0.000
C20:4 n6	13.85^{b}	19.38^{a}	15.41^{b}	14.82^{b}	0.587	0.003
C20:5 n3	101.20^{b}	151.76^{a}	117.81^{ab}	88.02^{b}	6.348	0.001
C22:5 n3	23.96^{b}	42.46^{a}	39.59^{a}	33.15^{ab}	1.849	0.001
C22:6 n3	107.59^{b}	164.70^{a}	154.99^{ab}	128.53^{ab}	7.065	0.013
∑ SFA	859.05^{b}	1101.69^{ab}	1213.25^{a}	899.19^{b}	41.428	0.005
∑ BCFA	54.80	62.54	61.03	56.25	1.988	0.467
∑ MUFA	1162.89^{b}	1576.44^{ab}	1633.93^{a}	1446.68^{ab}	60.278	0.028
∑ PUFA	351.28^{b}	564.24^{a}	524.71^{a}	506.84^{a}	21.537	0.002
∑ PUFA / ∑SFA	0.42^{c}	0.53^{ab}	0.43^{bc}	0.57^{a}	0.015	0.000
∑ n3	251.86^{b}	389.38^{a}	344.42^{ab}	280.10^{b}	15.261	0.003
∑ n6	99.42^{c}	174.87^{b}	180.29^{b}	226.74^{a}	8.865	0.000
∑ n3 / ∑ n6	2.51^{a}	2.29^{a}	1.87^{b}	1.24^{c}	0.084	0.000
TFA	2373.22^{b}	3242.37^{a}	3371.90^{a}	2852.71^{ab}	118.481	0.012

Abbreviations: HIM, *Hermetia illucens* larvae meal; VEG, vegetable protein based diet; SEM, standard error of the mean; *p*, probability; ww, wet weight; DM, dry matter; CP, crude protein; EE, ether extract; *c*, *cis*; *t*, *trans*; SFA, saturated fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids.
Values are reported as mean of duplicate analyses.
Different superscripts within a row indicate significant differences ($p \leq 0.05$).

Table 6. Apparent digestibility coefficients (ADC) of the experimental diets (n = 4).

	HIM0	HIM25	HIM50	VEG	SEM	*p*-value
ADC_{DM}	71.5^{b}	70.6^{b}	72.4^{b}	77.0^{a}	0.713	0.000
ADC_{CP}	88.5^{b}	86.5^{c}	86.6^{c}	90.4^{a}	0.436	0.000
ADC_{GE}	83.3^{b}	81.7^{b}	81.4^{b}	85.8^{a}	0.511	0.001

Abbreviations: HIM, *Hermetia illucens* larvae meal; VEG, vegetable protein based diet; SEM, standard error of the mean; *p*, probability; DM, dry matter; CP, crude protein; GE, gross energy.
Different superscripts within a row indicate significant differences ($p \leq 0.05$).

5.2 Could dietary Black Soldier Fly meal inclusion affect the liver and intestinal histological traits and the oxidative stress biomarkers of Siberian sturgeon (*Acipenser baerii*) juveniles?

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Simple Summary: Insect meal is a suitable alternative to fishmeal (FM) in aquaculture feed. In recent years, numerous authors have studied the effects of insect meal as a substitute for fishmeal on fish growth performance, while only a few papers investigated its influence on the physiology and morphology of the digestive system and the oxidative status. The present study evaluated the effects of dietary highly defatted *Hermetia illucens* larva meal (H) inclusion and a vegetable protein based diet (VEG) on histological traits of liver and distal intestinal and oxidative stress biomarkers of liver and kidney in Siberian sturgeon juveniles. The results show that both the VEG the H diets did not influence the liver and gut histology, but the highest inclusion level of H led to changes in oxidative stress biomarkers. Overall, these findings highlighted the possibility to include up to 18.5% of H as FM replacement in Siberian sturgeon diets without affecting the health status of fish.

Abstract: The trial investigates if a highly defatted *Hermetia illucens* larva meal (H) at two dietary inclusion levels and a vegetable protein based diet (VEG) influences the normal gut and liver histology and the oxidative stress biomarkers in liver and kidney of Siberian sturgeon juveniles. Fish were fed 4 diets: one control diet (H0) containing 70% of fishmeal (FM), two diets including 18.5% (H185) and 37.5% (H375) of highly defatted H in substitution for 25% and 50% of FM, and one vegetable protein based diet (VEG). At the end of a growth trial, 12 fish per treatment were sacrificed by over anesthesia to collect 12 liver and 5 distal intestine samples for histological analyses, as well as 12 liver and kidney samples for biochemical analyses. The H and VEG diets did not significantly affect the histology of liver and distal intestine, but alterations of the oxidative stress biomarkers were detected at the highest inclusion level of H (37.5%). In order to avoid unfavorable effects on the fish health, an

inclusion level up to 18.5% of H is recommended for Siberian sturgeon juveniles.

Keywords: Gut histochemistry; *Hermetia illucens;* insect meal; liver histology; oxidative stress biomarkers; Siberian sturgeon

1. Introduction

The constant increasing rate of aquaculture production leads to a consequent increase in volume of fish feeds. Currently, aquaculture is the 4% of the global feed production accounting for about 44 million tons [1] with a constant global growth of about 4% over the last years. To ensure a proper growth of farmed fish, the use of high quality feed ingredients in order to guarantee the optimal feed utilization and constant productivity is necessary. Proteins are the most important macronutrient for animals but represent the most expensive component in feed production [2]. For many years, fishmeal (FM) has been the primary protein source in feed for carnivorous fish, thanks to its high protein level, adequate aminoacid profile, high palatability and digestibility, [2].

In the last few years, the increasing price and low eco-sustainability of FM have prompted the focus towards new sustainable protein sources. The replacing of FM with vegetable proteins (VP) has been widely investigated [2]. The use of VP is advantageous due to their lower price and continuous availability but despite this, the imbalanced amino acid profile, the high carbohydrate content and the presence of antinutritional factors (ANFs), lead to a lower efficiency in feeds utilization, which makes these meals unsuitable for many species [2]. In some species, in particular carnivorous fish, it has been observed that the inclusion of VP as primary protein sources negatively affects growth performance [3,4] and can lead to intestinal dysfunction [5,6,7]. These effects have mainly been attributed to

the presence of ANFs as protease inhibitors, phytates, saponins and lectin able to decrease the nutrient utilization and induce an inflammatory response [2,8,9]. Additional FM substitutes have been identified in the terrestrial processed animal proteins (PAP), such as blood meal, poultry by-product meal, feather meal and meat and bone meal. PAPs are characterized by an optimal amino acid profiles and have shown a protein digestibility like that of FM [10]. Despite this, PAPs have a high variability due to the quality of raw materials and technological processes that limit their use in feeds production [11].

Starting from July 2017, the European Commission has approved the use of insect meals (IM) as protein sources in aquaculture [12]. Compared to FM and other alternative protein sources, the IM production has some advantages: insects can convert low value organic wastes into protein and fat [13], their production leads to lower emission of greenhouse gases and have a lower water footprint [14]. From a nutritional point of view, IM are rich in proteins, with a good essential amino acid (EAA) profile, and have a good content of fat, vitamins and minerals [15]. In the last few years, the potential of IM as partial or complete replacer of FM has been investigated [15] with interesting results both in freshwater fish such as rainbow trout (*Oncorhynchus mykiss*) [16,17], Siberian sturgeon (*Acipenser baerii*) [18,19], Jian carp (*Cyprinus carpio* var. Jian) [20], Yellow catfish (*Pelteobagrus fulvidraco*) [21], Nile tilapia (*Oreochromis niloticus*) [22,23] North African catfish (*Clarias gariepinus*) [24] and mandarine fish (*Siniperca scherzeri*) [25], and marine fish, as in European seabass (*Dicentrarchus labrax*) [26,27], gilthead sea bream (*Sparus aurata*) [28], Japanese seabass (*Lateolabrax japonicus*) [29], Atlantic salmon (*Salmo salar*) [30,31,32] and meagre (*Argyrosomus regius*) [33].

In addition to the evaluation of growth performance associated with a new feed ingredient, other aspects such as the physiology and morphology of the digestive system and the changes in oxidative status are also important parameters to investigate. Intestine is the main site of feed digestion and nutrient absorption, and its health status is capable of greatly affecting the utilization of dietary nutrients [16]. Therefore, a healthy digestive system is fundamental for the fish welfare and growth. Moreover, liver is one of the most important organs involved in nutrient metabolisms [34]. For the above-mentioned reasons, the assessment of the morphological alterations that may occur in these organs is crucial to guarantee the nutritional efficiency of the diet [16,35]. Another important aspect to consider is the role of the intestinal mucins, which are glycoproteins produced by the goblet cells (GC) of the gut mucosa. In particular, the neutral mucins promote the absorption and transportation of nutritive molecules in the plasma membrane [36], also performing an important role in the process of absorption of carbohydrates and fatty acids [37]. Histology together with other physiological indicators can be used to provide knowledge on the tolerance of fish species to new feed ingredients. Oxidative catabolism and nutritional factors may produce changes in levels of oxidative stress biomarkers [38]. Oxidative stress is generated by an imbalance between reactive oxygen species (ROS) and the antioxidant defenses of organism. Mechanisms that involve superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are important protective metabolic pathways and serve as biomarkers of oxidative stress. Both SOD and CAT enzymes catalyze the breakdown of ROS-generating O_2- and hydrogen peroxide (H_2O_2), respectively. The GPx enzyme reduces either H_2O_2 or organic peroxides, whereas glutathione reductase (GR) is involved in regeneration of reduced glutathione (GSH) from

oxidized glutathione (GSSG). The metabolites produced from exogenous compounds metabolism by the superfamily of enzymes of cytochrome P450 (CYP) of phase I are conjugates with polar endogenous constituents in phase II producing water-soluble conjugates that are easily excreted. These reactions are catalyzed by glutathione S-transferase (GST) enzyme. Nevertheless, it is already known that feeding behaviors, as well as environmental conditions may affect the oxidative homoeostasis in fish. Antioxidant defense in fish can vary changing from a marine-based to a plant-based diet; hence, lower transcriptional levels of SOD and reduced GSH concentration and CAT activity can be found in fish liver [39]. Although published data are available as far as the effects of vegetable oils or plant proteins on oxidative stress biomarkers of fish are concerned [39-43], there is still scanty information about the effects of insect inclusion [16,20,44-46].

This study aims firstly to investigate the effects of an highly defatted *Hermetia illucens* larva meal (HIM) dietary inclusion and vegetable protein based diet (VEG) on histological traits of liver and distal intestinal sections of sturgeons, and secondly to assess if changes in levels of biomarkers of oxidative stress (superoxide dismutase, catalase, glutathione peroxidases, glutathione reductase, ethoxyresorufin O-deethylase, and glutathione S-transferase, and malondialdehyde, an indicator of lipid peroxidation) in liver and kidney are related to H and VEG diets.

2. Materials and Methods

A growth trial was conducted at the experimental facility of the Department of Agricultural, Forest and Food Sciences (DISAFA) of the University of Torino (Italy) as reported in Caimi et al. [19]. The experimental protocol was designed according to the guidelines of the current European Directive (2010/63/EU) [47] on the protection of animals used for scientific purposes and approved by the Ethical Committee of the University of Turin (Italy) (protocol N° 143811).

2.1. Experimental Diets and Fish Management

The experimental design, diets and fish management are described in detailed by Caimi et al. [19]. Briefly, four isonitrogenous (crude protein – CP: 50.5 g 100g-1, as it), isolipidic (ether extract – EE: 12.4 g 100g-1, as it) and isoenergetic (gross energy – GE: 20.84 MJ kg-1, as it) diets were formulated (Table 1) as follows: one control diet (HIM0) containing a high level (70%) of FM, two diets contained increasing inclusion levels of highly defatted HIM (Hermetia Deutschland GmbH & Co. KG, Baruth/Mark, Germany in substitution for 25% (HIM25) and 50% (HIM50) of FM (corresponding to an inclusion level of 18.5% and 37.5%, as fed basis, respectively), and one vegetable protein based diet (VEG), formulated to mimic currently available commercial feeds (containing 32% of FM and 49% of plant protein sources).

Table 1. Ingredients and proximate composition of *H. illucens* larva meal and experimental diets (modified from Caimi et al., 2020).

Ingredients (%, as fed)	**Experimental diets**				
	HIM	**HIM0**	**HIM25**	**HIM50**	**VEG**
Fish meal (Chile, super prime)[a]	-	70.0	52.5	35.0	32.0
HI larva meal[b]	-	0	18.5	37.5	0
Wheat meal	-	14.0	12.0	10.0	0
Corn gluten meal	-	0	0	0	15.0
Soybean protein concentrate	-	0	0	0	20.0
Soybean meal	-	0	0	0	14.0
Starch gelatinized, D500	-	8.0	8.0	8.0	8.0
Fish oil	-	6.0	7.0	7.5	9.0
Vitamin mixture[c]	-	1.0	1.0	1.0	1.0
Mineral mixture[d]	-	1.0	1.0	1.0	1.0
Chemical composition (% as fed)[e]					
DM	94.94	96.41	96.39	96.29	97.37
CP	62.51	50.29	50.65	50.20	50.87
EE	4.03	12.68	12.62	12.10	12.81
Ash	8.20	13.15	11.71	10.24	9.91
Chitin[f]	4.97	nd	0.72	1.92	nd
NFE[g]	20.29	23.88	24.15	25.55	26.41
GE (MJ kg^{-1})	20.76	19.77	19.65	20.64	20.44

Note: HI, *Hermetia illucens*; HIM, *Hermetia illucens* larva meal; VEG, vegetable protein based diet; DM, dry matter; CP, crude protein; EE, ether extract; NFE, Nitrogen free extracts; GE, gross energy; nd, not detected. [a] Purchased from Corpesca S.A. (Santiago, Chile). Proximate composition (g $100g^{-1}$, as fed basis): 88.7 DM; 63.8 CP; 8.4 EE; 14.9 ash. [b] Purchased from Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, Germany). [c] Vitamin mixture (IU or mg kg^{-1}): DL-α tocopherol acetate, 60 IU; sodium menadionebisulphate, 5 mg; retinyl

acetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B_{12}, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg (purchased from Granda Zootecnica S.r.l., Cuneo, Italy). [d] Mineral mixture (g or mg kg^{-1}): dicalcium phosphate, 500 g; calcium carbonate, 215 g; sodium salt 40, g; potassium chloride, 90 g; magnesium chloride, 124 g; magnesium carbonate, 124 g; iron sulphate, 20 g; zinc sulphate, 4 g; copper sulphate, 3 g; potassium iodide, 4 mg; cobalt sulphate, 20 mg; manganese sulphate, 3 g; sodium fluoride, 1 g (purchased from Granda Zootecnica S.r.l., Cuneo, Italy). [e] Values are reported as mean of duplicate analyses. [f] Estimated as ADF – ADFN [57]. [g] Calculated as 100 − (CP + EE + Ash + Chitin).

To conduct the trial, a total of 440 fish were lightly anesthetised (MS222 100 mg L−1; PHARMAQ Ltd, Fordingbridge, UK), individually weighed and randomly allocated to sixteen tanks (22 fish per tank). Fish were fed in quadruplicate with the four experimental diets as detailed in Caimi et al. [19]. In the growth trial, an additional treatment where FM was completely substituted by including 75% of the insect meal (HIM100) was also formulated and distributed to the same number of tank replicates. However, due to a very low diet acceptance and in respect of the fish welfare, this treatment was stopped and excluded from any further investigations.

At the end of the growth trial (118 days), fish were fasted for 24h and three fish per tank (12 fish per treatment) were sacrificed by over anaesthesia (MS-222 - PHARMAQ Ltd, Fording bridge, UK; 300 mg L-1) to collect 12 portions of liver and 5 distal intestines (spiral valve) for histological analyses, and 12 portions of liver and kidney for biochemical analyses.

2.2. Sampling and Histological Investigations

Sections of liver and distal intestine (spiral valve) were collected and fixed in 10% buffered formalin from twelve and five fish/treatment, respectively. The collected tissues were dehydrated in a graded ethanol series and embedded in paraffin wax blocks. Sections of 4 ± 2 μm thicknesses were cut with a microtome (Leica SM2000R, Leica Biosystem), mounted on glass slides and stained with Mayer Haematoxylin & Eosin (HE). The HE liver sections were examined by means of light microscopy in order to evaluate the following histological changes: nuclear displacement, cytoplasm vacuolization, lymphocytic infiltrates and necrotic tissue areas. The observed histopathological findings on HE liver sections were assessed using a four-graded semi

quantitative scoring system as follows (Table 2): absence of alterations (score 0); mild alterations (score 1); moderate alterations (score 2); severe alterations (score 3).

Gut sections were stained with the periodic acid-Schiff (PAS), which identified the neutral mucins in magenta [49]. One slide per intestinal section was examined by light microscopy. Five randomly selected high power fields per each slide were captured with a Nikon DS-Fi1 digital camera to a Nikon microscope (Nikon Instruments Europe B.V., Italy) using a 10× objective lens and NIS-Elements Ar 4.50.0064 Software (Nikon Instruments Europe B.V., Italy) was used for image capturing. The villus height (Vh) was evaluated on 5 villi per captured field (25 villi/sample) and the total number of PAS-positive goblet cells (GC) was counted on the five captured fields, according to Baeza-Arino et al. [35] and Pryor et al. [50]. Gut sections were also stained with Mayer Haematoxylin & Eosin (HE) and examined by means of light microscopy in order to evaluate the lymphocytic infiltrates using the above-mentioned four-graded semi quantitative scoring system (Table 2).

Table 2. Semi quantitative scoring system adopted for the assessment of the liver and the intestinal histological traits.

Alteration	**Score 0**	**Score 1**	**Score 2**	**Score 3**
Liver				
Vacuolization	Absent	Mild, focal to multifocal vacuolization of the hepatocytes (<25% of the cells is affected)	Moderate, multifocal to diffuse vacuolization of the hepatocytes (>25%, but <50% of the cells is affected)	Severe, multifocal to diffuse vacuolization of the hepatocytes (>50% of the cells is affected)
Nuclear displacement	Absent	The nucleus of the hepatocytes is mildly displaced towards the cell membrane	The nucleus of the hepatocytes is moderately displaced towards the cell membrane	The nucleus of the hepatocytes is severely displaced towards (and adhered to) the cell membrane
Lymphocytic infiltrates	Absent	Mild, focal to multifocal interstitial and/or perivascular lymphocytic infiltrates (<25% of the liver parenchyma is affected)	Moderate, multifocal to diffuse interstitial and/or perivascular lymphocytic infiltrates (>25%, but <50% of the liver parenchyma is affected)	Severe, multifocal to diffuse interstitial and/or perivascular lymphocytic infiltrates (>50% of the liver parenchyma is affected)
Necrotic tissue area	Absent	Small, focal to multifocal areas of hepatocyte necrosis	Small to large, multifocal areas of hepatocyte necrosis	Large, multifocal to diffuse areas of hepatocyte necrosis

Spiral valve				
Lymphocytic infiltrates	Absent	Mild, focal to multifocal mucosal, submucosal and/or muscular lymphocytic infiltrates (<25% of the intestinal wall is affected)	Moderate, multifocal to diffuse mucosal, submucosal and/or muscular lymphocytic infiltrates (>25%, but <50% of the intestinal wall is affected)	Severe, multifocal to diffuse mucosal, submucosal and/or muscular lymphocytic infiltrates (>50% of the intestinal wall is affected)

2.3. Biochemical Analyses

Oxidative stress biomarkers were evaluated in liver and kidney of each sample by spectrophotometer analysis (Varian Cary spectrophotometer at constant temperature of 25°C) [16].

For malondialdehyde (MDA) analysis, sample was homogenized (1:7 p/v) in 20 mM Tris/HCl buffer pH 7.4, and 0.5 M butylated hydroxytoluene (BHT), centrifuged at 3000 x g for 10 minutes at 4°C. The supernatant was derivatized in 1-methyl-2-phenylindole (10.32 mM in acetonitrile/methanol diluted 4:1), concentrated HCl and dilution buffer (Tris/HCl, pH 7.4), sample or MDA standard (0–4 μM of 1,1,3,3-tetramethoxypropane). All samples were incubates for 60 minutes at 45°C and then centrifuged at 15000 x g for 10 minutes, 4°C. Concentration of MDA was read at 586 nm. Results were reported as nanomoles per gram of tissue.

For all enzyme analysis, except for ethoxyresorufin O-deethylase (EROD), tissue was homogenized by an Ultra Turrax homogenizer in 100mM potassium phosphate buffer (KP) pH 7.5 and 2.5% sodium chloride (NaCl), aprotinin 0,008 TIU/ml and 0.1 mg/ml bacitracin. Homogenates were centrifuged at 11000 x g for 45 min and then at 50000 x g for 90 min. Superoxide dismutase (SOD) activity was performed at 550 nm in 50mM Na2CO3, pH 10, 0.1 mM EDTA, 500mM cytochrome C and 1mM hypoxantine and xantine oxidase. The reduction of cytochrome C by the xantine/hypoxantine system was measured versus a standard curve of SOD units or 50 μl of sample (diluted 1:50). One unit of SOD is defined as the amount of enzyme necessary to inhibit 50% of the reduction of cytochrome C.

Catalase (CAT) activity was performed at 240nm (ε = - 0.04 $mM^{-1}cm^{-1}$) following the decrease in absorbance of H_2O_2. The assay was carried out

in a 100mM NaH_2PO_4 + Na_2HPO_4 buffer pH 7 and 24mM H_2O_2 and 10 µl of sample.

Glutathione peroxidase (GPx) activity was measured at 340nm (ε = - 6.22 $mM^{-1}cm^{-1}$) in a 100mM NaH_2PO_4 + Na_2HPO_4 buffer pH 7,5, 1mM EDTA, 0.12 mM NADPH (β-Nicotinamide adenine dinucleotide), 2 mM GSH, 1mM NaN_3, 1 U of GR (glutathione reductase) and 0.6mM H_2O_2 and 20 µl and 50 µl of sample for liver and kidney, respectively. The assay follows the oxidation of NADPH.

Glutathione S-transferase (GST) activity was performed at 340 nm (ε = 9.6 $mM^{-1}cm^{-1}$) using CDNB (1-chloro-2,4-dinitrobenzene) as substrate. The assay was measured at 340nm in 100mM NaH_2PO_4 + Na_2HPO_4 buffer 100mM pH 6.5, 2mM GSH and 2mM CDNB and 20 µl of sample.

Glutathione reductase (GR) was measured at 340nm (ε = - 6.22 $mM^{-1}cm^{-1}$) in 100mM NaH_2PO_4 + Na_2HPO_4 buffer 100mM pH 7, GSSG (oxidized glutathione) 1mM and NADPH 0.06mM and 25 µl and 50 µl of sample for liver and kidney, respectively. The assay measured the decrease in absorbance due to the oxidation of NADPH.

The EROD activity was assessed on S9 cytosolic fraction after the homogenization in 100 mM KP, pH 7.5 with 0.15 M KCl and 1mM EDTA (1:5). Enzyme activity was measured spectrofluorimetrically following resorufin production at 535/585 subsequent incubation in 100mM KP buffer, pH 7.5, 4 mM 7-ethoxyresorufin and 0.25 mM NADPH for 5 minutes and 100 µl of sample. The concentration of the protein in the cytosol was determined according to Lowry et al. [51] and was used to normalize the biomarker levels.

2.4. Statistical Analyses

Prior to statistical analysis, all the obtained data were evaluated for normality of distribution by Shapiro-Wilk's test and when the assumption was not satisfied a logarithmic data transformation was applied. The Vh, number of GC and biomarker data were analysed by one-way ANOVA using IBM SPSS Statistics v. 25.0 for Windows. The following model was used: $Y_{ij} = \square + D_i + \varepsilon_{ij}$, where Y_{ij} = observation; μ = overall mean; D_i = effect of diet (HIM0, HIM25, HIM50, VEG); ε_{ij} = residual error. Tukey's HSD test was performed to discriminate the differences between the experimental treatments and the control groups. The histopathological scores were analysed by means of the Kruskal-Wallis test (post-hoc test: Dunn's Multiple Comparison test). Results are given as mean and SD. The criterion for significance was set at $P < 0.05$.

3. Results

3.1. Histological Investigations

Dietary HIM inclusion did not significantly affect ($P > 0.05$) the liver histopathological changes (Table 3, Figure 1A-B). Analogously, the Vh and the number of GC showed no significant differences ($P > 0.05$) among the dietary treatments (Table 4, Figure 1C-D). The spiral valve also displayed no significant lymphocytic infiltrates in all the dietary treatments (Table 2).

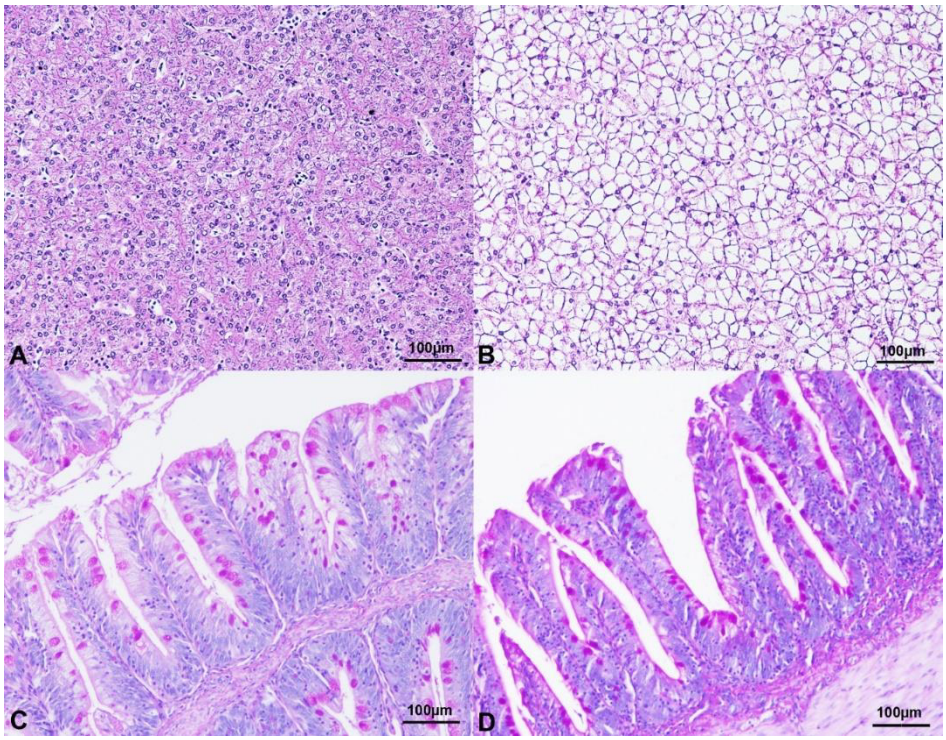

Figure 1. Histological and histochemical findings of Siberian sturgeon in the current trial. A) Liver, Haematoxylin & Eosin stain, 10× magnification. HIM0 diet. Occasional, multifocal vacuolization of the hepatocytes. B) Liver, Haematoxylin & Eosin stain, 10× magnification. Severe and diffuse vacuolization of the hepatocytes. VEG diet. C) Spiral valve, 10× magnification. Numerous goblet cells strongly reacting to periodic acid-Schiff (PAS) stain are evident along the spiral valve epithelium. HIM25 diet. D) Spiral valve, 10× magnification. Several PAS-positive goblet cells are identified in the spiral valve villi. VEG diet. Abbreviations: H, *Hermetia illucens* larva meal; VEG, vegetable protein based diet

Table 3. Effects of dietary *H. illucens* larva meal inclusion on liver histopathological findings of the sturgeons (n = 12).

Items	Experimental diets				SEM	*p*-value
	HIM0	HIM25	HIM50	VEG		
Liver						
Vacuolization	2.67	2.58	2.83	2.75	0.073	0.663
Nuclear displacement	1.50	1.83	1.92	2.08	0.100	0.215
Lymphocytic infiltrates	1.42	1.33	1.33	1.67	0.098	0.601
Necrotic tissue area	Absent	Absent	Absent	Absent		
Spiral valve						
Lymphocytic infiltrates	Absent	Absent	Absent	Absent		

Note: HIM, *Hermetia illucens* larva meal; VEG, vegetable protein based diet; SEM, standard error of the mean; *p*, probability. Data are expressed as mean of the histopathological scores.

Table 4. Effects of dietary *H. illucens* larva meal inclusion on gut morphology and neutral mucin-producing goblet cells of the sturgeons (n = 5).

Items	Experimental diets				SEM	*p*-value
	HIM0	HIM25	HIM50	VEG		
Vh (μm)	330.93	313.16	338.04	314.54	4.290	0.105
GC number	61.28	77.08	68.76	67.44	2.909	0.293

Note: HIM, *Hermetia illucens* larva meal; VEG, vegetable protein based diet; Vh, villus height; GC, Goblet cell; SEM, standard error of the mean; *p*, probability. Data are expressed as mean.

3.2. Oxidative Stress Biomarkers

The effects of experimental diets on biomarkers of oxidative stress of Siberian sturgeon are shown in Table 5.

The MDA concentrations in liver and kidney of control and HIM and VEG fed diets did not noticeably change ($P > 0.05$). The two HIM diets produced a dose-dependent increase (50%) of SOD activity in mainly kidney ($P=0.001$), whereas HIM50 caused a lower (30%) GPx activity in mainly liver ($P=0.027$). GR activity increased (35%) with the highest HIM inclusion in liver ($P=0.001$), while the same activity in kidney was higher (40%) at both HIM doses ($P =0.026$). Higher EROD activity was found in kidney of HIM25 (6 fold, $P<0.001$) and HIM50 (4 fold, $P=0.005$), whereas no changes were found for the enzyme level in liver of HIM groups ($P >0.05$). GST activity was higher (50%, $P=0.001$) in kidney of HIM50 and did not change in liver of both HIM groups, compared to own controls ($P >0.05$).

The levels of biochemical indicators in both tissues of sturgeons fed the VEG diet showed higher levels of CAT (40%) in both tissues and mainly in kidney ($P=0.003$), of GPx (40%, $P=0.003$) and GR (45%, $P=0.003$) in kidney and lower GST (35%, $P= 0.006$) activity in liver.

Table 5. Effects of dietary *H. illucens* larva meal inclusion and vegetable protein based diet on oxidative stress biomarkers in liver and kidney of sturgeons.

Items	Experimental diets				SEM	p- value
	HIM0	HIM25	HIM50	VEG		
Liver						
MDA	150.73	140.81	159.64	129.13	4.53	0.094
SOD	23.53^{b}	20.18^{b}	33.22^{a}	26.84^{ab}	1.25	0.001
CAT	614.13^{b}	644.23^{b}	734.23^{ab}	836.06^{a}	20.51	0.001
GPx	69.75^{a}	55.71^{ab}	50.53^{b}	57.16^{ab}	2.48	0.037
GR	30.07^{b}	30.42^{b}	46.63^{a}	34.35^{b}	1.59	0.001
EROD	13.22	17.96	10.60	15.01	1.06	0.077
GST	246.50^{a}	239.30^{a}	274.39^{a}	155.80^{b}	11.16	0.001
Kidney						
MDA	108.49	111.55	108.75	121.64	5.47	0.816
SOD	12.61b	18.51ab	22.95a	18.82ab	1.02	0.001
CAT	85.31b	81.10b	109.18ab	134.15a	5.59	0.003
GPx	53.55bc	67.78b	36.24c	83.64a	4.18	0.001
GR	16.64b	26.63a	25.94a	29.52a	1.36	0.003
EROD	6.34c	38.07a	22.42b	12.32bc	3.49	0.001
GST	48.05b	62.55b	95.22a	52.60b	4.86	0.001

Abbreviations: HIM, *Hermetia illucens* larva meal; VEG, vegetable protein based diet; Data are reported as mean; SEM, standard error of the mean (n=12). *p*, probability. Different letters (a, b, c) indicate statistically significant differences (Tukey's multiple comparisons test) between the experimental groups (HIM or VEG). MDA (nmol/g tissue); SOD (U/mg prot), CAT (µmol/min/mg prot), GPx, GR and GST (nmol/min/mg prot), EROD (pmol/min/mg prot).

4. Discussion

4.1. Histological Investigations

One of the limiting factors of including insect meal in fish feed is their chitin content, that is able to determine gut and liver histological changes [16]. In particular, chitin could affect liver lipid accumulation [20] and may induce intestinal inflammation [52]. The histological effects on liver and gut by HIM utilization in diets for different fish species have already been characterized, with different results being obtained till now [16,20,24,31]. Our results showed that up to 37.5% of highly defatted HIM can be included in Siberian sturgeon feed without any negative effects on spiral valve and liver histology.

No histopathological alterations were observed in the liver of Siberian sturgeons fed HIM based diets. This result is in agreement with those observed in Japanese seabass [29], Atlantic salmon [30,31], zebrafish (*Danio rerio*) [52] and rainbow trout [16] fed with HIM meal. On the contrary, Li et al. [20] observed significant alterations in liver (decrease hepatopancreas fat deposition and increase in mild necrosis and apoptosis of hepatocytes) in Jian carp (*Ciprinus carpio* var. Jian) fed 7.9% inclusion level of HIM. Another one recent study shows that inclusion level up to 40% of mopane worm meal (*Imbrasia belina*) cause worsened in liver degradation, probably caused by the high fiber content in the mopane worm [24].

In most fish species, the dynamics of absorption decrease proceeding towards the posterior tract of the intestine. Since in sturgeon the maximum nutrient absorption takes place into the posterior intestine (spiral valve) [53], this segment was herein considered. In the present study, no significant differences were observed between the Vh and the number of GC between HIM0, VEG and HIM-fed sturgeons. This is in agreement

with what was reported in rainbow trout [16,54], post smolt Atlantic salmon [30] and Japanese seabass [29] feed with HIM meal. Another study conducted in Siberian sturgeon fed with HIM-based diet [18], show that in the proximal intestine, the inclusion of 15% of full-fat HIM did not affect the villus height, but the authors reported a reduction in the thickness of mucosa and increase in muscle layer thickness; changes that has been associated with an enhanced of the digestion and absorption process.

An increase in the number of GC in the distal intestine is usually associated with an immune reaction during the inflammation process and a decrease in nutrient absorption [35]. This gut mucin dynamics are frequently related to high inclusion levels of VP sources in fish diets [7,35]. In particular, the use of VP may cause morphological alterations capable of affecting the optimal nutrient absorption [6,55]. However, the effects of VP usually vary on varying the species, the protein source and the level of inclusion [35,56-58]. Based on the authors' knowledge, the effects of VP on the liver and gut histology of sturgeon have been only characterized by Kittel et al. [59]. In particular, the authors formulated four experimental diets for shovelnose sturgeon (*Scaphirhynchus platorhynchus*) containing increasing levels of soybean meal (from 0 to 51.23%) as partial replacement of FM and highlighted histopathological signs of distal enteritis in fish fed soybean meal-based diets confirming what was reported by previous studies [6,34,55].

4.2. Oxidative Stress Biomarkers

The dietary inclusion of HIM meal in substitution of 25% and 50% of FM and VEG diet did not significantly alter the histological traits of liver and distal intestine sections suggesting no adverse effect on digestive capacity of Siberian sturgeons. However, all the experimental diets (HIM25,

HIM50 and VEG) have led to a disturb of some oxidative stress indicators in liver and kidney of sturgeons.

Both kidney and liver tissues chosen for this study have high potential for ROS production and response of the oxidative stress biomarkers in fish is tissue-specific [60-63].

Each biomarker, under chemical or physical pressure, is prone to change and, within the different treatments, the response of all biochemical parameters can be dissimilar. In order to deepen the knowledge on the effects of HIM and VEG diets on sturgeons, a panel of physiological and biochemical markers was thus applied. Fish fed the HIM (25% and 50% substitution) and VEG diets showed increased levels of several biomarkers in liver and especially in kidney. Although HIM25 diet led to sporadic changes in renal enzymes activity, altered detoxifying response occurred more frequently in sturgeon fed HIM50. The GPx showed lowest value in both liver and kidney of specimens following HIM50 diets. However, no marked lipid peroxidation was observed through the experiment.

In the present study, the altered activity of several antioxidant enzymes, mainly in sturgeon fed HIM50, may be related to the diets composition. Hepatic increased levels of antioxidant enzymes, such as CAT, GPx and GR, have been previously associated to protein- or lipid-rich FM diets in Adriatic sturgeon *Acipenser naccarii* [64]. Although HIM25 and HIM50 were isoproteic, isolipidic and isoenergetic, differences in fatty acid profile were found compared to HIM0 diet [19]. Indeed, the levels of total fatty acids (TFA) were consistently higher in HIM50 compared to HIM0 (8025.84 *vs* 6276.23 mg/100 g dry matter (DM), respectively), as well as saturated fatty acids (SFA - 2794.26 *vs* 1989.84 mg/100 g DM, respectively), and monounsaturated fatty acids (MFA -3580.15 *vs* 2545.82 mg/100 g DM, respectively). Moreover, the ratio PUFA/SFA in HIM50

diet was found lower than control diet (0.59 *vs* 0.87 mg/100 g DM, respectively). Sturgeon fed HIM50 also showed a reduced feed consumption compared to fish fed HIM0 (2823.15 and 3003.04 g DM, respectively), resulting in a diminished final body weight (141.94 g and 159.32 g, respectively), weight gain (117.73 g and 135.12 g, respectively), and specific growth rate (1.48 and 1.59 %/day, respectively) [19]. Starvation was previously associated with a triggered antioxidant capacity in Siberian sturgeon, mainly increasing the activity of SOD [65]. Similarly, in our study a higher SOD activity was found in specimen fed HIM50. We may thus assume that the raised SOD and GR levels in liver and kidney of *A. baerii* may be related to the different lipid composition and lower HIM consumption.

Also the polysaccharide chitin, one of the centerpieces of insect exoskeleton and present at higher levels (up to 1.92 g/kg) in HIM substituted diets, deserves attention. Chitin has been recognized as an antioxidant molecule, also preventing deleterious effects in various diseases [66,67] and boosting the SOD activity in orange-spotted grouper (*Epinephelus coioides*) [68]. Chitin role is very complex and, as for other antioxidant, such as selenium [69-70], may act as pro-oxidant when the optimal threshold is encompassed. In the present study, significantly higher chitin dose (1.92 g/kg) were found in HIM50 diet and may be related to the lower GPx activity in both liver and kidney of sturgeon. This outcome is not surprising and was previously discussed in rainbow trout (*Oncorhynchus mykiss*) as associated with the ability of polysaccharide to bind selenium present in the diets, both in inorganic (selenite and selenite) and organic (selenomethionine and selenocysteine) form [16]. In particular, selenocysteine is essential for GPx functioning since contains a

selenocysteine residue in its structure and its unavailability following chitin bond may have reduced the enzyme activity.

In the present study, GST was affected by both the included diets (HIM25, HIM50) and VEG, although different responses were observed. Similarly to our previous results in rainbow trout [16], GST was enhanced only in kidney of sturgeon fed the higher substituted diet. This outcome and the concomitant increase of EROD suggest a strengthening of the detoxifying ability in groups fed HIM50 and an important role in the biotransformation of lipophilic compounds. Increase in GST activity has been previously reported in African catfish liver fed with cricket meal (*Grillus bimaculatus*) [46], while no changes were measured in carp fed with domestic fly larva meal [44]. On the contrary, the sturgeon fed VEG showed a decreased GST level. Previous study showed that β-conglycinin, one of the major allergenic proteins present in soybean [72] can decrease GST activity in intestine and enterocytes of carp, concomitant with an increased expression of the main antioxidant genes [73]. The dropped hepatic GST levels measured in our study, may suggest a pointing scenario, since the reactive intermediates produced by the phase I enzyme (CYP) may not be adequately removed.

Nevertheless, the unchanged MDA levels in both liver and kidney in sturgeon fed with both the categories of substituted diets indicate preserved antioxidant efficiency. The lack of lipid peroxidation following vegetable meal is not surprising. Peng et al. [74] measured a reduction in lipid peroxidation in fish fed with soybean meal, probably correlated with an increased storage of hepatic α-tocopherol. Likewise, Wang et al. [29] find a lower concentration in MDA in Japanese seabass fed H revealing that this diet may improve the antioxidant status of fish. On the contrary, Ji et al. [75] showed that the amino acid deficiencies in diets substituted

with higher silkworm pupae doses caused lipid peroxidation and oxidative damage, also altering the intestinal microvilli and hepatocytes structure in juvenile Jian carp. This outcome suggests that lipid peroxidation may be related to fish species and/or protein source.

In conclusion, the inclusion of a highly defatted HIM and VP does not significantly affect the histology of liver and distal intestine of Siberian sturgeon. Nevertheless, as unfavorable effects on antioxidant response were reported at 37.5% of HIM inclusion, an inclusion level up to 18.5% is recommended for sturgeons.

Considering the high longevity of the sturgeons, further investigations are required to observe the long-time effects of insect and vegetable meals on gut and liver histology and the oxidative stress biomarkers of Siberian sturgeon.

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5.3 Partially defatted *Tenebrio molitor* larva meal in diets for grow-out rainbow trout, *Oncorhynchus mykiss* (Walbaum): effects on growth performance, diet digestibility and metabolic responses

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Simple Summary: The current developments in the aquaculture sector have highlighted the need to find sustainable ingredients to replace fishmeal as a protein source in fish feeds. The use of insect meals may be a valid option, due to their good nutritional values and low ecological footprint. In this study, we evaluated the effects of a progressive fishmeal substitution with increasing concentrations of a partially defatted yellow mealworm meal in rainbow trout diets. We observed that the total substitution of fishmeal with insect meal is feasible and that there are no negative effects on fish growth or on the digestibility of most nutrients. The activities of hepatic enzymes involved in the amino acid metabolism and lipid synthesis were also evaluated. The enzymatic activities were not negatively influenced by insect meal inclusion in the diets. These results are of practical application for feed manufacturers and farmers, as they support the inclusion of insect meals in fish diets to obtain sustainable feeds that able to support an increase in aquaculture production.

Abstract: Insect meals are good candidates to replace fishmeal as a new protein sources in aquafeeds. This study evaluated the effects of fishmeal replacement with different dietary inclusion levels of a partially defatted *Tenebrio molitor* (L.) larva meal (TM) on the growth, diet digestibility, and hepatic intermediary metabolism of rainbow trout (*Oncorhynchus mykiss* Walbaum). A 154-day growth trial was performed with 252 rainbow trout (78.3 ± 6.24 g). The trout were randomly divided into twelve tanks and fed four experimental diets containing increasing levels of TM: 0% (TM0), 25% (TM25), 50% (TM50), and 100% (TM100), corresponding to TM dietary inclusion levels of 0%, 5%, 10%, and 20%, respectively, in substitution of fishmeal. A digestibility trial in which 180 rainbow trout (94.6 ± 7.31 g) were fed with the experimental diets used in the growth trial. The growth parameters were not affected by the TM

dietary inclusion. As far as the evaluated apparent digestibility coefficients (ADC) are concerned, only the ADC of crude protein was affected, and showed the following trend: TM0 = TM25 > TM50 > TM100. The activities of key hepatic amino acid catabolic and lipogenic enzymes were not affected by the dietary composition. The obtained results suggested that a partially defatted TM could be used to totally replace fishmeal in commercial rainbow trout diets without any negative effects on fish performance.

Keywords: defatted insect meal; yellow mealworm; carnivorous fish; productive traits; apparent digestibility coefficient; hepatic enzyme

1. Introduction

Aquaculture production is growing faster than any other major food sectors. Indeed, with 110.2 million tons harvested in 2016, it will provide the most reliable supply of seafood in the upcoming years [1]. Fish diets, and those for carnivorous species in particular, have traditionally incorporated for incorporating a large amount of fishmeal (FM), which represents a high-quality source of protein with a well-balanced essential amino acids (EAA) and fatty acid (FA) profiles, high digestibility, and good palatability [2].

FM production depends on the catches of small pelagic wild stocks such as menhaden, herring, anchovies and sardines, which are processed to obtain different products [3]. Unfortunately, the unrestrained use of FM over the last few decades has put wild stocks under critical pressure with no prospect of rapid recovery [4]. As a result, the aquaculture industry has to face the problem of a limited FM supply and a consequent increase in its cost. Over the last few years, researchers and feed manufacturers have focused their efforts on reducing FM inclusion levels in commercial fish

diets while, at the same time, maintaining fish health and the nutritional quality of the final products.

Many advances have been made in the partial replacement of FM with alternative protein sources in aquafeeds [5]. The amount of FM used in diets for carnivorous species has shown a clear decreasing trend toward a more selective use of FM as a strategic ingredient at lower levels, depending on the fish life-cycle stage and species of the fish [6]. Likewise, the amount of FM in feeds for omnivorous fish has also been reduced, especially in grow-out feeds [5]. However, overall, the use of FM use in the aquafeed sector has continued to increase as a consequence of the growth in aquaculture production and the related consumption of aquafeeds [5]. A further reduction of FM inclusion in aquafeeds is thus mandatory.

Plant protein sources are the most common alternatives used to replace FM. Unfortunately, they have shown adverse effects, such as an extremely variable protein content, EAA imbalances, and anti-nutritional factors, which limit their use in diet formulations [6]. Insects have recently been considered promising alternative protein candidates, to substitute FM in aquafeeds, thanks to their interesting nutritional values, in terms of balanced amino acids (AA) profile, and their lipid, vitamin and mineral contents [7]. Interest in insects as an innovative aquafeed ingredients has grown rapidly within the scientific community and among stakeholders and their use in aquafeeds was approved by the European Commission (Annexe II of Regulation 2017/893 of 24th May 2017), which authorized the use of insect-derived processed animal proteins from seven insect species (two flies, two mealworms and three crickets). Compared to conventional livestock, the rearing of insects to produce animal feeds offers several ecological and economic advantages, because insects grow

and reproduce easily, generate low greenhouse gas emissions and can be reared on discarded organic by-products [8,9]. Moreover, rearing insects on bio-waste and organic side streams meets the recycling principles of the Circular Economy, thus reflecting the efforts of the EU to develop a sustainable, resource-efficient, low carbon and competitive economy [10,11].

Yellow mealworm, *Tenebrio molitor* (L.) is one of the seven insect species authorized by the European Union. It is a worldwide distributed beetle; and its larvae can easily be reared on low-nutritive plants and can efficiently convert food wastes and agricultural by-products into high-quality biomass [12]. They are rich in proteins (44.1-60.3% on a dry matter – DM – basis) and lipids (16.6-43.1% DM) and their AA and FA profiles make them suitable for their inclusion in animal feeds [13].

The use of *Tenebrio molitor* larvae meal (TM) as a partial substitute for conventional protein sources has been studied for different aquaculture species, and promising results have been observed for fish growth performance, diet digestibility, and immune system parameters [14-21]. However, in the majority of these studies, the experimental diets were characterized by just a few ingredients (fewer than those normally used in commercial fish diets formulations) and by high levels of FM inclusion (up to 75% as fed). Thus, these diets are not truely representative of the commercial diets currently used in aquaculture.

It should also be considered that, over the last few years, insect manufacturers have increased the production of defatted insect meals. The defatting process allows insect meals to be obtained with larger amounts of crude protein (CP) and better resistance to degradation than full-fat insect meals. Indeed, the latter contain a high lipid content, which in turn makes the extrusion process difficult. Therefore, a defatting process could

provide a useful product to reach an adequate feed composition [22]. To date, only a few studies have been performed to evaluate the use of defatted TM in the diets of different fish species [23,24]. Further investigations should be performed to assess the effects of partially defatted TM dietary inclusion in commercial diets.

The present research was designed to assess the effects of a progressive FM substitution (0, 25, 50 and 100%) with a partially defatted TM (corresponding to dietary inclusion levels of 0, 5, 10 and 20%) in commercial diets on the growth performance, somatic indexes, nutrient digestibility and liver activity of key enzymes of lipogenic and amino acid catabolic pathways in grow-out rainbow trout, *Oncorhynchus mykiss* (Walbaum).

2. Materials and Methods

A growth trial and a digestibility trial were conducted at the Experimental Facility of the Department of Agricultural, Forest and Food Sciences (DISAFA) of the University of Turin (Italy). The experimental protocol was designed according to the guidelines of the current European and Italian laws on the care and use of experimental animals (European directive 86 609/EEC, put into law in Italy with D.L. 116/92). The experimental protocol was approved by the Ethical Committee of DISAFA (protocol n° 143811).

2.1. Experimental diets

The used TM was supplied by Ÿnsect (Evry, France). The larvae had been raised on plant by-products and partially defatted using a mechanical process. No other information was given by the producer about either the

rearing substrate or the processing methodologies, as this information is considered confidential.

Four experimental diets were formulated, in accordance with SPAROS LDA (Olhão, Portugal) and the TM producer, to be isonitrogenous (CP: about 42.5% as fed), isolipidic (ether extract – EE: about 24.2% as fed), and isoenergetic (gross energy – GE: about 23.8 MJ/kg). The diets were prepared including, as fed basis, increasing levels of a partially defatted TM in substitution of 0% (TM0), 25% (TM25), 50% (TM50) and 100% (TM100) of FM, corresponding to dietary inclusion levels of TM equal to 0%, 5%, 10% and 20%, respectively. In order to ensure that the experimental diets remained isonitrogenous, isolipidic and isoenergetic, and because of the different chemical compositions of TM and FM, the amounts of some other dietary ingredients (i.e. wheat gluten, wheat meal and sardine oil) were modified slightly with the dietary increase of TM inclusion. Moreover, AA supplementation was included in the diet formulations to meet the EAA requirements of the fish.

In order to prepare the experimental extruded diets (SPAROS LDA), all the powder ingredients were mixed according to the target formulation in a double-helix mixer (500L, TGC Extrusion, France) and ground (below 400 µm) in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Germany). The diets (pellet size: 4.0 mm) were manufactured using a twin-screw extruder (BC45, Clextral, France) with a screw diameter of 55.5 mm. The extruded pellets were dried in a vibrating fluid bed dryer (DR100, TGC Extrusion, France). After cooling, oils were added by means of vacuum coating (PG-10VCLAB, Dinnissen, The Netherlands). Immediately after coating, the diets were packed in sealed plastic buckets and shipped to the research site. The composition of the ingredients of the experimental diets is shown in Table 1.

Table 1. Ingredients (% as fed) of the experimental diets.

	TM0	TM25	TM50	TM100
Fishmeal 65 (Peruvian)	20.00	15.00	10.00	0
Tenebrio molitor larva meal	-	5.00	10.00	20.00
Soy protein concentrate	18.00	18.00	18.00	18.00
Wheat gluten	7.75	7.40	7.40	7.06
Corn gluten	8.00	8.00	8.00	8.00
Soybean meal 48	7.00	7.00	7.00	7.00
Wheat meal	14.23	14.00	14.23	13.80
Sardine oil	4.30	4.26	4.20	4.10
Soybean oil	8.60	8.52	8.40	8.20
Rapeseed oil	8.60	8.52	8.40	8.20
Soy lecithin	0.50	0.50	0.50	0.50
Vit-Min Premix	1.00	1.00	1.00	1.00
Antioxidant	0.20	0.20	0.20	0.20
Sodium propionate	0.10	0.10	0.10	0.10
Monocalcium phosphate	0.52	0.92	0.92	1.72
L-Arginine	-	-	-	0.10
L-Lysine	-	0.30	0.30	0.60
L-Tryptophan	0.05	0.08	0.10	0.12
DL-methionine	0.15	0.20	0.25	0.30
Celite®	1.00	1.00	1.00	1.00

Abbreviations: TM, *Tenebrio molitor* larva meal.

2.2. Chemical analyses of feed

Feed samples were ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland) and analyzed for DM (AOAC #934.01), CP (AOAC #984.13) and ash (AOAC #942.05) contents according to AOAC International [25]; EE (AOAC #2003.05) was analyzed according to AOAC International [26]. The GE content was determined using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany). The proximate composition of the experimental diets is shown in Table 2. Chitin was estimated according to Finke [27] by correction considering the AA content of the acid detergent fiber (ADF) fraction and assuming that the remainder of the ADF fraction was chitin. The FA composition analysis of the experimental diets was performed as reported in Renna et al. [28]. The fatty acid methyl esters (FAME) were separated, identified and quantified as reported by Ravetto Enri et al. [29]. The results reported in Table 3 are expressed as mg/100 g DM.

All the chemical analyses of the feeds were performed in duplicate.

Table 2. Proximate composition of the experimental diets (g/100g as fed, unless otherwise stated).

	TM0	TM25	TM50	TM100
DM	93.77	93.83	94.13	94.41
CP	42.08	43.07	43.38	44.25
EE	22.63	22.95	22.44	22.36
Ash	7.57	7.09	6.49	5.60
Chitin	-	0.43	0.78	1.49
NFE[1]	21.49	20.29	21.05	20.71
GE (MJ/kg as fed)	22.24	22.71	22.75	22.55

Abbreviations: DM, dry matter; CP, crude protein; EE, ether extract; NFE, nitrogen-free extract; GE, gross energy. [1] Calculated as 100 – [(100 – DM) + CP + EE + Ash + Chitin]. All values are reported as mean of duplicate analyses.

Table 3. Fatty acid composition (mg/100 g DM) of the experimental diets.

	TM0	TM25	TM50	TM100
C10:0	71.17	88.22	85.80	126.97
C14:0	303.11	296.23	299.63	309.09
C16:0	2418.11	2479.54	2517.68	2685.00
C16:1 *c*9	288.62	287.85	282.39	262.44
C18:0	651.44	674.13	674.11	734.35
C18:1 *c*9	6307.94	6654.64	6717.97	7095.02
C18:1 *c*11	448.42	465.82	457.72	434.23
C18:2 n6	5650.96	6007.19	6153.86	6517.83
C18:3 n3	1021.00	1058.46	1041.25	1049.31
C20:0	94.35	87.46	81.51	86.24
C20:1 *c*11	259.29	261.22	217.80	179.48
C20:2 n6	53.55	47.33	50.62	48.32
C20:3 n6	79.29	72.43	75.73	70.41
C20:5 n3	375.85	411.60	348.32	315.80
C22:0	48.13	51.95	52.19	54.66
C22:1 n9	95.98	84.80	56.26	12.00
C22:6 n3	102.36	139.92	159.95	101.11
Other FA[1]	326.12	330.95	318.66	265.68
Σ SFA	3719.26	3812.03	3844.38	4112.18
Σ BCFA[2]	62.23	63.04	62.74	63.55
Σ MUFA	7477.13	7831.43	7812.49	8052.37
Σ PUFA	7420.41	7877.44	7956.88	8204.94
Σ PUFA / Σ SFA	2.00	2.07	2.07	2.00
Σ n3 FA	1579.06	1688.61	1627.05	1525.86
Σ n6 FA	5841.34	6188.84	6329.83	6679.08
Σ n3 / Σ n6 FA	0.27	0.27	0.26	0.23
TFA	18616.80	19520.90	19613.74	20369.49

Abbreviations: DM, dry matter; TM, *Tenebrio molitor* larva meal; *c*, *cis*; *t*, *trans*; FA, fatty acids; SFA, saturated fatty acids; BCFA, branchedchain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA,

total fatty acids. [1] All less than 50 mg/100 g DM for each treatment: C12:0 + C15 *iso* + C15 *aiso* + C14:1 *c*9 + C15:0 + C16 *iso* + C17:0 + C17 *iso* + C17 *aiso* + C17:1 *c*9 + C18:1 *t* + C18:1 *c*12 + C18:1 *c*14 + C18:1 *t*16 + C18:3 n6 + C18:4 n3 + C20:1 *c*9 + C20:4 n6 + C22:5 n3. [2] C15 *iso* + C15 *aiso* + C16 *iso* + C17 *iso* + C17 *aiso*. All values are reported as mean of duplicate analyses.

2.3. Growth trial

2.3.1. Fish and rearing conditions

Two hundred and fifty-two grow-out rainbow trout purchased from a private fish hatchery ("Troticoltura Bassignana"; Cuneo, Italy) were used to carry out a 154-day trial after a two-week period of acclimation during which the fish were fed a commercial diet (42% CP and 22% EE, Skretting Italia Spa, Mozzecane (Vr), Italy).

At the beginning of the trial, the fish were anesthetized slightly (MS-222 - PHARMAQ Ltd, UK; 60 mg/L), individually weighed (78.3 ± 6.24 g) and randomly distributed into twelve 400-L tanks (three replicate tanks per diet, twenty-one fish per tank). Artesian well water (constant temperature of 13 ± 1°C) was supplied in a flow-through open system (tank water inflow: 8 L/min). Dissolved oxygen levels were measured every two weeks and they ranged between 7.6 and 8.7 mg/L. The fish were fed 1.6 % of the tank biomass for the first 8 weeks and then, according to the fish growth and water temperature, the daily quantity of distributed feed was decreased to 1.4%. The fish were fed twice a day (08:00 and 15:00) six days per week and the feed intake was monitored at each administration. In order to update the daily feeding rate, the biomass tanks were weighed in bulk every 14 days. Mortality was checked every day.

2.3.2. Growth performance

At the end of the trial, the fish were left unfed for one day, anesthetized slightly (MS-222 - PHARMAQ Ltd, UK; 60 mg/L) and weighed

individually (KERN PLE-N v.2.2; KERN & Sohn GmbH, Balingen-Frommern, Germany; d: 0.001). The following performance indexes were calculated:

Mortality (%) = 100 - [(number of dead fish / number of fish at start) × 100];

Individual weight gain (iWG, g) = iFBW (individual final body weight, g) – iIBW (individual initial body weight, g);

Specific growth rate (SGR, % day^{-1}) = [(lnFBW - lnIBW) / number of days] × 100;

Feed conversion ratio (FCR) = total feed supplied (g, DM) / WG (g);

Protein efficiency ratio (PER) = WG (g) / total protein fed (g, DM).

Feed intake (FI) = total amount of feed consumed (g, DM) / ((final number of fish + initial number of fish) / 2) / days.

SGR, FCR, PER and FI were calculated per tank.

2.3.3. Condition factor and somatic indexes

Fifteen fish per treatment (five fish per tank) were killed by over anaesthesia (MS-222; PHARMAQ Ltd., UK; 500 mg/L). The fish were weighed individually (KERN PLE-N v.2.2; KERN & Sohn GmbH, Balingen-Frommern, Germany; d: 0.001) and the total length of the fish was measured to determine the Fulton's condition factor (K). The fish were slaughtered to calculate the hepatosomatic index (HSI), the viscerosomatic index (VSI), and the coefficient of fatness (CF). The condition factor and the somatic indexes were calculated as follows:

- K = [fish weight (g) / (body length)³ (cm)] × 100;
- HSI (%) = [liver weight (g) / fish weight (g)] × 100;
- VSI (%) = [gut weight (g) / fish weight (g)] × 100;
- CF (%) = [perivisceral fat weight (g) / fish weight (g)] × 100.

2.4. Digestibility trial

An *in vivo* digestibility trial was performed to assess the apparent digestibility coefficients (ADCs) of the diets. One hundred and eighty rainbow trout (94.6 ± 7.31 g) were divided into twelve 250-L cylindroconical tanks connected to the same open water system as that of the growth trial (three replicate tanks per diet, fifteen fish per tank). After fourteen days of acclimation to the experimental diets, the fish were fed by hand to apparent visual satiety twice a day (08:00 and 15:00), six days per week. The ADCs were measured using the indirect acid-insoluble ash method. To this aim, 1% celite® (Fluka, St. Gallen, Switzerland) was added to the diets as an inert marker in substitution of 1% of wheat meal. The faeces were collected daily from each tank for four consecutive weeks, using a continuous automatic device (Choubert's system), as described by Palmegiano et al. [30]. The feces were freeze-dried and frozen (-20°C) until analyzed. The ADCs of the DM (ADC_{DM}), CP (ADC_{CP}), EE (ADC_{EE}) and GE (ADC_{GE}) were calculated as reported by Caimi et al. [31] and expressed as a percentage.

2.5. Hepatic enzyme activities

Liver samples were collected (nine replicates per treatment) and stored at -80°C to measure the alanine aminotransferase (ALAT; EC 2.6.1.2), aspartate aminotransferase (ASAT; EC 2.6.1.1) and glutamate dehydrogenase (GDH; EC1.4.1.2) activities. The liver samples were homogenized (dilution 1:10) in an ice-cold buffer (30 mM 4-(2hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.25 mM saccharose, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM K_2HPO_4, 1 mM dithiothreitol (DTT); pH 7.4). After being centrifuged at 1,000 g for 10 min at 4°C, the supernatants were sonicated for 1 min (pulse

1 s, amplitude 50) and centrifuged again at 15,000 g for 20 min at 4°C. The resultant supernatant was collected for enzyme activity measurements. GDH activity was measured using 10 mM of L -glutamic acid, as described by Bernt and Bergmeyer [32]. ALAT and ASAT were assayed using Spinreact kits (ALAT/GPT, ref. 41282; ASAT/ GOT, ref. 41272) according to the manufacturer's instructions.

For glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49), malic enzyme (ME; EC 1.1.1.40), and fatty acid synthetase (FAS; EC 2.3.1.38) activities, liver samples were homogenized (dilution 1:5) in ice-cold buffer (0.02 M Tris-HCl; 0.25 M sucrose; 2 mM EDTA; 0.1 M sodium fluoride; 0.5 mM phenyl methyl sulphonyl fluoride (PMSF); 0.01 M β-mercapto ethanol; pH 7.4) and the homogenate was centrifuged at 30,000 g for 20 min at 4°C. G6PD activity was measured according to Bautista et al. [33], ME activity was measured according to Ochoa [34] and FAS activity according to Chang et al. [35], as modified by Chakrabarty and Leveille [36].

All the enzyme activities were expressed per mg of hepatic soluble protein (specific activity). Protein concentration was determined according to Bradford [37] using Sigma-Aldrich protein assay kit (ref. B6916) with bovine serum albumin as standard. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 µmol of substrate per min at the assay temperature (37°C). All the enzyme assays were carried out using a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China). All the reagents used for the enzymatic analysis were purchased from Sigma-Aldrich (Química, S.L., Sintra, Portugal).

2.6. Statistical analyses

The obtained data were analyzed by means of one-way ANOVA, using IBM SPSS Statistics v. 25.0 for Windows. The following model was used: $Y_{ij} = \mu + D_i + \varepsilon_{ij}$, where:

Y_{ij} = observation; μ = overall mean; D_i = effect of diet (TM0, TM25, TM50, TM100); ε_{ij} = residual error.

The assumption of normality was checked using the Kolmogorov–Smirnov test. The Levene's homogeneity of variance test was used to assess homoscedasticity. If such an assumption did not hold, the Brown-Forsythe statistic was applied to test the equality of group means instead of the F one. Pairwise multiple comparisons were performed to test the difference between each pair of means (Tukey's test and Tamhane's T2 in the cases of assumed or not assumed equal variances, respectively). The results were expressed as the mean and pooled standard error of the mean (SEM). Significance was set at $p \leq 0.05$.

3. Results

3.1. Diets

The fish willingly accepted all the experimental diets and all the supplied feeds were consumed without rejection or loss. The proximate composition analyses conducted at the DISAFA laboratories revealed that all the analyzed parameters (DM, CP, EE and ash) were comparable among the experimental diets (Table 2), according to the formulation provided by the producer. The GE values were also verified to be similar for all the diets (about 22.56 MJ/kg as fed).

3.2. Growth trial

3.2.1. Growth performance

Table 4 presents the mortality (%), growth performance and feed utilization of the fish fed the experimental diets. Mortality ranged from 7.94% (TM100) to 11.11% (TM0 and TM50) and was not significantly different among treatments ($p > 0.05$). At the end of the trial, the body weight of the fish from all the treatments had more than tripled. No significant differences among treatments were observed for any of the consiedererd growth performance parameters ($p > 0.05$).

Table 4. Mortality and growth performances of rainbow trout fed the control (TM0) and *Tenebrio molitor* larva meal (TM) experimental diets (n = 3).

	TM0	TM25	TM50	TM100	SEM	*p*-value
Mortality (%)	11.11	9.52	11.11	7.94	2.332	0.965
iIBW (g)	78.24	78.19	78.34	78.25	0.259	0.224
iFBW (g)	390.48	421.90	408.81	431.69	11.822	0.699
IWG (g)	312.24	343.71	330.47	353.44	11.813	0.698
SGR (% day^{-1})	1.04	1.09	1.07	1.11	0.019	0.688
FCR	1.07	1.09	1.11	1.02	0.022	0.594
PER	2.09	2.00	1.96	2.09	0.040	0.652
FI (g DM $fish^{-1}$ day^{-1})	2.69	2.44	2.84	2.97	0.096	0.242

Abbreviations: SEM, standard error of the mean; *p*, probability; iIBW, individual initial body weight; iFBW, individual final body weight; IWG, individual weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; FI, feed intake.

3.2.2. Condition factor and somatic indexes

No significant differences among treatments were observed for the condition factor or the somatic indexes. The only exception was HSI,

which resulted higher ($p < 0.05$) in the fish fed diet TM100 than the fish fed diet TM0, while the fish fed diets TM25 and TM50 showed intermediate values (Table 5).

3.3. Digestibility trial

ADC_{CP} showed the following decreasing trend with the increase of TM in the diets: TM0 = TM25 > TM50 > TM100. Moreover, ADC_{DM}, ADC_{EE} and ADC_{GE} did not show any significant differences among treatments (Table 6).

Table 5. Condition factor (K) and somatic indexes of rainbow trout fed the control (TM0) and *Tenebrio molitor* larva meal (TM) experimental diets (n = 15).

	TM0	TM25	TM50	TM100	SEM	*p*-value
K	1.17	1.21	1.17	1.24	0.013	0.153
HSI	0.90^{b}	1.08^{ab}	0.93^{ab}	1.11^{a}	0.027	0.008
VSI	12.66	12.60	12.96	12.60	0.190	0.895
CF	3.51	3.45	3.66	3.42	0.171	0.963

Abbreviations: SEM, standard error of the mean; *p*, probability; K, Fulton's condition factor; HSI, hepatosomatic index; VSI, viscerosomatic index; CF, coefficient of fatness. Different letters within a row indicate significant differences ($p \leq 0.05$).

Table 6. Apparent digestibility coefficients (ADC) of dry matter, crude protein, ether extract and gross energy of rainbow trout fed the control (TM0) and *Tenebrio molitor* larva meal (TM) experimental diets (n = 3).

	TM0	TM25	TM50	TM100	SEM	*p*-value
ADC_{DM}	94.69	94.43	94.32	94.16	0.197	0.108
ADC_{CP}	98.48^{a}	98.50^{a}	97.98^{b}	97.25^{c}	0.145	0.000
ADC_{EE}	98.84	98.45	98.36	98.33	0.182	0.212
ADC_{GE}	96.71	97.34	96.59	96.15	0.163	0.179

Abbreviations: SEM, standard error of the mean; *p*, probability; ADC_{DM}, apparent digestibility coefficient of dry matter; ADC_{CP}, apparent digestibility coefficient of crude protein; ADC_{EE}, apparent digestibility coefficient of ether extract; ADC_{GE}, apparent digestibility coefficient of gross energy. Different letters within a row indicate significant differences ($p \leq 0.05$).

3.4. Hepatic enzyme activities

The activity of both hepatic amino acid catabolic (ALAT, ASAT, and GDH) and lipogenic enzyme (G6PD, ME, and FAS) was not significantly affected by the dietary treatment ($p > 0.05$) (Table 7).

Table 7. Hepatic amino acid catabolic and lipogenic enzyme activities (mU mg $protein^{-1}$) in rainbow trout fed the control (TM0) and *Tenebrio molitor* larva meal (TM) experimental diets (n=9).

	TM0	TM25	TM50	TM100	SEM	*p*-value
Amino acid catabolizing enzymes						
ALAT	312.7	326.3	359.0	361.4	9.374	0.173
ASAT	635.0	487.0	522.4	634.3	22.655	0.069
GDH	38.0	33.4	36.7	37.2	1.166	0.531
Lipogenic enzymes						
G6PD	228.7	195.8	208.5	226.7	7.702	0.392
ME	78.0	63.4	65.5	76.8	2.518	0.071
FAS	4.5	5.3	3.8	4.5	0.231	0.169

Abbreviations: SEM, standard error of the mean; *p*, probability; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; GDH, glutamate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; ME, malic enzyme; FAS, fatty acid synthase.

4. Discussion

4.1. Growth performance

TM is considered a good alternative ingredient for the partial replacement of FM in the diets of several fish species [14,15,18,20]. However, high dietary inclusion levels (usually higher than 25-30%) have been reported to negatively affect fish performance, usually due to a deficiency of some nutrients, such as some EAAs [7,13]. Unlike the results found in literature, the results of the present study have revealed that the inclusion of TM in rainbow trout diets did not affect either the fish growth performance or feed utilization for any of the considered inclusion levels. These results can be partly justified by considering the composition of the experimental diets. The control diet in the present study in fact had a low FM inclusion level (20%), in order to reflect the current levels used in commercial diets. Therefore, the full dietary replacement of FM (TM100 diet) corresponded to an inclusion level of TM equal to 20%. Such TM inclusion level is much lower than the inclusion levels (25% and 50%) used in previous studies [16,20]. It could be argued that such a low dietary TM content was unable to affect rainbow trout growth negatively. Another concurrent reason for the lack of diet effect on trout growth could be the EAA supplementation of the experimental diets. Similarly, the growth performance of common yellow catfish (*Pelteobagrus fulvidraco* Richardson) juveniles was not affected by diets containing amounts of TM of up to 75% in replacement of FM, as demonstrated by Su et al. [18]. In addition, the control diet in the above-mentioned study, was formulated with a low FM concentration (24% of inclusion), to reflect the levels in commercial diets for that species.

The present study is one the first to evaluate the effects of total FM replacement with partially defatted TM in rainbow trout diets. Rema et al.

[23] have recently described the effects of total FM replacement with defatted TM larva meal in rainbow trout diets. The TM substitution levels were 20%, 30%, 60% and 100%, that is corresponding to 5%, 7.5%, 15% and 25% of TM inclusion, respectively, and thus similar to those used in our study. Rema et al. [23] observed that the gradual dietary increase of the TM level led to a significant and progressive increase in FBW, SGR and PER, and to a significant reduction in FCR, when compared to the control diet. Conversely, no improvement in fish growth performance has been observed in our study. However, it should be highlighted that Rema et al. [23] performed a trial with rainbow trout fingerlings (IBW: 5.01 g), while grow-out rainbow trout (IBW: 78.3 g) were used in our trial. Considering that the fish specific growth rate decreases as body size increases and that the dietary fat content in the two studies was comparable, the substantial disparity in the initial weight of the fish could reliably explain the differences observed in the fish growth performance.

In agreement with the findings of Rema et al. [23], Ido et al. [24] observed that both the partial and total replacement of FM in diets with a defatted TM positively promoted the growth of red seabream (*Pagrus major* Temminck and Schlegel). WG increased in the fish fed a diet characterized by a 100% replacement of FM with TM, while FCR was not affected by the presence of insect meal in the diet. Therefore, these authors assumed that the TM diets were remarkably preferred by the fish [24].

Among the evaluated somatic indexes, the HSI was significantly higher in the fish fed the TM100 diet than the fish fed the TM0 diet. In contrast, Belforti et al. [14] observed a decrease in the HSI level in rainbow trout fed diets with increasing levels of full-fat TM. Considering that the experimental diets did not affect the fish WG, these authors related this outcome to a voluntary reduction of fish intake due to the high quantity of

fat present in the full-fat TM that was used. It is well known that the liver is a key organ of the fish metabolism and that HSI is an index that is usually utilized to investigate the effects of diets on liver functionality [38]. HSI values that exceede a standard range (between 1 and 2%) could reflect disorders in the glucose and lipid metabolism, the existence of an oxidized feed, or even a vitamin deficiency [39]. In our study, the HSI values for all treatments fell within the normal physiological range. Therefore, the differences observed between fish fed the control diet and the TM100 diet were not supposed to have caused any negative effects on fish health.

Almost all insect meals are considered low in lysine and tryptophan, with TM also being limited in sulfur amino acids [13]. The absence of any adverse effects on fish growth observed in our study could also be related to the fact that all the experimental diets were supplemented with some EAA (L-Arginine, L-Lysine, L-Tryptophan and DL-methionine), were supplemented to all experimental diets, in order to allow the EAA requirements of rainbow trout to be fully met.

4.2. Digestibility trial

All the experimental diets presented high values of ADC, thereby supporting the positive growth performance herein observed.

The most relevant outcome was observed for ADC_{CP}, which significantly decreased when the fish were fed diets supplemented with the two highest inclusion levels of TM. Indeed, the ADC_{CP} of the TM100 diet was significantly lower than that of the TM50 diets. The decrease in ADC_{CP} could be related to the increasing amount of chitin in the TM-based diets (Table 2). Belforti et al. [14] found comparable results for TM-fed rainbow trout while Renna et al. [40] found comparable results for black soldier fly (*Hermetia illucens*)-fed rainbow trout. The digestibility of insect protein

has been reported to vary and to depend on the amount of protein linked to chitin that negatively influences protein digestion [27]. Nevertheless, it is known that some chitinase activity has been identified in the digestive tract of some fish species, thus suggesting that certain fish, such as marine carnivorous teleosts, may possess chitinase and degrade chitin [41]. On the contrary, chitinase activity is low or completely absent in rainbow trout [22], and this could explain the reduction in ADC_{CP} in the fish fed the highest inclusion levels of TM. However, it should be highlighted that the reduction in protein digestibility observed in our study, although statistically significant, was of low amplitude, and this may explain why it did not negatively affect the overall growth performance of the fish.

As previously shown by other authors [14,23,40], ADC_{DM}, ADC_{EE}, and ADC_{GE} were not impaired by the inclusion of insect meals in the diets of rainbow trout.

4.3. Hepatic enzyme activity

To the best of our knowledge, the effect of TM dietary inclusion on the hepatic intermediary metabolism of rainbow trout has not yet been reported in any published literature. TM dietary inclusion did not influence the activity of the AA catabolic enzymes, ALAT, ASAT, and GDH. The nutritional regulation of the AA metabolism has already been reviewed extensively [42-44]. It is well known that dietary protein levels exert little effects on the liver AA catabolism, whereas there is a relatively good response of these enzymes to AA intake [44]. As mentioned above, the experimental diets were supplemented with EAA to supply a balanced amino acid profile in the TM diets in order to be comparable to the FM-containing control diet. This could explain the absence of any significant differences in the ASAT, ALAT, and GDH activities among the diets.

Similarly, a previous study performed on gilthead sea bream (*Sparus aurata* L.) demonstrated that partial or total substitution of FM by a mixture of plant protein sources balanced with EAA did not modify the hepatic activity of AA catabolic enzymes [45]. Furthermore, a recent study performed by Guerreiro et al. [46] has observed that feeding meagre (*Argyrosomus regius* Asso) juveniles with increasing dietary levels of *Hermetia illucens* larva meal did not affect the activity of the hepatic amino acid catabolic enzymes.

Regarding lipogenic enzymes activities, several studies have demonstrated the primary role of fish liver in *de novo* FA synthesis [47-50]. Indeed, the activity of such hepatic lipogenic enzymes as FAS (a multi-enzyme complex which, together with acetyl-CoA carboxylase, catalyzes de novo FA synthesis), G6PD and ME (the suppliers of NADPH, which is essential for FAS activity) [51], has usually been evaluated. It has already been demonstrated that the activity of these lipogenic enzymes is negatively affected when fish are fed high-fat diets [48]. Different FA compositions in fish diets could also affect the activity of lipogenic enzymes. In particular, dietary polyunsaturated omega-3 fatty acids (such as C20:5n3 or C22:6n3, together with high levels of C18:3n3) have been reported to inhibit FAS and G6PD activities in hepatocyte rainbow trout cultures and in gilthead sea bream, respectively [52,53]. In the present study, the activity of the hepatic lipogenic enzymes has not been influenced by the dietary of TM inclusion as the experimental diets were formulated to be isolipidic. Moreover, comparable levels of C18:3n3, C20:5n3 and C22:6n3 were found in the experimental diets.

As far as ME is concerned, previous studies have demonstrated that its activity is related to growth rate variations than to different dietary treatments [48,54]. In the current trial, SGR and the other growth indices

did not vary among the experimental groups, thus explaining the similar ME activity among treatments.

5. Conclusions

This study has evaluated, for the first time, the effects of the dietary inclusion of a partially defatted *Tenebrio molitor* larva meal on growth performance, diet digestibility, and hepatic intermediary metabolism of practical diets for on-growing rainbow trout. The obtained results have shown that, in the current typical commercial diets that contain about 20% of FM and a well-balanced EAA profile, FM could be substituted completely by TM, without any negative effects on fish growth, the condition factor or the activity of hepatic amino acid catabolizing and lipogenic enzymes. Among the digestibility coefficients, only ADC_{CP} resulted to be negatively affected by the inclusion of dietary insect meal, but it should also be highlighted that, in absolute values, the ADC remained high in all the treatments.

These results are of practical application for feed manufacturers and farmers. The inclusion of insect meals in fish diets could lead to sustainable feeds that would be able to support an increase of in aquaculture production without a massive use of conventional protein sources, which are characterized by strong environmental impacts.

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5.4 Dietary inclusion of a partially defatted black soldier fly (*Hermetia illucens*) larva meal in low fishmeal-based diets for rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Background: Recent investigations highlighted that *Hermetia illucens* (BSF) larva meal can be a valuable alternative protein source for aquafeed production. In this study, in substitution of fishmeal, we used increasing inclusion levels of a partially defatted BSF larva meal in low fishmeal-based diets for rainbow trout (*Oncorhynchus mykiss* Walbaum) and we evaluated the related implications in terms of growth performance, physical characteristics, proximate and fatty acid (FA) compositions of the fillets, gut and liver histology, and diet digestibility. In a 131-day trial, 576 fish (100.1 ± 9.29 g) were randomly allotted to 24 tanks (24 fish/tank, 4 replicates/treatment). Six experimental diets were produced to have partial replacement of fishmeal (0%, 10%, 20%, 30%, 40% and 50%) by increasing levels of BSF meal (0% [BSF0], 3% [BSF3], 6% [BSF6], 9% [BSF9], 12% [BSF12] and 15% [BSF15] on as fed basis, respectively).

Results: No differences were observed among the treatments for all the considered growth performance parameters. The viscero and hepato-somatic indexes showed significant differences among the treatments, with the highest values observed in the BSF15 group. No differences were recorded in terms of fillet's physical characteristics, dry matter (DM), crude protein (CP) and ether extract (EE) contents. Total saturated and monounsaturated FA increased, while polyunsaturated FA (particularly n3 FA) decreased while increasing the HI meal inclusion in the diet. Histopathology of liver and gut was not affected, whereas, in posterior gut, villi were higher in BSF6 and BSF9 compared to BSF3 fish. The apparent digestibility of DM, CP, EE and gross energy did not vary among the treatments.

Conclusions: These results suggest that a partially defatted BSF meal could be included up to 15% in low fishmeal-based diets for rainbow trout

with no adverse effects on growth performance, fillet's physical characteristics, gut and liver health, and diet digestibility. On the contrary, the fillet FA composition worsened while increasing the level of BSF meal in the diet.in the diet.
Keywords*:* Apparent digestibility coefficient; Chemical and physical characteristics of fillets; Fatty acid profile; Fishmeal substitution; *Hermetia illucens* meal; Histopathology; Performance

Background

In the next few years, there will be an increase in the global demand of protein because of the constant increase in the world population [1]. Aquaculture has the fastest growing in the food production, with an average annual rate above 5.5% per year [2] and, for this reason, is considered as one of the livestock sectors able to support the global demand of animal products [3]. The growing in fish production leads to an increase in the demand of aquaculture feeds. For many years, fishmeal (FM) has been the preferred protein source for the production of aquafeeds due to its valuable content of protein and fatty acids (FA), amino acid profile, high digestibility and palatability [4]. However, the constant increase of the demand of aquaculture feeds has led to a rapid growth in the FM price and to a negative impact on the marine ecosystem [5]. In order to maintain the correct percentage of protein to meet the nutritional requirements of fish, in the last 20 years FM has been partially replaced with alternative raw materials, such as vegetable protein sources and processed animal proteins (PAPs), leading to a reduction in the dietary FM inclusion in the feeds.

Recently, in order to replace FM, the attention has been focused on the use of insect-derived PAPs. Insects can be used to produce high quality

ingredients, rich in protein and fat, starting from waste biomass and with low environmental impact [6, 7]. One of the insect species with the highest potential to be used in fish feeds is the black soldier fly (*Hermetia illucens* L.) (BSF). The larvae of this fly can be reared on low value organic waste, with low water demand and generating low greenhouse gas emissions [8]. Generally, the meals obtained from BSF show a protein content ranging from 37 to 63%, and a fat content from 7 to 39% on a dry matter (DM) basis [9, 10].

Despite the nutritional value of BSF meal, its successful inclusion level in aquafeeds depends also on their effect on gut health and liver integrity, which are fundamental for nutrient digestion and absorption and thus for growth performances [11]. For this reason, gut histomorphometry and liver histopathology are usually taken into account when alternative ingredients are investigated in animal nutrition [12]. Nutritional studies on the total and partial substitution of FM with BSF meal have been previously conducted in rainbow trout (*Oncorhynchus mykiss* Walbaum). A recent study by Cardinaletti et al. [12] showed that in a control diet containing 42% of FM, the 50% of the FM could be replaced including a dietary inclusion up to 21% of a full-fat BSF meal without negative effects on the growth performance of rainbow trout. However, a decrease in villus height and an increase in liver lipid accumulation was observed in trout fed BSF meal. Compared to a control diet with a FM dietary inclusion of 60%, Renna et al. [13] showed that an inclusion up to 40% of partially defatted BSF meal could replace up to 50% of FM without any adverse effects on growth performance, gut and liver histomorphometry. On the contrary, studies by Dumas et al. [14] and St-Hilaire et al. [15], using a defatted BSF meal (up to 26.4% of inclusion) and a full-fat BSF meal (up to 29.8% of inclusion), respectively, showed a worsening of the growth performance

of trout at increasing BSF inclusion levels. The growth performance and the gut and liver histopathology reported in these studies showed contradictory results probably due to several factors, such as the nutritional composition and inclusion level of the insect meal, and fish size.

As we can see in literature, most of the studies performed in fish nutrition replacing FM with BSF meal have been conducted using control diets with high levels of FM, usually higher than 30% [13, 16-20]. However, due to the current price of FM, only 10 – 20% of FM is currently included in commercial diets [2, 21].

Therefore, the aim of this study was to determine the potential of six inclusion levels of a partially defatted BSF larva meal as a partial replacer of FM in low FM-based diet, on growth performance, somatic indexes, fillet's physical characteristics, proximate composition and FA profile, histopathological investigation of gut and liver, and digestibility in rainbow trout.

Methods

The experimental protocol was designed according to the guidelines of the current European Directive on the protection of animals used for scientific purposes (2010/63/EU) and approved by the Ethical Committee of the University of Turin (Italy) (protocol n° 143811). The trial was carried out at the Experimental Facility of the Department of Agricultural, Forest and Food Sciences (DISAFA) of the University of Turin (Italy).

Experimental Diets

Six experimental diets were formulated to be isonitrogenous (crude protein – CP: about 45.8 g/100g as fed), isolipidic (ether extract – EE: about 15.2 g/100g as fed), and isoenergetic (gross energy – GE: about 22.6 MJ/kg as

fed). The six diets were obtained including, as fed basis, increasing levels of a partially defatted BSF larva meal – 0%, 3%, 6%, 9%, 12% and 15% - corresponding to a substitution of 0% (BSF0), 10% (BSF3), 20% (BSF6), 30% (BSF9), 40% (BSF12) and 50% (BSF15) of FM. The BSF larva meal used in this study was provided by MUTATEC (Caumont-sur-Durance, France). The larvae had been raised on plant by-products and partially defatted using a mechanical process. Unfortunately, no other information was given by the producer about either the rearing substrate or the processing methodologies, as this information is considered confidential. The experimental diets were prepared at the Experimental Facility of DISAFA. All the ground ingredients and oils were individually weighed (KERN PLE-N v.2.2; KERN & Sohn GmbH, Balingen-Frommern, Germany; d: 0.01) and mixed with a blender (Brevetti S.A.G.A., Milano, Italy). To facilitate the pelleting process, an amount of 250 to 500 mL/kg of water was added to the mixture. The pelletizing was performed using a meat grinder (LABOR 32; Rheninghaus Factory, San Mauro Torinese, Italy). The pellets (3.0 mm) were subsequently dried (50°C for 48h) and stored in black bags at -20°C until used. The ingredients of the experimental diets are reported in Table 1.

Table 1. Ingredients and proximate composition of the experimental diets and HI larva meal.

	BSF	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15
Ingredients (%)							
Fishmeal[a]		20.0	18.0	16.0	14.0	12.0	10.0
Hermetia illucens larva meal[b]			3.0	6.0	9.0	12.0	15.0
Wheat gluten		13.0	13.0	13.0	13.0	13.0	13.0
Soybean meal		20.0	20.0	20.0	20.0	20.0	20.0
Swine haemoglobin		9.2	9.0	8.8	8.6	8.4	8.2
Wheat starch		23.4	22.6	21.8	21.0	20.2	19.4
Fish oil		7.0	7.0	7.0	7.0	7.0	7.0
Soybean oil		7.0	7.0	7.0	7.0	7.0	7.0
Minerals[c]		0.25	0.25	0.25	0.25	0.25	0.25
Vitamins[d]		0.20	0.20	0.20	0.20	0.20	0.20
Chemical composition [e]							
Dry matter, g/100g	94.0	97.2	97.2	96.9	96.8	96.9	96.6
Ash, g/100g as fed	10.2	5.8	5.9	5.8	5.7	5.5	5.5
Crude protein, g/100g as fed	56.9	45.6	46.1	45.6	46.0	45.7	46.1
Ether extract, g/100g as fed	7.0	14.9	15.9	15.8	15.7	14.6	14.3
Gross energy, MJ/kg as fed	20.2	22.4	22.7	22.6	22.7	22.7	22.6
Chitin, g/100g	6.3	0.00	0.18	0.37	0.56	0.75	0.93

Abbreviations: BSF, *Hermetia illucens*. [a] Purchased from Skretting Italia S.p.A. (Località Vignetto, 17, 37060 Mozzecane VR, Italy). Proximate composition (g/100g, as fed): DM 91.0, CP 67.6, EE 8.3, Ash 16.4. [b] Provided by MUTATEC (Caumont-sur-Durance, France). [c] Mineral mixture (g/kg or mg/kg diet): dicalcium phosphate, 500 g; calcium carbonate, 215 g; sodium salt 40, g; potassium chloride, 90 g; magnesium chloride, 124 g; magnesium carbonate, 124 g; iron sulphate, 20 g; zinc sulphate, 4 g; copper sulphate, 3 g; potassium iodide, 4 mg; cobalt sulphate, 20 mg; manganese sulphate, 3 g; sodium fluoride, 1 g (purchased from Granda Zootecnici S.r.l., Cuneo, Italy). [d] Vitamin mixture (IU/kg or mg/kg diet): DL-α tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15 000 IU; DL-cholecalciferol, 3000 IU; Stay C Roche (vitamin C), 90mg; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B_{12}, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg (purchased from Granda Zootecnici S.r.l., Cuneo, Italy). [e] Values are reported as mean of duplicate analyses.

Chemical analyses of BSF meal and experimental diets

The proximate composition and the energy level of the BSF meal and experimental diets were measured in duplicate at the DISAFA laboratories. Feed samples were ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland) and analysed for DM (AOAC #934.01), CP (AOAC #984.13) and ash (AOAC #942.05) contents according to AOAC International [22]; EE (AOAC #2003.05) was analysed according to AOAC International [23]. The GE content was determined using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany). The chitin content of BSF meal was estimated according to Finke [24] by correction considering the AA content of the acid detergent fiber (ADF) fraction and assuming the remainder of the ADF fraction is chitin. Due to the presence of vegetable raw material in the diets, the method proposed by Finke [24] cannot be applied to calculate the chitin content of the diets. For this reason, the amount of chitin of the experimental diets was estimated based on the chitin content of the BSF meal and its inclusion level in the diets.

The AA determination of BSF larva meal and experimental diets was performed according to the method described in De Marco et al. [25]. After

a 22 h hydrolysis step in 6 N HCl at 112 °C under a nitrogen atmosphere, the AA content in hydrolysate was determined by means of HPLC after postcolumn derivatization. Performic acid oxidation occurred prior to acid hydrolysis for methionine and cysteine. Tryptophan was not determined. The AA composition is shown in Table 2.

A combined direct trans-esterification and solid-phase extraction was carried out for the determination of the fatty acid profile of the BSF meal and experimental diets, using eptadecanoic acid as internal standard, as reported in Dabbou et al. [26]. Fatty acid methyl esters (FAME) were separated, identified and quantified as reported in Dabbou et al. [27]. The results are expressed as mg/100g DM and are reported in Table 3.

All the chemical analyses of feed were performed in duplicate.

Fish and rearing conditions

A 131-day growth trial was carried out with rainbow trout purchased from a private fish hatchery ("Troticoltura Bassignana", Cuneo, Italy). An acclimatization period of two weeks was provided during which the fish were fed a commercial diet (42 g/100g as fed of CP; 22 g/100g as fed of EE; Skretting Italia Spa, Mozzecane (VR), Italy). Then a total of 576 fish were lightly anesthetised (60 mg/L MS-222 - PHARMAQ Ltd, Fordingbridge, Hampshire, UK), individually weighed (mean individual initial body weight – iIBW: 100.1 ± 9.29 g; KERN PLE-N v.2.2; KERN & Sohn GmbH, Balingen-Frommern, Germany; d: 0.01) and randomly divided into 24 fiberglass 200-L tanks (four replicate tanks per diet, twenty-four fish per tank). Artesian well water (constant temperature of 13 ± 1°C) was supplied in flow-through open system with each tank having a water inflow of 8 L/min. Dissolved oxygen was measured every week and ranged between 7.6 and 8.7 mg/L. Feed was distributed by hand twice a

day, six days per week (number of feeding days = 121). The fish were fed 1.4% of the tank biomass. Feed intake was checked at each administration and feed administration was stopped as soon as the fish stopped eating. In order to update the daily quantity of feed, the fish were weighed in bulk every 14 days. Mortality was checked every day.

Growth performance

At the end of the trial, after 24 h of fasting, all the fish were lightly anesthetised and individually weighed. The following performance indexes were calculated:

- Mortality (%) = (number of dead fish / initial number of fish) × 100
- Individual weight gain (iWG, g) = iFBW (average individual final body weight, g) – iIBW (average individual initial body weight, g)
- Specific growth rate (SGR, %/day) = [(InFBW - InIBW) / number of feeding days] × 100
- Feed conversion ratio (FCR) = total feed supplied (g, DM) / WG (g)
- Protein efficiency ratio (PER) = WG (g) / total protein fed (g DM).

Individual initial and final body weight were used to calculate the iWG while SGR, FCR and PER were calculated per tank.

Somatic indexes, carcass yield and coefficient of fatness

At the end of the trial, twenty-eight fish per treatment (seven fish per tank) were sacrificed by over anaesthesia (MS-222 - PHARMAQ Ltd, Fordingbridge, Hampshire, UK; 500 mg/L). The fish were individually weighed and then slaughtered to calculate the carcass yield (CY), the hepatosomatic index (HSI), the viscerosomatic index (VSI), and the coefficient of fatness (CF). The following formulas were used:

- CY (%) = [total weight without gut and gonad (g) / fish weight (g)] × 100
- HSI (%) = [liver weight (g) / fish weight (g)] × 100
- VSI (%) = [gut weight (g) / fish weight (g)] × 100
- CF (%) = [perivisceral fat weight (g) / fish weight (g)] ×100.

Physical characteristics, proximate composition and fatty acid profile of fillets

Nine fish per treatment were filleted, and the right fillets were weighed, packaged in a plastic bag and then refrigerated at +4°C. After 24 h at +4°C, the right fillets were gently dried with paper to remove excess moisture, and then weighed. Subsequently, the muscle pH (pH_{24}) and flesh color were assessed on the inside portion of the cranial, medial and caudal region of each fillet. The pH_{24} measurement was performed using a Crison MicropH 2001 (Crison Instruments, Barcelona, Spain) equipped with a combined electrode and an automatic temperature compensator. The flesh color was analysed using a bench colorimeter Chroma Meter CR-400 (Konica Minolta Sensing Inc., Osaka, Japan). The results were expressed in terms of lightness (L*), redness (a*) and yellowness (b*) in the CIELAB color space model [28].

The water holding capacity was calculated as follows:

- Drip loss (DL; %) = [(raw fillet weight (g) – raw fillet weight after 24h (g)) / raw fillet weight (g)] x 100.

The fillets were then individually vacuum-packaged in a plastic bag and stored at -20°C. After total freezing, the fillets were thawed at +4°C, removed from the bags, dried with paper, and weighed to calculate the thawed loss (TL) as follows:

- Thawing Loss (TL; %) = [(raw fillet weight (g) – thawed fillet weight (g)) / raw fillet weight (g)] x 100.

The same fillets were then vacuum-packaged in a plastic bag and cooked in a fish kettle for 10 min at 80°C (core temperature of the fillets: 75°C). After cooking, the bags were removed from the fish kettle and cooled in fresh water for 15 min to stop the cooking process. Then, the fillets were removed from the bags, dried with paper and weighed again to calculate the cooking loss (CL), as follows:

- Cooking Loss (CL; %) = [(raw fillet weight (g) – cooked fillet weight (g)) / raw fillet weight (g)] x 100.

Following cooking loss determination, a cooked fish sample (1.5 cm x 1.5 cm) from each fillet was sheared perpendicular to the fibre direction using the Instron 5543 Universal Testing Machine (Instron Corporation, Canton, Massachusetts, USA) equipped with a straight edged shear blade (crosshead speed of 30 mm/min). The maximum peak force recorded during the analysis was reported as Newton (N) shear force. The nine left fillets per treatment were frozen, finely ground with a knife mill (Grindomix GM200; Retsch GmbH, Haan, Germany) and freeze-dried (Edwards MF 1000, Milan, Italy) to determine their proximate composition (DM, CP, EE, and ash), according to the same procedures implemented for feed analyses [23, 24]. The freeze-dried and ground samples of the fish fillet were also used to assess their FA composition. After dichloromethane-methanol extraction of total lipids from fillets, a basic saponification and a BF_3 esterification were used for the determination of the fatty acid composition, adding tridecanoic acid as internal standard, as reported by Renna et al. [29]. FAME were separated using the same analytical instruments and temperature program previously reported for the FA analysis of feeds. Peaks were identified by injecting

pure FAME standards as reported in Renna et al. [30]. The results were expressed as mg/100g wet weight (ww). All chemical analyses were performed in duplicate.

Morphometric investigation

Eight fish per treatment were submitted to morphometric and histopathological evaluation. Samples of anterior and posterior gut were excised and flushed with 0.9% saline solution to remove all the content. Liver samples were also collected. The collected samples were fixed in 10% buffered formalin solution, routinely embedded in paraffin wax blocks, sectioned at 5 μm thickness, mounted on glass slides and stained with Haematoxylin & Eosin. One slide per each intestinal segment was examined by light microscopy and captured with a Nikon DS-Fi1 digital camera (Nikon Corporation, Minato, Tokyo, Japan) coupled to a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germania) using 2.5× objective lens. NIS-Elements F software was used for image capturing. Morphometric analysis was performed by Image®-Pro Plus software (6.0 version, Media Cybernetics, Maryland, USA) on 10 well-oriented and intact villi chosen from each gut segment. The evaluated morphometric index was villus height (Vh, from the villus tip to the crypt bottom). The observed histopathological findings were evaluated in all the organs using a semi-quantitative scoring system as follows: absent (score = 0), mild (score = 1), moderate (score = 2) and severe (score = 3). Gut histopathological findings were separately assessed for mucosa (inflammatory infiltrates) and submucosa (inflammatory infiltrates and Gut-Associated Lymphoid Tissue activation) for each segment. The total score of each gut segment was obtained by adding up the mucosa and submucosa scores. All the slides were blind assessed by two independent

observers and the discordant cases were reviewed, using a multi-head microscope, until unanimous consensus was reached.

Digestibility trial

In parallel with the growth trial, an *in vivo* digestibility experiment was performed to determine the apparent digestibility coefficients (ADC) of the diets. Two hundred and forty rainbow trout (mean individual body weight: 100.6 ± 8.53 g) were divided into twelve 250-L cylindroconical tanks (two replicates per treatment, twenty fish per tank) connected to the same open water system of the growth trial. After 14 days of acclimatization with the experimental diets, the fish were fed by hand to visual satiation two times per day (at 8:00 and 15:00 h), six days per week. The feces were collected daily from each tank for three consecutive weeks, using a continuous automatic device (Choubert' system) as described by Palmegiano et al. [31]. To ensure the correct level of replications per treatment (i.e. n = 4), the experiment was conducted over two blocked events using the same batch of fish for both blocks. Before new fecal collection commenced, the fish were allowed to acclimatize to their new diet for a period of 10 days [32]. The feces were pooled within tank, kept frozen (-20°C) before being freeze dried and then refrigerated (+4°C) until analysed. The ADCs were measured using the indirect acid-insoluble ash method. For this purpose, the fish were fed the same experimental diets of the growth trial, added with 1% Celite® (Fluka, St. Gallen, Switzerland) as an inert marker in substitution of 1% of wheat gluten meal. The ADC of DM, CP, EE, and GE were calculated as reported by Caimi et al. [19] and expressed as a percentage.

Statistical analyses

Data were analysed using IBM SPSS Statistics v. 25.0 for Windows. One way-ANOVA or Kruskall Wallis tests were used to compare data among the experimental groups. The assumption of normality was checked using the Kolmogorov–Smirnov test. The assumption of homoscedasticity was assessed by Levene's homogeneity of variance test. If such an assumption did not hold, the Brown-Forsythe statistic was applied to test the equality of group means instead of the F one. Pairwise multiple comparisons were performed to test the difference between each pair of means (Tukey's test and Tamhane's T2 in the cases of equal variances assumed or not assumed, respectively [one-way ANOVA], or Dunn's test [Kruskall-Wallis test]). The results were expressed as the mean and pooled standard error of the mean (SEM) or median and interquartile range (IR), depending on data distribution. Significance was set at $P < 0.05$.

Results

Diets

As expected, the DM, ash, CP, EE and GE contents were comparable among the experimental diets (Table 1). Table 2 shows the AA composition of the BSF meal and the experimental diets. Leucine, valine and tyrosine were the most represented essential AA (EAA). Excepted for histidine, methionine and tyrosine, all EAA decreased with the increase of BSF inclusion. However, all diets covered the fish AA requirements. As far as the FA composition is concerned (Table 3), the concentration of total SFA increased at the increase of the insect meal in the diet. In particular, C12:0 showed a 42-fold higher concentration in BSF15 than in BSF0. Noticeable decreases were observed for total PUFA. Omega-3 FA

drastically decreased and reached the absolute lowest concentration in the BSF15 diet.

Growth performance

The mortality ranged from 0 (BSF0 and BSF12) to 2.1% (BSF15) and was not affected by the dietary treatment (Table 4). No differences were observed for the considered growth performance traits ($P > 0.05$).

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Table 4. Effect of the dietary inclusion of BSF meal on growth performance of rainbow trout (n = 4)

	BSF0	**BSF3**	**BSF6**	**BSF9**	**BSF12**	**BSF15**	**SEM**	***P*-value**
Mortality, %	0.00	1.04	1.04	1.04	0.00	2.08	0.512	0.708
iIBW, g	100.1	100.1	100.4	100.0	100.4	100.0	0.388	0.999
iFBW, g	278.8	282.3	284.9	278.4	272.9	267.0	2.06	0.179
iWG, g	178.8	182.3	184.6	178.5	172.6	167.3	3.536	0.818
SGR, %/day	0.84	0.84	0.84	0.83	0.82	0.78	0.010	0.675
FCR	1.08	1.09	1.09	1.12	1.13	1.18	0.013	0.398
PER	2.02	1.99	2.00	1.94	1.94	1.83	0.024	0.339

Abbreviations: BSF, *Hermetia illucens*; SEM, standard error of the mean; iIBW, individual initial body weight; iFBW, individual final body weight; iWG, individual weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio.

Somatic indexes, carcass yield and coefficient of fatness

The results concerning the somatic indexes, carcass yield and coefficient of fatness are reported in Table 5. The HSI showed an increasing trend with the increase of BSF meal inclusion in the diet. Specifically, HSI values were higher in BSF15 fish than BSF0 and BSF3 fish, while the other treatments showed intermediate values ($P < 0.001$). A similar trend was also observed for VSI, except for BSF0 that showed comparable values as BSF6, BSF9 and BSF12 ($P < 0.01$).

Table 5. Effect of the dietary inclusion of BSF meal on condition factor, somatic indexes, carcass yield and coefficient of fatness of rainbow trout (n = 28).

	BSF0	**BSF3**	**BSF6**	**BSF9**	**BSF12**	**BSF15**	**SEM**	***P*-value**
HSI	1.20^{b}	1.22^{b}	1.37^{ab}	1.37^{ab}	1.39^{ab}	1.47^{a}	0.019	0.000
VSI	11.2^{ab}	11.0^{b}	11.6^{ab}	12.1^{ab}	12.3^{ab}	12.6^{a}	0.143	0.008
CY	87.9	87.7	87.7	87.0	86.8	89.1	0.341	0.448
CF	3.65	3.64	3.64	3.82	3.84	3.24	0.093	0.622

Abbreviations: BSF, *Hermetia illucens*; SEM, standard error of the mean; HIS, hepatosomatic index; VSI, viscerosomatic index; CY, carcass yield; CF, coefficient of fatness. Different superscripts within a row indicate significant differences ($p < 0.05$).

Physical characteristics, proximate composition and fatty acid profile of fillets

The dietary treatment did not affect either color or pH_{24} of the fillets (Table 6). The fillet's physical characteristics were also unaffected by diet (Table 7). The DM, CP and EE contents of the fillets did not show differences among the dietary treatments. The ash content was higher in BSF0 compared to BSF9, BSF12 and BSF15, while the fish fed with BSF3 and BSF6 showed intermediate values.

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Table 6. Effect of the dietary inclusion of BSF meal on flesh color and pH_{24} of cranial, medial and caudal regions of rainbow trout fillets (n = 9).

	BSF0	**BSF3**	**BSF6**	**BSF9**	**BSF12**	**BSF15**	**SEM**	***P*-value**
Cranial region								
L*	42.3	46.1	43.5	45.0	43.2	43.4	0.799	0.788
a*	0.35	0.42	- 0.32	2.06	- 0.35	0.33	0.255	0.069
b*	6.25	8.06	6.32	8.17	6.17	6.60	0.258	0.051
pH_{24}	6.49	6.31	6.38	6.32	6.37	6.42	0.020	0.101
Medial region								
L*	42.0	45.1	44.3	47.2	43.6	45.0	0.778	0.562
a*	1.57	3.73	1.58	2.70	2.88	1.87	0.316	0.292
b*	7.93	9.66	8.26	9.86	7.60	8.51	0.314	0.204
pH_{24}	6.40	6.26	6.33	6.28	6.31	6.35	0.019	0.119
Caudal region								
L*	44.3	49.6	47.1	47.0	45.8	47.3	0.743	0.490
a*	2.95	2.14	2.93	3.92	4.81	2.31	0.579	0.784
b*	9.08	10.30	9.68	10.54	7.98	8.60	0.337	0.203
pH_{24}	6.37	6.26	6.29	6.25	6.28	6.34	0.017	0.143

Abbreviations: BSF, *Hermetia illucens*; L*, lightness; a*, redness; b*, yellowness; SEM, standard error of the mean.

Table 7. Effect of the dietary inclusion of BSF meal on the physical characteristics of rainbow trout fillets (n = 9).

	HI0	**HI3**	**HI6**	**HI9**	**HI12**	**HI15**	**SEM**	**p-value**
Drip Loss, %	2.89	3.32	2.76	3.22	2.80	3.04	0.114	0.658
Thawed Loss, %	9.50	9.47	8.98	10.52	10.18	9.35	0.240	0.470
Cooking Loss, %	16.6	17.6	16.6	18.8	18.3	17.7	0.373	0.439
Shear Force, N	28.79	26.02	21.13	25.78	26.69	22.71	1.142	0.434

Abbreviations: BSF, *Hermetia illucens*; SEM, standard error of the mean.

As for FA contents, total SFA and total MUFA showed a clear increasing trend, while a decreasing trend was observed for total PUFA, as the level of BSF larva meal increased in the diet (Table 8). Consequently, the Σ PUFA / Σ SFA ratio of the fillets progressively decreased, ranking in the following order: BSF0 = BSF3 = BSF6 > BSF9 > BSF12 > BSF15. The fish fed with BSF15 showed a higher content of total SFA when compared to the fish fed with BSF0 (+ 29%), while the fish fed with the other treatments showed intermediate values ($P < 0.05$). Regarding individual SFA, C12:0 was about 19-fold higher in BSF15 when compared to BSF0 (1.73 *vs* 0.09 g/100g TFA, respectively; $P < 0.001$). An increasing trend was also observed for C14:0 with the dietary increase of the insect meal, while other individual SFA (i.e., C16:0, C17:0, C18:0 and C20:0) only showed higher values in BSF15 when compared to the other treatments. Various branched chain fatty acids (BCFA) were detected. The rates of all of them, with the exception of C18 *iso*, were significantly affected by the diet. The majority of BCFA showed the absolute highest content when the fish were fed the BSF15 diet. Total MUFA and C18:1 *c*9 were about 1.3-fold higher in BSF15 when compared to BSF0. As previously described for individual SFA and BCFA, some individual MUFA (i.e., C14:1 *c*9 – which coeluted with C15:0 in the chromatograms -, total C18:1 *t* and C18:1 *c*11) showed higher values in BSF15 when compared to the other treatments. The observed decrease of total PUFA was substantial (-58% in BSF15 when compared to BSF0) and regarded both total n3 and total n6 FA ($P < 0.001$). The decrease was more marked for FA belonging to the n3 (-81% considering total n3-FA) than the n6 (-43% considering total n6-FA) series. Consequently, the Σ n3 / Σ n6 FA ratio also significantly decreased while increasing BSF meal in the diet, following the order BSF0 > BSF3 = BSF6 = BSF9 > BSF12 > BSF15. All individual n6 FA showed

significantly lower values in BSF15 when compared to the other treatments. Similar results were obtained for C18:3 n3 and C20:3 n3, while the decreasing trend was gradual for long-chain n3 PUFA (C20:5 n3, C22:5 n3 and C22:6 n3).

Morphometric investigation

No differences were found for morphometry at the anterior gut, whereas villi were higher in the posterior gut of BSF6 and BSF9 groups when compared to BSF3 (Table 9).

Table 9. Effect of the dietary inclusion of BSF meal on morphometric traits of anterior and posterior gut of rainbow trout (n = 8).

	BSF0	**BSF3**	**BSF6**	**BSF9**	**BSF12**	**BSF15**	**SEM**	***P*-value**
Vh anterior gut	0.54	0.51	0.54	0.50	0.54	0.49	0.015	0.906
Vh posterior gut	0.66^{abc}	0.53^{c}	0.66^{a}	0.63^{ab}	0.63^{abc}	0.53^{bc}	0.013	0.007

Abbreviations: BSF, *Hermetia illucens*; SEM, standard error mean; Vh, villus height. Different superscripts within a row indicate significant differences ($p < 0.05$).

Regarding the histopathological alterations of liver, absent to mild multifocal lymphoplasmacytic inflammatory infiltrates were observed with absent/mild vacuolar degeneration (Table 10). The anterior and posterior gut showed from absent to mild mucosal lymphoplasmacytic infiltration. However, BSF meal inclusion did not affect the severity of the observed histopathological alterations ($P > 0.05$).

Table 10. Effect of the dietary inclusion of BSF meal on histopathological alterations of liver and gut of rainbow trout (n = 8).

	BSF0	**BSF3**	**BSF6**	**BSF9**	**BSF12**	**HI15**	***P*-value**
Liver	0.5	0.0	0.0	0.0	0.0	0.0	0.057
Inflammation, *median (IR)*	(0.0-1.0)	(0.0-0.4)	(0.0-0.0)	(0.0-1.0)	(0.0-0.0)	(0.0-0.0)	
Degeneration, *median (IR)*	0.0	0.0	0.25	1.25	0.25	0.0	0.071
	(0.0-0.4)	(0.0-0.5)	(0.0-1.0)	(0.50-1.9)	(0.0-1.0)	(0.0-1.0)	
Gut	0.0	0.0	0.0	0.0	0.0	0.0	0.180
Anterior, *median (IR)*	(0.0-0.0)	(0.0-0.5)	(0.0-0.9)	(0.0-0.0)	(0.0-0.8)	(0.0-0.0)	
Posterior, *median (IR)*	0.0	0.0	0.0	0.0	0.0	0.0	0.345
	(0.0-0.8)	(0.0-0.0)	(0.0-0.0)	(0.0-0.0)	(0.0-0.0)	(0.0-0.0)	

Abbreviations: BSF, *Hermetia illucens*; IR, interquartile range.

Digestibility trial

The apparent digestibility of DM, CP, EE and GE was not influenced by diet ($P > 0.05$) (Table 11). The ADC of DM ranged between 86.8% (BSF6) and 87.7% (BSF9). Overall, the ADC of CP and EE were higher than 94% and 98%, respectively. Results concerning ADC of GE ranged between 92.1% (BSF9) and 93.1% (BSF15).

Table 1. Apparent digestibility coefficients (ADC) of the experimental diets (n = 4).

	BSF0	**BSF3**	**BSF6**	**BSF9**	**BSF12**	**HI15**	**SEM**	***P*-value**
$ADC_{DM,}$ %	87.3	87.1	86.8	87.7	86.9	87.5	0.332	0.988
$ADC_{CP,}$ %	94.7	94.2	94.4	94.6	94.2	94.6	0.163	0.967
$ADC_{EE,}$ %	98.8	98.7	98.7	98.8	98.6	98.6	0.054	0.903
$ADC_{GE,}$ %	92.9	92.5	92.7	92.1	92.6	93.4	0.240	0.947

Abbreviations: BSF, *Hermetia illucens*; SEM, standard error of the mean; DM, dry matter; CP, crude protein; EE, ether extract; GE, gross energy. Different superscript letters within a row indicate significant differences ($p < 0.05$).

Discussion

Growth performance

Starting from the first day of trial, all the fish promptly accepted all the experimental diets. After 131 days of trial, there were no significant differences among the groups in terms of growth performance and feed utilization. The results obtained in the present study showed that, in current typical commercial diets for trout, it is possible to substitute up to the 50% of FM with a partially defatted BSF larva meal (corresponding to a maximum of 15% dietary inclusion level) without adverse effects on the fish growth performance. Such results are consistent with previous trials in which other fish species were fed diets with inclusion levels up to 20% of full-fat [33], partially defatted [21, 34] and highly defatted BSF meals [19, 35]. Indeed, inclusion levels of BSF meal higher than 20% can induce

a stress response in rainbow trout [36]. Published studies show that dietary inclusion levels of about 33% and 26% of full-fat BSF meals [16, 37] or of a partially defatted BSF meal [15] determined a worsening of the WG and feed utilization. Similar results were also observed in meagre (*Argyrosomus regius*) when fed increasing amounts of a partially defatted BSF meal [20]. However, other trials showed that it could be possible to include up to 40% of a partially defatted BSF larva meal [14] or up to 50% of a full-fat BSF prepupae meal [13] in substitution of FM, without negative effects on the growth performance of adult and juvenile rainbow trout, respectively.

Somatic indexes, carcass yield and coefficient of fatness

In literature, HSI values between 1% and 2% (as obtained in the current trial) are indicators of proper liver function [39]. Published data on the effect of insect meals on HSI values in rainbow trout are inconsistent. Similarly to what obtained in our trial, Dumas et al. [15] and Chemello et al. [22] also reported an increase of HSI in rainbow trout fed with partially defatted BSF and *Tenebrio molitor* meals, respectively. On the contrary, Sealey et al. [37] recorded a decrease in HSI in fish fed with diets characterized by increasing levels of BSF meal. Renna et al. [14] observed that inclusion levels up to 40% of a partially defatted BSF larva meal did not influence the HSI in rainbow trout. The dissimilarity of results found in literature could be related to several factors, such as the insect species and the nutritional composition of the insect meal, whereas the chemical composition of BSF meal, especially its fat content and FA profile, could be influenced by the rearing substrate of the larva [9, 40, 41] and the defatting method [15, 42]. In addition, results can also differ due to fish species, size and physiological status.

Physical characteristics, proximate composition and fatty acid profile of fillets

The pH of fish fillets provides a measure of the stress status of the fish and the flesh freshness. Changes in pH could be due by multiple factors, such as high density in tank, stress in pre-killing phase and dietary treatments [43]. An increase of the pH during storage could also reflect the production of bacterial metabolites and therefore a decrease of the shelf life of the product [44]. In the currently trial, the pH of the fillets was not affected by diet, and the recorded values are in line with those previously reported for rainbow trout fed with BSF larva meals [14, 45] and *Tenebrio molitor* larva meals [39]. The results obtained in our study also showed that an inclusion of partially defatted BSF meal up to 15% did not influence the color of the raw fillets. Such results confirm those recently obtained by Secci et al. [45] in rainbow trout fed up to 40% of partially defatted BSF larva meal. The lack of influence of BSF meal on fillet color should be considered as an advantage. In fact, color is an important quality parameter of the fillet, as it directly influences the perception of freshness, being also used by consumers to evaluate product quality [46].

We observed no differences in terms of fillets DL, TL, CL and SF among the experimental groups. Similarly, Secci et al. [45] and Borgogno et al. [47] reported no significant variations for the SF and CL when rainbow trout were fed with BSF meals. In our trial, both SF and CL showed higher values when compared to those obtained by the above-mentioned authors. Such difference may be due to different factors, such as sampling procedure, preservation and storage of samples and analytical methods used.

The lack of effects of BSF meal on fillet DM, CP and EE contents confirms previous findings obtained by Renna et al. [14], Mancini et al. [48] and

Reyes et al. [49], when BSF larva meals were included in rainbow trout diets up to inclusion levels of 25%. In our trial, the ash content of the fillets showed a decreasing trend following increasing levels of BSF larva meal inclusion in the diet. When compared to FM, BSF meal showed noticeably lower phosphorous levels [50]. Such difference may have determined the observed significant decreases in fillet ash content at increasing BSF levels in the diet. However, in young grass carp (*Ctenopharyngodon idella* Valenciennes), Wen et al. [51] observed decreased levels of ash in fillets while increasing the dietary level of available phosphorous. As no information can be found in literature on rainbow trout, further studies are needed to clearly understand the obtained results.

Fish contain high amounts of long-chain n-3 PUFA, well known to exert beneficial effects on human health [52]. Usually, the FA composition of fish fillets reflects that of the administered diet [14]. The FA content and composition of insect larvae (and derived meals) depend on the considered insect species, rearing substrate, developmental stage and processing (e.g., defatting methods) [53]. Differently from FM, insects are generally rich in SFA and poor in PUFA [54]. In particular, BSF larval fat consists mainly of C12:0 and other SFA [9, 53], as confirmed by the analyses of the tested BSF meal. Even if the BSF meal used in this trial was partially defatted (EE: 7.0 g/100g as fed), this led to a noticeable increase of C12:0 in the BSF-containing diets when compared to the FM-control diet (BSF0), as already observed by other authors [14, 20]. In recent trials where high inclusion levels of insect meals were evaluated in aquafeed for various fish species, significant alterations of the FA composition of fish whole body and fish fillets were observed [21, 48, 55]. The most frequently reported modifications were increasing levels of SFA associated with reductions of PUFA contents, particularly when considering long-chain PUFA of the n3

series (i.e., C20:5 n3 and C22:6 n3), which also led to undesirable decreases of the PUFA/SFA and n3/n6 PUFA ratios of the product [19, 20, 45]. Our results confirm such findings and, in addition, clearly show that the FA composition of trout fillets could be negatively affected even in case of low inclusion levels of BSF meal as a replacement of FM in typical commercial diets.

Morphometric investigation

Regardless the dietary treatment, Vh showed a proximo-distal increasing gradient from anterior to posterior gut. In literature, the majority of the studies conducted in mammals and poultry reported a proximo-distal decreasing gradient from anterior to posterior gut [56]. This is due to the different intensity of nutrient digestion and absorption processes along the gut. Only few publications are available on the morphometry of rainbow trout and they mainly concentrated on the anterior gut [57] as it is the most important site for nutrient absorption, receiving physical, chemical and hormonal stimuli caused by the presence of the diet in the lumen [58, 59]. The lack of differences for Vh in the anterior gut among the dietary treatments is a positive finding and it is in accordance with Renna et al. [14] who did not record any morphological changes in the intestine of trout fed up to 40% of BSF meal. Moreover, the unaffected morphometry of the anterior gut could also explain the unchanged growth performances and diet digestibility recorded for the trout of the present study, suggesting good nutrient absorption and utilization with BSF meal dietary inclusion levels up to 15%. On the contrary, BSF meal influenced Vh in the posterior gut, being lower in BSF3 diet compared to the other treatments. This is one of the first studies describing posterior gut in trout and further study are needed to better investigate this upward trend. Regarding the

histopathological alterations of liver and gut, they varied from absent to mild in all the organs. The absence of adverse effects related to dietary BSF meal inclusion observed in liver and gut of the present study is in agreement with published literature [36, 60, 61].

Digestibility trial

The ADC values of nutrients and energy were high and comparable among the experimental groups. Such results suggest that BSF larva meal is well digested by rainbow trout and its inclusion up to 15% in the diet does not adversely affect the fish growth performance. In fact, studies conducted on rainbow trout with BSF larva meal [14] and *Tenebrio molitor* larva meal [22, 62], showed that dietary inclusion levels up to 25% did not affect the apparent digestibility of nutrients and energy.

Conclusions

The findings of this study suggest that partially defatted black soldier fly larva meal can be considered a suitable ingredient in low fishmeal-based diets for rainbow trout. In this study, we did not observe any adverse effect on growth performance, somatic indexes, fillet physical quality parameters and chemical composition, and diets digestibility. However, the FA composition of fillets worsened while increasing the level of BSF meal in the diets. In particular, a decrease of PUFA, and an increase of total SFA and total MUFA was observed. The commercial use of insect meal for aquaculture feed is a good approach to reduce the environmental impact and support a sustainable increase in aquaculture production.

Abbreviations

ADC, apparent digestibility coefficient; ADF, acid detergent fiber; BCFA, branched chain fatty acids; BSF, *Hermetia illucens*; *c*, *cis*; CF, coefficient of fatness; CL, cooking loss; CP, crude protein; CY, carcass yield; DL, drip loss; DM, dry matter; EE, ether extract; FA, fatty acid; FAME, fatty acid methyl ester; FBW, final body weight; FCR, feed conversion ratio; FM, fish meal; GE, gross energy; HSI, hepatosomatic index; IBW, initial body weight; IR, interquartile range; MUFA, monounsaturated fatty acids; nd, not detected; PAPs, processed animal proteins; PER, protein efficiency ratio; PUFA, polyunsaturated fatty acids; SEM, standard error of the mean; SF, shear force; SFA, saturated fatty acids; SGR, specific growth rate; *t*, *trans*; TFA, total fatty acids; TL, thawed loss; Vh, villus height; VSI, viscerosomatic index; WG, weight gain; ww, wet weight.

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Availability of data and materials

The datasets analysed in the current study are available from the corresponding author on request.

Authors' contributions

CC, IB, AS and LG conceived and designed the experiment. CC, IB, GC, SBO, FG, AS, AT, MR and LG prepared the diets, performed the growth and digestibility trial and collected the experimental data. AB and CC performed the fillet physical quality parameters. CC, CL, VM and AT carried out the chemical analyses. MTC and EC performed the histomorphometric investigations and analysed the data. CC, AB, IB and MR performed the statistical analysis. CC, FG, MR and LG analysed and interpreted the data. CC, MR and LG wrote the first draft of the manuscript. All the authors critically reviewed the manuscript for its intellectual content and gave their approval for the final version to be published.

Ethics approval and consent to participate

The experimental protocol was approved by the Ethical Committee of the University of Turin (Italy) (protocol n° 143811).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table 2 Amino acid (AA) concentration (g/100g of protein) of BSF meal and experimental diets.

	BSF	BSF0	BSF3	BSF6	BSF9	BSF12	BSF15
Essential AA							
Arginine	3.9	5.3	5.2	5.2	5.1	5.0	5.0
Histidine	2.2	2.8	2.8	2.8	2.8	2.7	2.9
Isoleucine	3.3	3.5	3.4	3.4	3.3	3.3	3.3
Leucine	5.2	7.7	7.5	7.5	7.3	7.2	7.1
Lysine	3.8	5.9	5.6	5.5	5.3	5.2	5.0
Methionine	2.6	2.5	2.5	2.5	2.5	2.5	2.6
Cysteine	1.3	1.3	1.2	1.1	1.1	1.0	1.3
Phenylalanine	3.0	4.6	4.6	4.5	4.5	4.4	4.3
Tyrosine	4.8	3.0	3.0	3.1	3.1	3.2	3.2
Threonine	3.1	7.6	7.6	7.6	7.6	7.6	7.6
Valine	4.9	3.5	3.5	3.4	3.4	3.3	3.3
Non essential AA							
Alanine	6.2	4.8	4.8	4.8	4.8	4.8	4.8
Aspartic acid	6.7	8.1	7.9	7.9	7.7	7.7	7.6
Glycine	4.2	2.7	2.8	2.9	3.0	3.1	3.2
Glutamic acid	8.8	16.0	15.9	16.1	16.1	16.3	16.2
Proline	5.5	8.5	8.2	8.1	7.8	7.6	7.4
Serine	3.7	3.7	3.6	3.7	3.6	3.6	3.6

Abbreviations: BSF, *Hermetia illucens*.

Table 3. Fatty acid profile (mg/100g DM) of *H. illucens* larva meal and experimental diets.

	BSF	**BSF0**	**BSF3**	**BSF6**	**BSF9**	**BSF12**	**BSF15**
C10:0	67.8	43.8	60.3	73.4	91.0	49.7	63.2
C12:0	1898.7	6.94	64.7	101.1	177.9	228.4	291.4
C14:0	483.0	413.6	436.2	446.7	465.6	450.9	475.8
C15 *iso*	4.51	13.2	14.3	13.5	13.3	13.4	13.4
C15 *aiso*	5.74	4.62	4.63	4.54	4.12	4.24	4.34
C14:1 *c*9 + C15:0	27.0	39.0	41.3	41.2	40.8	39.0	41.1
C16 *iso*	1.90	1.50	3.19	1.92	2.34	2.16	2.10
C16:0	1111.4	1901.7	1980.2	2072.9	2058.6	1966.5	2060.0
C17 *iso*	2.83	37.1	40.8	31.5	29.4	29.1	30.0
C17 *aiso*	29.8	11.5	18.6	7.66	4.75	5.85	6.20
C16:1 *c*9	174.5	488.8	504.4	365.8	281.3	287.0	316.5
C17:1 *c*9	8.16	14.9	18.5	10.3	8.06	8.38	9.06
C18:0	246.8	548.9	562.0	553.9	537.9	513.9	538.7
C18:1 *t*	14.5	11.0	11.0	9.43	9.24	9.13	10.9
C18:1 *c*9	1216.0	2078.2	2171.7	1658.9	1278.1	1332.4	1530.6
C18:1 *c*11	27.8	273.8	281.1	209.8	161.8	162.4	179.4
C18:1 *c*12	0.32	6.22	3.49	1.25	1.43	1.00	1.16
C18:1 *c*14 + *t*16	4.42	11.41	8.85	5.38	4.79	4.16	5.01
C18:2 n6	403.4	2381.2	2486.6	533.1	398.4	387.7	418.5
C18:3 n3	26.0	241.3	244.8	30.4	24.3	22.7	21.7
C18:3 n6	1.56	10.0	10.1	1.89	2.05	1.01	0.28
C20:0	20.0	34.6	34.2	40.0	35.1	37.7	41.8
C20:1 *c*9	4.34	67.1	65.4	55.3	44.5	43.7	47.1
C20:1 *c*11	n.d.	2.02	2.08	0.10	0.54	0.19	0.35
C20:2 n6	n.d.	62.3	59.3	6.37	4.53	4.75	3.91
C20:3 n6	n.d.	2.44	3.49	0.60	0.17	0.87	0.33

C20:3 n3	n.d.	13.4	8.40	4.90	2.89	3.22	2.89
C20:4 n6	1.11	25.3	24.0	4.28	2.75	2.76	1.89
C20:5 n3	n.d.	298.8	275.8	47.3	35.6	35.9	30.00
C22:0	11.5	31.9	26.7	27.8	28.4	26.6	26.4
C22:1 n9	n.d.	8.39	8.30	5.87	4.31	4.63	4.38
C22:5 n3	n.d.	32.9	29.7	2.15	1.47	1.70	2.88
C22:6 n3	n.d.	119.3	151.5	35.9	23.7	21.9	19.8
Σ SFA	3866.0	3020.3	3205.5	3357.2	3435.2	3312.8	3538.4
Σ MUFA	1450.0	2961.7	3075.0	2322.1	1794.1	1853.0	2104.5
Σ PUFA	436.6	3186.9	3293.7	666.8	495.8	482.4	502.2
Σ PUFA / Σ SFA	0.11	1.06	1.03	0.20	0.14	0.15	0.14
Σ n3	26.0	705.8	710.2	120.5	87.9	85.3	77.3
Σ n6	406.0	2481.2	2583.5	546.3	407.9	397.1	424.9
Σ n3 / Σ n6	0.06	0.28	0.27	0.22	0.22	0.21	0.18
TFA	5797.4	9236.9	9655.7	6405.3	5779.0	5702.8	6201.1

Abbreviations: DM, dry matter; BSF, *Hermetia illucens*; *c*, *cis*; *t*, *trans*; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; n.d., not detected. Values are reported as mean of duplicate analyses.

Table 8. Effect of the dietary inclusion of BSF meal on fillet proximate composition (g/100g ww) and fatty acid profile (g/100g of TFA) of rainbow trout (n = 9).

	BSF	BSF0	BSF3	BSF6	BSF9	BSF12	SEM	*P*-value
Proximate composition								
DM	25.8	26.8	27.2	25.5	26.1	26.6	0.303	0.643
CP	19.2	20.0	18.3	19.2	19.6	20.5	0.323	0.493
EE	4.44	4.97	5.34	4.74	5.13	4.76	0.223	0.897
Ash	2.06^{a}	1.68^{ab}	1.54^{ab}	1.43^{b}	1.28^{b}	1.22^{b}	0.065	0.010
Fatty acid composition								
C12:0	0.09^{e}	0.28^{de}	0.44^{d}	0.75^{c}	1.16^{b}	1.73^{a}	0.081	0.000
C13 *iso*	0.07^{ab}	0.07^{ab}	0.06^{ab}	0.07^{a}	0.05^{ab}	0.04^{b}	0.003	0.024
C14:0	2.93^{c}	3.00^{bc}	2.91^{c}	3.16^{bc}	3.35^{b}	4.36^{a}	0.078	0.000
C15 *iso*	0.07^{b}	0.07^{b}	0.07^{b}	0.06^{b}	0.07^{b}	0.09^{a}	0.001	0.000
C15 *aiso*	0.02^{b}	0.02^{b}	0.02^{b}	0.02^{b}	0.03^{ab}	0.04^{a}	0.002	0.000
C14:1 *c*9 + C15:0	0.33^{b}	0.33^{b}	0.32^{b}	0.34^{b}	0.34^{b}	0.43^{a}	0.007	0.000
C16:0	18.6^{b}	19.1^{b}	18.8^{b}	19.6^{b}	20.2^{b}	25.4^{a}	0.361	0.000
C17 *iso*	0.21^{b}	0.22^{b}	0.21^{b}	0.20^{b}	0.22^{b}	0.28^{a}	0.005	0.000
C17 *aiso*	0.43^{cd}	0.42^{d}	0.42^{d}	0.50^{bc}	0.52^{b}	0.64^{a}	0.013	0.000
C16:1 *c*9	4.88^{c}	5.14^{bc}	5.03^{bc}	5.29^{bc}	5.52^{b}	6.31^{a}	0.085	0.000
C17:0	0.29^{b}	0.28^{b}	0.29^{b}	0.27^{b}	0.28^{b}	0.40^{a}	0.008	0.000
C18 *iso*	0.12	0.11	0.10	0.12	0.12	0.12	0.004	0.421
C17:1 *c*9	0.19	0.18	0.20	0.23	0.21	0.21	0.006	0.217
C18:0	5.11^{b}	5.13^{b}	5.10^{b}	5.32^{b}	5.44^{b}	6.53^{a}	0.078	0.000
C18:1 *t*6-10	0.09^{b}	0.10^{b}	0.09^{b}	0.10^{b}	0.11^{ab}	0.12^{a}	0.002	0.000
C18:1 *t*11-12	0.03	0.03	0.02	0.02	0.02	0.03	0.001	0.104
C18:1 *t*13-14+*c*6-8	0.12^{b}	0.12^{b}	0.11^{b}	0.13^{b}	0.12^{b}	0.16^{a}	0.003	0.000
C18:1 *c*9	23.3^{d}	24.0^{cd}	24.9^{bcd}	25.4^{bc}	26.6^{b}	31.3^{a}	0.397	0.000

C18:1 *c*11	2.88^{b}	2.93^{b}	2.92^{b}	2.93^{b}	2.99^{b}	3.50^{a}	0.038	0.000
C18:2 n6	21.0^{a}	21.6^{a}	21.8^{a}	20.9^{a}	20.7^{a}	12.3^{b}	0.513	0.000
C18:3 n3	2.51^{a}	2.62^{a}	2.51^{a}	2.41^{a}	2.22^{a}	0.93^{b}	0.089	0.000
C18:3 n6	0.32^{a}	0.31^{a}	0.34^{a}	0.36^{a}	0.29^{a}	0.12^{b}	0.014	0.000
C20:0	0.16^{b}	0.17^{b}	0.18^{b}	0.18^{b}	0.18^{b}	0.23^{a}	0.004	0.000
C20:1 *c*9	1.17^{b}	1.15^{b}	1.20^{b}	1.22^{b}	1.26^{ab}	1.44^{a}	0.024	0.003
C20:1 *c*11	0.17^{a}	0.16^{ab}	0.15^{ab}	0.15^{ab}	0.14^{b}	0.05^{c}	0.006	0.000
C20:2 n6	0.98^{a}	0.93^{a}	1.01^{a}	0.96^{a}	0.95^{a}	0.66^{b}	0.025	0.000
C20:3 n3	0.15^{a}	0.15^{a}	0.16^{a}	0.14^{a}	0.14^{a}	0.07^{b}	0.005	0.000
C20:3 n6	0.63^{a}	0.59^{a}	0.64^{a}	0.70^{a}	0.60^{a}	0.20^{b}	0.025	0.000
C20:4 n6	0.75^{a}	0.72^{a}	0.73^{a}	0.76^{a}	0.62^{a}	0.22^{b}	0.031	0.000
C20:5 n3	2.96^{ab}	3.14^{a}	2.68^{ab}	2.53^{b}	1.82^{c}	0.54^{d}	0.128	0.000
C22:0	0.10	0.10	0.12	0.12	0.11	0.11	0.002	0.055
C22:1 n9	0.14	0.14	0.15	0.16	0.17	0.17	0.006	0.517
C22:5 n3	0.92^{ab}	1.03^{a}	0.92^{ab}	0.80^{b}	0.55^{c}	0.12^{d}	0.046	0.000
C22:6 n3	8.19^{a}	5.56^{b}	5.35^{bc}	4.18^{cd}	2.97^{d}	1.11^{e}	0.326	0.000
Σ SFA	27.7^{b}	28.4^{ab}	28.2^{ab}	29.7^{ab}	31.0^{ab}	35.7^{a}	0.821	0.043
Σ BCFA	0.91^{bc}	0.91^{bc}	0.86^{c}	0.97^{b}	1.00^{b}	1.22^{a}	0.018	0.000
Σ MUFA	33.0^{d}	34.0^{cd}	34.8^{bcd}	35.6^{bc}	37.2^{b}	43.3^{a}	0.519	0.000
Σ PUFA	38.4^{a}	36.7^{ab}	36.1^{ab}	33.8^{bc}	30.8^{c}	16.3^{d}	1.089	0.000
Σ PUFA / Σ SFA	1.39^{a}	1.29^{a}	1.28^{a}	1.14^{b}	1.00^{c}	0.39^{d}	0.047	0.000
Σ n3	14.7^{a}	12.5^{b}	11.6^{bc}	10.1^{c}	7.71^{d}	2.78^{e}	0.559	0.000
Σ n6	23.7^{a}	24.2^{a}	24.5^{a}	23.7^{a}	23.1^{a}	13.5^{b}	0.590	0.000
Σ n3 / Σ n6	0.62^{a}	0.51^{b}	0.47^{b}	0.43^{b}	0.34^{c}	0.19^{d}	0.020	0.000

Abbreviations: BSF, *Hermetia illucens*; ww, wet weight; TFA, total fatty acid; SEM, standard error of the mean; DM, dry matter; CP, crude protein; EE, ether extract; *c*, *cis*; *t*, *trans*; SFA, saturated fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
Values are reported as mean of duplicate analyses.
Different superscripts within a row indicate significant differences ($P < 0.05$).

6. Discussion & Conclusion

This PhD project evaluated the effect of the inclusion of insect meal in diets for freshwater fishes.

The first and second study, evaluated the inclusion of a highly defatted black soldier fly larvae meal in diets for Siberian sturgeon juveniles. Results highlight that inclusion up to 18.5% of HI meal can replace up to 25% of the FM without any adverse effect on the growth performance and fish health.

In the third study was evaluated the inclusion of a partially defatted *Tenebrio molitor* larvae meal in diets for on-growing rainbow trout. Results shown that inclusion up to 20% of FM with TM meal could be applied without negative effect on growth performance, diets' digestibility and metabolic response of the fish.

The fourth study was also performed on rainbow trout, but the objective was the evaluation of the use of a partially defatted black soldier fly larvae meal in low fishmeal-based diets. Obtained results suggest that HI meal could be included up to 15% replacing 50% of the FM without compromising growth performance, diets digestibility and fillets' physical characteristics.

In Siberian sturgeon, inclusion level up to 15% of *Tenebrio molitor* meal (Józefiak et al., 2019b) did not influence fish performances and health status; on the contrary, the influence of BSF meal show contradictory results. In a study conducted by Caimi et al. (2020a), inclusion level higher than 18.5% affect growth performance while a refusal of consumption was observed in fish fed with the total substitution of FM with BSF meal (level of 75% of the diet). In a second study (Zarantoniello et al., 2021), a lower

palatability of the diet with 50% of full-fat BSF was observed. As a result, the fish entered a fasting condition that could negatively affect growth performance and survival. On the contrary, studies performed by Rawsky et al. (2020) and Józefiak et al. (2019b), inclusion level up to 50% of full-fat BSF meal did not affect feed consumption and performance.

Studies on the feeding behaviour of sturgeon shown a connection between feed's physical property, protein composition and feed acceptance (Kasumian & Taukik, 1994; Gawlica et al., 2002; Kasumyan & DÖving, 2003; Kasumian, 2018). Comparing the dietary formulation and preparation methods of the previously cited studies (Józefiak et al. 2019b; Rawsky et al. 2020; Caimi et al, 2020a), results suggest that BSF meal may have a negative effect when used as a single protein sources in diets or an adverse effect could be due to the defatting process or the physical quality of the feed.

About rainbow trout, results obtained in the two studies conducted by Chemello et al. (2020) and Caimi et al. (2021) agree with the more recent literature. Indeed, growth performance and health status are not influenced by the inclusion up to 50% of *Tenebrio molitor* (Belforti et al., 2015; Rema et al., 2019; Chemello et al., 2020; Jeong et al., 2020) or *Hermetia illucens* meal (Renna et al., 2017; Dumas et al., 2018; Cardinaletti al., 2019; Jozefiak et al., 2019a; Caimi et al. 2021). In this specie, positive results were obtained both with full-fat and partially defatted meal.

Comparing literature about the use of IM in freshwater and marine fish, freshwater fish are more likely to utilize insect protein sources. In studies conducted with seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), inclusion level less than 25% of IM are recommended to prevent negative effect on growth performance and digestibility (Gasco et al., 2016; Piccolo et al., 2017; Basto et al., 2020; Reyes et al., 2020). This

results could be due to difference about feeding habits and physiology between marine and freshwater fish. One of the most limiting factor on the use of IM in marine species, is the natural absence of n3 PUFAs. As opposed to freshwater fish, marine fish have a lower capacity to convert α-linolenic acid into EPA and DHA (Sargent et al. 1995; Tschirner & Kloas, 2017), which limits the possibility to replace FM with alternative protein sources. The actual level of FM included in aquafeeds for marine aquaculture ranging from 20% to 50% (Tschirner & Kloas, 2017; Arru et al., 2019; Mulazzani et al., 2021). From an economic point of view, comparing the current price of FM (1.3 €/kg in 2018, in Italy) and IM in European Union (3.5 €/kg) (Mulazzani et al., 2021), IM is not a competitive protein source for aquaculture feeds. The main cause of this problem is the lower supply of this raw material, but according to the forecast, considering the constant increase of BSF meal production, IM could be competitive with FM only in 2023 (Hilkens & De Klerk, 2016; IPIFF, 2018; Hua et al., 2019).

Results obtained during the three years of the PhD, allow to increase the knowledge about the use of IM in aquaculture feeds. The presence of contradictory results in literature underlines the importance of more research to improve the knowledge on this issue. Considering the growing interest on IM, other aspects that must be investigated concern the economic effects on the aquaculture feeds and the possible use of IM not only as a protein source but also additive. Moreover, further research are necessary to highlight how the manufacturing techniques (i. e. drying methods, defatting process) could influence the results in order to standardize the production process and ensure the growth performance and good health of farmed fish

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