



Fakulta rybnářství  
a ochrany vod  
Faculty of Fisheries  
and Protection  
of Waters

Jihočeská univerzita  
v Českých Budějovicích  
University of South Bohemia  
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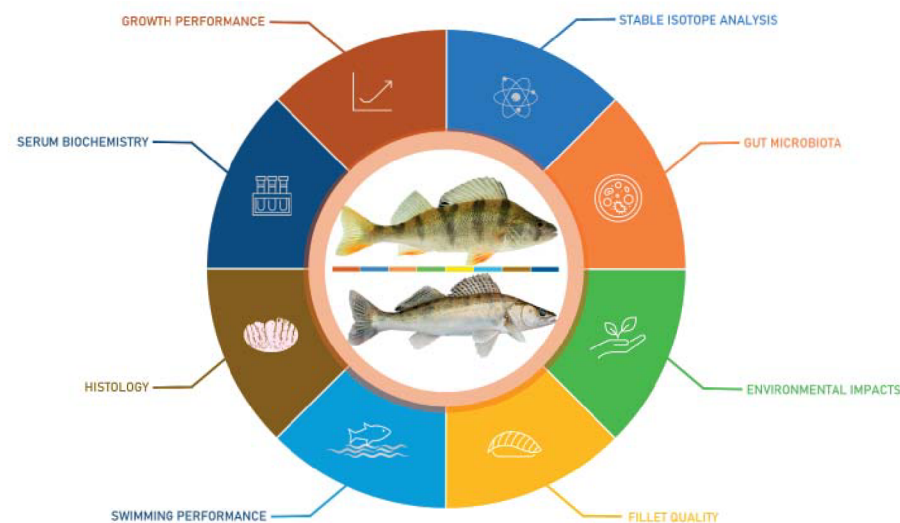


Doctoral thesis

## Insect meal as feed source in nutrition of percids, Eurasian perch (*Perca fluviatilis*) and pikeperch (*Sander lucioperca*)

Hmyzí moučka jako zdroj krmiv ve výživě okounovitých ryb, okouna říčního (*Perca fluviatilis*) a candáta obecného (*Sander lucioperca*)

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Doctoral thesis by  
Tran Quang Hung

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*Doctoral thesis by Tran Quang Hung*

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## **CHAPTER 1**

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### **GENERAL INTRODUCTION**

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## 1.1. Potential alternative protein sources in aquafeeds

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Aquaculture plays a significant role in providing aquatic food production and nutrition security for the world population (Edwards et al., 2019; FAO, 2020b). The contribution of aquaculture to global seafood production was 48% in 2018 and is expected to reach 53% by 2030, outpacing that of capture fisheries (FAO, 2020b). The past twenty years have witnessed a substantial expansion of aquaculture industry regarding live weight production and cultured species diversity. Aquaculture production was recorded at 36 million metric tons (MT) in 1997, encompassing approximately 300 cultivated species. By 2017, the output has climbed to 112 million MT, diversifying to around 425 species (Naylor et al., 2021). Aquaculture has become the fastest expansion in the food-producing sectors and is expected to play a central role in the global fish supply budget (Béné et al., 2015; FAO, 2020b; Kobayashi et al., 2015). Aquatic plants, algae, and molluscs were the most dominant contributors, accounting for almost 44% of overall aquaculture production in 2017, followed by freshwater fish (40%) (Naylor et al., 2021). The sector's fast growth by 2030 will be fuelled mainly by a steady increase in tilapia and shrimp production, as well as salmon, carp, catfish, and other freshwater species (Kobayashi et al., 2015).

Farming of these key species required significant inputs of compound feeds. In 2018, the vast majority of aquaculture species were fed-species, accounting for around 70% of the total; the remainder were non-fed species (FAO, 2020b). Among aquaculture species, salmon requires 100% of fed inputs, whereas more than half of carp, tilapia catfish production was supplemented with compound feeds (Naylor et al., 2021). Fed aquaculture has transitioned from the reliance on by-products from agriculture for extensive and semi-intensive pond-based farming to pelleted feed for intensive techniques (Fry et al., 2016; Henriksson et al., 2021). In response to the rapid growth of aquaculture, aquafeed production increased more than 100% between 2000–2008, reaching 51.23 million MT in 2017, and an additional 21 million MT are expected to add to the current volume by 2025 (Tacon, 2020). The combination of various components, including marine sources, terrestrial crop products, animal by-products, and micronutrients, are commonly used in aquafeeds (Fry et al., 2016; Henriksson et al., 2021). Fishmeal derived from pelagic forage fish has been typically used in aquafeeds as an ideal protein source to satisfy nutrient requirements and palatability of farmed fish (Cottrell et al., 2020). It was estimated that a total of roughly 13 million MT of wild fish was used to obtain fishmeal and fish oil in aquafeed, and approximately 3 million MT of fishmeal was included to feed aquaculture species in 2017 (Naylor et al., 2021). Since 2000 aquaculture has become a primary user of the global fishmeal budget, exceeding that destined for other animal-producing industries; salmon, marine fish, and shrimp are the leading consumers of fishmeal (Froehlich et al., 2018a; Jannathulla et al., 2019).

Since the 2000s, the rising demand for fishmeal for animal feed, in conjunction with a static supply of capture fisheries production, has resulted in a continuous surge in fishmeal prices on the global market (Naylor et al., 2021). In 2020, the price of fishmeal was about 1500 US dollars per MT, having more than quadrupled since the early 2000s and outpricing other terrestrial plant ingredients (IndexMundi, 2022). Approximately 90% of the worldwide marine fish resources have been fully exploited, and the maximum stock boundary will be reached in the following decades (El Abbadi et al., 2022; Froehlich et al., 2018a). Furthermore, using bycatch fish as feed may harm the marine ecosystem and biodiversity (Zhang et al., 2020). The development of aquaculture business to fulfil global seafood demand, accompanying greater aquafeed inputs, necessitates prudent management of marine protein sources to achieve long-term sustainability (Naylor et al., 2000; Naylor et al., 2009). Aquaculture has been considered to significantly contribute to global food security by 2050 if reducing reliance on fishmeal and fish oil (Béné et al., 2015). Over the last two decades, feed formulation for aquaculture has effectively reduced the inclusion levels of these finite marine proteins, as illustrated by a continuous decrease in dietary fishmeal and fish oil

(Tacon et al., 2011; Tacon and Metian, 2008). Currently, the incorporation of fishmeal derived from the wild is less than 10% in the diet for aquaculture species (except for eel, with a current level of 25%), and that of fish oil is less than 6%. Notably, wild-originated fish oil is no longer included in carp, catfish, and tilapia diets (Naylor et al., 2021).

In order to satisfy the protein and lipid gap at the expense of marine sources in aquafeeds, alternative ingredients originating from terrestrial agriculture products have been increasingly used (Froehlich et al., 2018b; Tacon et al., 2011; Troell et al., 2014). Approximately 30 million MT of plant ingredients are currently used in aquafeeds, mostly soybean, rapeseed, maize, and wheat; by 2050, this figure might rise to nearly 91 million MT (Troell et al., 2014). These terrestrial ingredients have become the prevalent components in aquafeed more than other analogues (Tacon et al., 2011). Although such transition significantly lifts aquaculture from large dependence on marine fisheries resources (Liland et al., 2013) and boosts resilience to the global food system (Troell et al., 2014), the widespread use of plant ingredients may have consequences associated with the physiology of fed animals and environments. Plant-based diets have been shown to impair production performance and health status, as well as alter intestine morphology and gut microbiota of fed aquatic animals (Colombo, 2020). High dietary plant feedstuff in aquafeeds also generates significant fecal losses (Naylor et al., 2009) and has an indirect impact on environmental and human health by altering the nutritional properties of fish food (Fry et al., 2016). Furthermore, the environmental consequences of employing plant ingredients in aquafeeds, such as ecotoxicity, pressures on natural resources, and biodiversity, should be considered (Foley et al., 2011).

Crop by-products and food-graded materials (e.g., wheat by-products, distillers' grains, peanut cake) contributed significantly to aquaculture feed input, in addition to plant feedstuff (Troell et al., 2014). Rendered animal products (e.g., meat and bone meal, poultry by-products, and blood meal) have also piqued the aquafeed industry's interest in filling the protein shortfall (Hua et al., 2019; Tacon et al., 2011). Consumer acceptance and EU legislation, on the other hand, may constrain their wide adoption in aquafeeds (Woodgate et al., 2022). Trimmings from fisheries and aquaculture are increasingly being used in aquafeeds (Olsen et al., 2014; Stevens et al., 2018), thus reducing wild fisheries demand (Froehlich et al., 2018a). In 2017, over 8 million MT of trimming products were used in aquafeeds, with marine fish, salmon, and shrimp being the prevalent destinations (Naylor et al., 2021). The estimated share of these by-products to global fishmeal and fish oil was 25–30% in 2018 (FAO, 2020b). Recycling these side-stream materials into aquafeed has significantly contributed to the resilience of the global food system (Fraga-Corral et al., 2022; Kusumowardani and Tjahjono, 2020).

The high demand for aquafeeds, as well as the need for more environmentally sustainable performance of aquaculture in coming decades, have pushed the sector to investigate more potential materials to sustain the long-term growth of the aquatic food system (Béné et al., 2015; Cottrell et al., 2020; Tacon, 2019). Diversifying ingredient options may also help maintain the economic viability and flexibility of aquafeeds when the price of any commodity fluctuates (Hua et al., 2019; Troell et al., 2014). On the other hand, the single option is unlikely to be adequate to fill the protein deficit of aquafeed and satisfy the nutritional requirement of fed organisms; complementarity among innovative resources will offer more robust solutions (Estévez et al., 2022; Ren et al., 2018; Turchini et al., 2019). Many ingredients have recently been proposed in the aquafeed shortlist, including insect meals, single-cell protein, macroalgae, food waste, among others; and it has been suggested that insect meals are preferable to others in supplying protein sources for future aquafeed (Cottrell et al., 2020; Gasco et al., 2020a; Hua et al., 2019).

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## 1.2. Insect meals as potential alternatives to fishmeal in aquafeed

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Insect meals are superior in terms of nutritional composition and environmental performance. They contain good nutritional properties with high macronutrient content, such as protein and lipid (Makkar et al., 2014; Nogales Mérida et al., 2019). Notably insect meals of black soldier fly (*Hemeticia illucens*) contain a high concentration of lauric acids (C12:0) – a saturated fatty acid with anti-microbial properties (Gasco et al., 2018; Gasco et al., 2021). In contrast, insect meals have a limited quantity of lysine and methionine and some minerals, such as calcium, which must be supplemented when incorporated into the diet of fish (Makkar et al., 2014). Furthermore, insect meals contain non-protein nitrogen, chitin, which modulates gut microbiota diversity and enhances the immunological response of fed organisms (Henry et al., 2018; Huyben et al., 2019). Regarding environmental concerns, the production of *H. illucens* requires less water and land input than plant ingredients of soy, pea, and whey concentrate (Smetana et al., 2019). When included in the diet for Icelandic Arctic char, *H. illucens* significantly reduced environmental impacts associated with acidification, eutrophication, carbon footprint (Smarason et al., 2017), and fecal phosphorus load (Weththasinghe et al., 2021). The use of insect meals in aquafeeds has an additional environmental advantage, as demonstrated by the fish in fish out ratio. Dietary insect meals significantly reduced this indicator in Siberian sturgeon (*Acipenser baerii*) farming (Rawski et al., 2020), implying that fewer marine wild fish was used to produce farmed fish. In consideration with circular economy principle, bioconversion of waste into insect meal biomass, subsequently used as feed ingredient could be a powerful approach to manage the waste and ensure sustainable food production (Fowles and Nansen, 2020; Gasco et al., 2020b). In contrast, the use of insect meals of *Tenebrio molitor* and *H. illucens* has been shown to increase nitrogen waste outputs and impart higher burdens on environmental indicators (Le Féon et al., 2019; Weththasinghe et al., 2021).

Among a wide range of insect meals, *H. illucens*, *T. molitor*, and common house fly (*Musca domestica*) have received great attention from academia and private producers (Hua, 2021; Mastoraki et al., 2020). In general, insect meal can be included at as high as 25–30% in diets without compromising the growth performance of the fed organisms (Liland et al., 2021). This inclusion level, on the other hand, is insect species-specific (Hua, 2021). A recent review on using insect meal in animal, particularly aquatic animals, diets (Gasco et al., 2019) demonstrated that dietary insect meal significantly affects nutrient digestibility, which is attributed to the presence of chitin in insect meal. The meat quality of insect-fed organisms was significantly altered, particularly omega-3 content was decreased in insect-containing diets. According to this investigation, fish fed insect meal exhibited negligible or no change in sensory characteristics. The gut microbiota investigation is received interest from academia for aquatic animals fed dietary insect meal. Research has shown that dietary insect meals significantly enhance microbiota diversity and abundance (Foyosal and Gupta, 2022; Huyben et al., 2019; Józefiak et al., 2019). Many inefficiencies associated with nutritional aspects of insect meals, such as imbalanced amino acids, a lack of essential fatty acids, and the presence of an excessive amount of chitin, have been identified to impede their extensive inclusion in feed formulas (Liland et al., 2021), processing (defatting) and manipulating feeding substrates could be practical approaches to enhance nutrient availability of insect meals for aquatic animals (Basto et al., 2020; St-Hilaire et al., 2007). The economic aspect is another bottleneck that challenges the expansion of insect meal in fish diets. Arru et al. (2019) reported that incorporating insect meal (*T. molitor*) in the diet for European seabass increased the feed costs due to outprice of insect meal relative to fishmeal. A similar finding was reported for Siberian sturgeon fed dietary *H. illucens* (Rawski et al., 2020). On the other hand, considering insect meals in conjunction with resultant environmental advantages and fish health improvement could further promote their role in aquafeeds (Arru et al., 2019; Rawski et al., 2020).

Overall, while evidence from the literature supports the validity of fishmeal substitution with insect meal is valid, broader aspects remain under further scrutiny, particularly encompassing more fish species, insect species, and their derivatives, as well as insights on nutrient complementarity among insect meals and other feed ingredients. The emphasis should also be laid on emergent traits, such as environmental benefits, meat traits, and effective microbiota modulations, among others, of aquaculture species fed dietary insect meals.

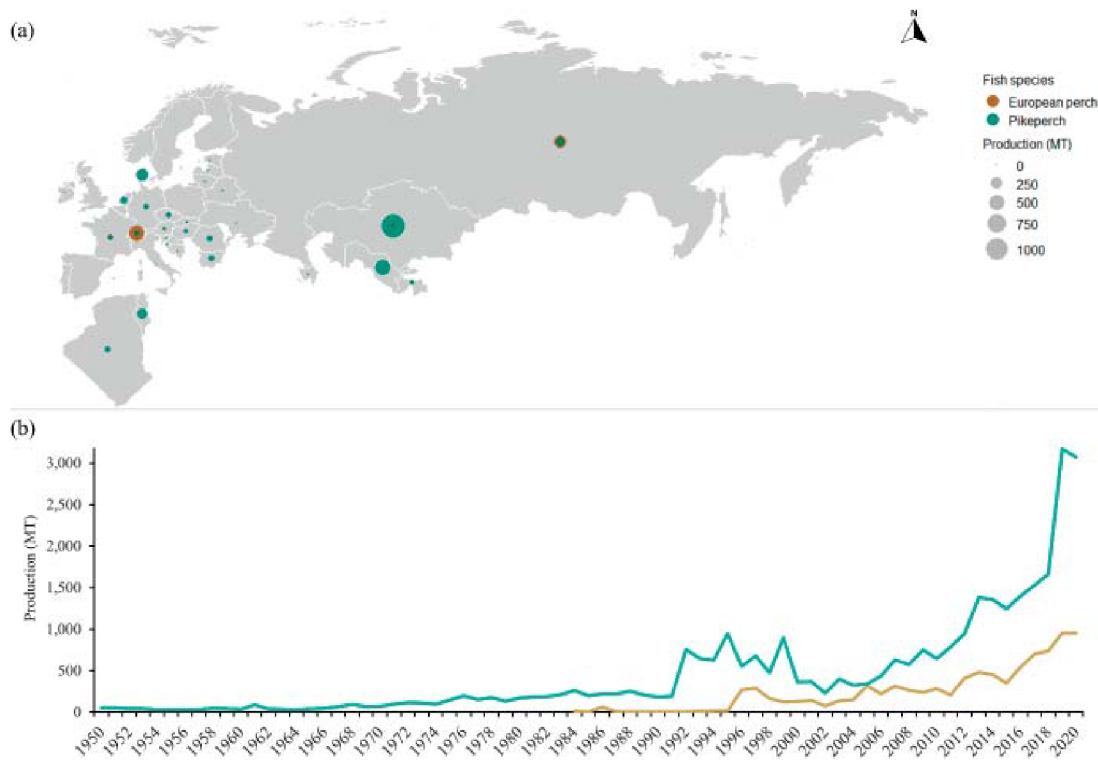
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### 1.3. Aquaculture and nutrition of Eurasian perch (*Perca fluviatilis*) and pikeperch (*Sander lucioperca*)

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Percid fish species, notably Eurasian or European perch (*Perca fluviatilis*) and pikeperch (*Sander lucioperca*), have the potential for aquaculture production in Europe in terms of delivering nutritious food items and market development (Policar et al., 2019). The production of perch and pikeperch, from wild capture and aquaculture, was estimated to be around 59 thousand MT in 2020, with the former accounting for more than 94%, and only a minor fraction was derived from farming (approximately 4 thousand MT) (FAO, 2020a). While the capture fisheries of perch and pikeperch have remained static since the 1990s (FAO, 2020a), farmed output has significantly increased (Fig. 1). Farmed-based pikeperch was documented at 50 MT in 1950, while the first data on Eurasian perch aquaculture was reported in 1993 in the Czech Republic with 12 MT. The output of both percid fishes has dramatically increased during the 2000s, and in 2020, pikeperch had reached 3074 MT, significantly above that of Eurasian perch (953 MT) (Fig. 1). Regarding production distribution, percid farming is mainly undertaken in Eurasian nations, but has expanded to African countries, such as Algeria and Tunisia, which contributed more than 200 MT pikeperch in 2020. Switzerland and Russia are the major producers, accounting for about 90% of total Eurasian perch yields, while Kazakhstan and Uzbekistan contribute more than 50% of world pikeperch outputs (Fig. 1).

Percid aquaculture is expected to pique the attention of a wide range of stakeholders and many farms will be established in Europe in the near future. Many challenges have been identified as needing to be addressed by the sector to assure future success, including feed for all stages of farmed fish, investments, reproduction programs, and marketplaces, among others (Overton et al., 2015). Feed is an important factor driving the success and benefit of any aquaculture practices. It is well adopted that feed costs account for 70–80% of the overall production cost for carnivorous fish in the recirculated aquaculture system (RAS) (Asiri and Chu, 2020; Hoerterer et al., 2022). The vast majority of perch and pikeperch production comes from RAS, using compound feeds (Bochert, 2020; European Commission, 2021; Hough, 2022; Wang et al., 2009). Despite the availability of commercial compound feeds developed for percid fish (such as Aller Aqua, Biomar, Le Gouessant Aquaculture), salmonid- and sturgeon-dedicated feeds are still extensively used in percid aquaculture (Bochert, 2020; Król et al., 2021; Schafberg et al., 2018).



**Figure 1.** Aquaculture production of Eurasian perch (*Perca fluviatilis*) and pikeperch (*Sander lucioperca*) in 2020. Global distribution (a) and production over time series (b). The size of the symbols denotes production (MT). Data retrieved from FAO (2020a).

Studies on the nutrient requirement of percid fish remain scarce all throughout the world. As elicited in Table 1, carnivorous fishes of perch and pikeperch require a high level of dietary nutrients. Dietary protein required by Eurasian perch and pikeperch varied from 47–57% and 43–55%, respectively, and is life-stage-dependent. Dietary lipid requirement for both fish species was reported to be as high as 17–19.3%; however, a low amount of lipid of 10% was also confirmed for pikeperch juvenile (Table 1). Kestemont et al. (2001) reported that dietary lipid of 12% also supports the growth performance of Eurasian perch. The prior study, investigating the effect of different commercial feeds on growth production and meat quality of Eurasian perch, found that Eurasian perch growth favours dietary protein levels greater than 50% (Bochert, 2020).

A recent review suggested that amino acid requirements are comparable between perch and pikeperch as they have close amino acid profiles in the whole body (Geay and Kestemont, 2015). Until recently, research on the amino acid requirement for perch and pikeperch has been universally limited. Langeland et al. (2014) suggested that Eurasian perch fed dietary lysine of 1.83–2.43% dry matter resulted in better growth performance. Authors also predicted dietary essential amino acid requirements of this fish species, which provides a groundwork for future percid feed formulation. Given the scarce data on these key nutrients for percids, Geay and Kestemont (2015) reported that Eurasian perch and rainbow trout had relatively similar dietary requirements for leucine, threonine, and valine. Therefore, to achieve precise and suitable feed for percid fish, additional investigation into comprehensive nutritional requirements for these species is required. Meanwhile, the available information from literature specific to other percid relatives could provide valuable references.

The investigation of carbohydrate requirement of percid fish is poorly reported, with the exception of a recent study encompassing pikeperch. Accordingly, the plausible levels of

carbohydrate ranged from 15 to 20% (Nyina-wamwiza et al., 2005). The study on carnivorous fish, Atlantic salmon (*Salmo salar*), revealed that 5–22% dietary starch supported growth performance; exceeding this range caused a detrimental effect (Hemre et al., 1993). Carbohydrates can promote the growth performance of carnivorous fish by avoiding amino acids from oxidative pathways (Hemre et al., 2002).

**Table 1.** Nutrient requirements (% as fed) of Eurasian perch (*Perca fluviatilis*) and pikeperch (*Sander lucioperca*).

Common name	Body weight (g)	Nutrient requirements*			References
		Protein	Lipid	CHO	
Eurasian perch	2.9	43.6–56.5			Fiogbé et al., 1996
	35	46.6	16.3		Mathis et al., 2003
	22.7		12		Kestemont et al., 2001
	34.2		19.3		Xu et al., 2001
Pikeperch	1.05	54.9	10		Schulz et al., 2007
	1.35–1.4	47	17		Schulz et al., 2008
		54	17		
	51.1	43	10		Nyina-wamwiza et al., 2005
			16		
		50			
				15–20	
210		10		Zakes et al., 2004	
22.1				Molnár et al., 2006	

\*The nutrient levels to which better growth performance of fish was reported; CHO: carbohydrate.

Mineral and vitamin requirements for percid fish are lacking in the literature. For many fish species, the study on micromineral availability received limited attention compared to macro-elements (Watanabe et al., 1997). In general, micro-and macro-minerals in the whole body and tissues of various fish species did not differ substantially (Antony Jesu Prabhu et al., 2016). Recently, the mineral requirement of some aquatic animal species has been defined (Davis and Gatlin, 1996; NRC, 2011). On the other hand, data for some fish species could be predicted through methods described earlier (Antony Jesu Prabhu et al., 2016; Schwarz, 1995; Shearer, 1995). It is worth noting that waterborne minerals occurred in many aquatic animals may change their dietary requirement (Antony Jesu Prabhu et al., 2016). A recent study indicated that dietary vitamin C and E supplementation enhanced growth performance and semen quality of yellow perch (*Perca flavescens*), a species belonging to the *Perca* genus (Lee and Dabrowski, 2004). However, the optimum level of vitamins in percid fish is missing from the literature. Therefore, more emphasis remained on a further investigation concerning minerals and vitamins requirements.

Above all, the nutrition study on percid fish in general, particularly perch and pikeperch, has been left a gap in academia. The future progress in this area will be essential groundwork to develop suitable feed formulation to support the emergence of Eurasian percid aquaculture.

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#### 1.4. Alternative protein sources for Eurasian perch (*Perca fluviatilis*) and pikeperch (*Sander lucioperca*) and suitability of insect meals

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Since commercial feed targeted to percid fishes has been recently developed in response to the global rise of percid aquaculture. Exploring a broader spectrum of aquafeed ingredients may safeguard the aquaculture industry's sustainable growth, and preserve flexibility and cost-effect in feed formulation (Naylor et al., 2009). While lipid replacement in perch and pikeperch diets has been thoroughly studied and described previously (Geay and Kestemont, 2015), the interest in alternative ingredients for fishmeal has received limited attention. Langeland et al. (2016) evaluated the nutrient digestibility of different protein-rich ingredients, including yeast (*Saccharomyces cerevisiae*), extracted yeast (*S. cerevisiae*), micro-fungus (*Rhizopus oryzae*), and mussel meal (*Mytilus edulis*) for use in Eurasian perch diets. The study demonstrated that all tested ingredients are efficiently digested by perch and that no significant difference in nutrient digestibility values was found across ingredients. Since nutritional composition and apparent digestibility of ingredients are essential evaluation before incorporating any ingredient into aquatic animal diets (Glencross et al., 2007), this study established framework for further feed formulation of Eurasian perch using novel ingredients. Recent study revealed possibility of substituting fishmeal and fish oil by innovative mix of algae-yeast in diets for pikeperch regarding feed palatability, growth performance, fish health and meat traits (Schafberg et al., 2018, 2021). In details, the mixture of microorganism, including cyanobacterium (*Arthrospira* sp.), algae (*Cryptocodinium cohnii*) and yeast (*Rhodotorula glutinis*), was used at the expense of fishmeal and fish oil in diets. It was concluded that these novel ingredients in the mixture was not favourable for pikeperch, which is ascribed to limited capacity of pikeperch to digest cell wall in these ingredients. The authors suggested the further optimization of feed composition for pikeperch is warranted. Recently, many novel feed ingredients, including fish by-products, insect meals, animal processed protein, sing-cell protein, among others, have been proposed in the aquafeed shortlist to sustain high-pace aquaculture growth (Cottrell et al., 2020; Hua et al., 2019; Naylor et al., 2009). Optimizing feed formulation for percid fishes based on alternative protein sources is essential for the future success of emerging-potential perch and pikeperch aquaculture.

Prior works speculated the suitability of insect meals in diets for many fish species, attributing it to the natural feeding behaviour of fish foraging on aquatic insects (Henry et al., 2015; Nogales Mérida et al., 2019). Some aquatic invertebrates, especially insects, are found to be prevalent in the digestive tracts of pikeperch and perch in their natural habitats. These dominant insects belong to the orders Chironomidae, Ephemeroptera, and Trichoptera (Akin et al., 2011; Vašek et al., 2018). However, the shifting diets from aquatic to terrestrial insect feed components may confront issues connected with the acceptance of percid fish species, due to nutritional deficiency, such as the relatively lower essential fatty acids in the latter than in the former ones (Fontaneto et al., 2011). Therefore, an *in vivo* feeding trial is necessary to investigate the physiological response of percid fish to the incorporation of terrestrial insect meals. The evidence from a recent study revealed that although having no adverse effect on survival rate, a dietary mixture of insect meals, including cricket (*Acheta domestica*) and superworm (*Zophobas morio*), compromised growth performance and feed efficiency of Eurasian perch juvenile (Tilami et al., 2020). These authors attributed this inefficiency to several factors, including the unpalatability of an insect-containing diet, and the presence of non-favourable components, oxidized fat, and chitin. That study also highlighted that dietary insect meals did not alter the fatty acid profile of fish fillets, with the exception of n-3 highly polyunsaturated fatty acids and linoleic acid (C18:2n-6), where insect-containing diet caused depletion of the former but elevation of the latter one compared to insect-free diet. It was further suggested that different combination of

insects should be warranted. This preliminary investigation underpinned important information on the use of insect as feed for percid fish, with emphasis placed not only on nutritional characteristics but also on palatability and anti-nutrient components.

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### 1.5. The objectives of the thesis

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The present thesis aims to provide a multidisciplinary investigation of the effect of insect meals on the physiological responses of Eurasian perch (*P. fluviatilis*) and pikeperch (*S. lucioperca*). In detail, the investigation included:

1. Overview of the environmental consequence and production performance of aquaculture fish fed dietary insect meals.
2. Effect of dietary black soldier fly (*Hermetia illucens*) and yellow mealworm (*Tenebrio molitor*) on growth performance, feed utilization, fillet quality of Eurasian perch.
3. Gut microbiota, histomorphology, and antioxidant biomarkers of pikeperch fed dietary black soldier fly (*H. illucens*).
4. Swimming capacity and physiological responses of Eurasian perch (*P. fluviatilis*) fed dietary yellow mealworm (*T. molitor*).
5. An insight into the proportional contribution of feed ingredients to muscle construction of Eurasian perch (*P. fluviatilis*) with and without the inclusion of dietary yellow mealworm (*T. molitor*).



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## **CHAPTER 2**

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### **SYSTEMATIC REVIEW AND META-ANALYSIS OF PRODUCTION PERFORMANCE OF AQUACULTURE SPECIES FED DIETARY INSECT MEALS**

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REVIEW

# Systematic review and meta-analysis of production performance of aquaculture species fed dietary insect meals

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## Abstract

The present work employed a systematic review and meta-analysis to quantify the overall effects of various types of insect meal on special growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) of aquatic animals. A total of 107 studies published from 1990 to 2021, targeting 23 freshwater and 17 marine fish species, employing 17 insect species as a replacement for fishmeal, was compiled. Overall, a significantly higher Hedges' *g* value for SGR and lower FCR was found in aquatic animals fed dietary larval defatted mealworm *Tenebrio molitor* and pupal full-fat silkworm *Bombyx mori* compared with fishmeal diet. The majority of dietary insect meals had a negative linear correlation with Hedges' *g* of growth performance, except larval fly *Chrysomya megacephala*, which had a positive linear relationship, and of prepupal defatted black soldier fly *Hermetia illucens*, which had a negative quadratic relationship. Some insect meals, including *G. bimaculatus*, adult grasshoppers of *Oxya fuscovittata* and *Zonocerus variegatus* and larval full-fat *Cirina butyrospermi*, supported adequate growth of aquatic animals at plausible inclusion levels. At as low as 2.2%, insect-derived chitin supported growth performance and improved feed utilization of marine fish species. In the quest to minimize fishmeal in aquafeeds, insect meal holds enormous potential but is not the sole option; rather, integrating insect meal and novel/conventional materials is more strategic. The present study lays the groundwork for further multidisciplinary considerations for the effective use of insect meal as an alternative aquafeed protein with the goal of long-term sustainability.

## KEYWORDS

chitin, growth performance, insect meal, meta-analysis, sustainability, systematic review

## 1 | INTRODUCTION

Blue foods, commonly known as aquatic foods derived from marine/freshwater capture or aquaculture,<sup>1</sup> have played a significant role in human consumption. In 2018, of 178 million tonnes of blue foods, approximately 90% were destined for humans, the remaining 10% was allocated for non-food applications, mainly reduced fishmeal and fish oil.<sup>2</sup> Rising population, wealth development and pescatarian-shifted behaviours will drive increasing global blue food demand in the following decades.<sup>2</sup> The top fish consuming nations'

(e.g. China, India) demand will nearly double from 80.7 million tonnes to 154.6 million tonnes between 2015 and 2050.<sup>1</sup> Given that capture fisheries, if applying technological and institutional innovations, might achieve maximum production of 57.4 million tonnes by 2050 (a 16% increase from the current level),<sup>3</sup> the growing demand for global fish foods will be mainly met by aquaculture production.<sup>4</sup> Aquaculture has been the fastest-growing food-producing sector, with an average annual growth rate of 5.3% in the period 2001–2018, the sector contributed approximately 46% in 2018 and will rise to 50% by 2030 to the global blue foods budget.<sup>2</sup> Within aquaculture,

fed aquaculture, which provided farmed organisms with formulated feeds or with processed/non-processed fish, has significantly contributed to annual aquaculture production in 2018 (69.5%) and outperformed non-fed aquaculture (contributed 30.5%).<sup>2</sup> Aquafeeds for fed aquaculture have traditionally relied on marine resources and, nowadays, increasingly on terrestrial crop ingredients.<sup>5-7</sup> The continuous growth of aquaculture, as driven by the rise in blue food demand, will accomplish the rising demand for aquafeed supply. Tacon<sup>8</sup> estimated that total aquafeed production used in 2017 was 51.23 million tonnes and would reach approximately 73.15 million tonnes in 2025, implying that a fill of 22 million tonnes will be required to support the global growth of aquaculture by 2025.

Given that forage fish from marine resources will reach their ecological stock boundary by 2037,<sup>9</sup> further use of this finite resource in aquaculture is not long-term sustainable,<sup>10</sup> and that intensification of terrestrial crop production for aquafeed inputs could raise concerns about environmental burdens, such as arable land use, water use, deforestation,<sup>11,12</sup> searching for alternative ingredients for future aquaculture growth is a priority to ensure continuous growth of the sector. Among a broad range of novel ingredients developed for future aquafeed inputs, insect meals exhibit the great feasibility of meeting the protein requirements for aquafeeds in the coming decades.<sup>6,13,14</sup>

Insect meals possess advantageous properties, such as favourable nutritional composition (mainly high protein content and balanced amino acid profiles),<sup>15</sup> health improvement for fed organisms,<sup>16</sup> and environmental benefits of insect-containing diets associated with economic fish in fish out, land use and solid phosphorus waste compared with insect-free diets,<sup>17</sup> the use of these insects for aquatic animals, therefore, has received increasing interest from research and industrial practitioners over last years. By the end of 2019, a total of 32 published articles investigating fishmeal replaced by insect meal was retrieved.<sup>13</sup> In the following year, by applying different screening and search criteria, 84 and 91 relevant records were compiled, respectively, by Tran et al.<sup>17</sup> and Liland et al.<sup>18</sup> At the commercial aspect, for example in Europe, the production of insect protein for feeds is predicted to reach 60,000 tonnes by 2025 and 200,000 tonnes by 2030.<sup>19</sup> Accordingly, the share of insect meal ingredients used in aquaculture will reach 40% by 2030, exceeding that in pet food (30%).<sup>20</sup>

Although some narrative reviews have summarized the effects of insect meals as replacement for fishmeal on the production performance<sup>21-23</sup> and found mixed results on fed organisms, the overall efficacy of insect meals, as well as the interplay of other explanatory variables rather than insect meal inclusion level on growth parameters, remain fragmented. Therefore, a quantitative approach is more appropriate to address mentioned inefficiency of the past qualitative works. Recently, meta-analysis has been used to synthesize the overall effect of dietary insect meals on growth performance and feed utilization of aquaculture species.<sup>9,13,18</sup> The tendency to which the growth parameters vary with the inclusion levels of insect meal was also emphasized. These works, on the other hand, considered insect meals a generic concept, despite the fact that insect meals varied greatly in terms of life stage (e.g. larvae, prepupa, adult) and degree of the defatted process (full-fat, partial defat and defat),

presence of chitin, among others. These forms of insect meals could cause a substantial discrepancy in nutritional compositions between and within insect meal(s), which subsequently have a significant impact on nutrient digestibility and growth performance of examined organisms.<sup>15,24-27</sup> Therefore, findings from the past systematic reviews could remain ambiguous or lead to a different interpretation when evaluating a specific type of insect meal.

In the present study, we combined systematic review and meta-regression analysis to investigate (i) current studies on the use of insect meal as replacement for fishmeal in aquatic animal diets; (ii) the overall effect of various insect meal categories on the effect size of production performance (specific growth rate, SGR; feed conversion ratio, FCR and protein efficiency ratio, PER); (iii) correlation between each insect category and production parameters; (iv) interactive of multiple explanatory variables on effect sizes indices; and (v) effect of insect-derived chitin levels on effect sizes indices. Further discussions were brought to tackle challenges that hinder the broader adoption of insect meal in aquafeeds.

## 2 | MATERIALS AND METHODS

### 2.1 | Literature search

The PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines<sup>28</sup> was used to compile relevant literature from Web of Science (1965–2021), PubMed (1975–2021) and Scopus (1970–2021) until 24 July 2021 (Figure 1). The following search strings in the title, keywords, abstract were used to retrieve records:

("insect meal" OR "*Tenebrio molitor*" OR "Insecta" OR "*Hermetia illucens*" OR "*Musca domestica*" OR "*Imbrasia belina*" OR "Chironomids" OR "*Gryllus bimaculatus*" OR "*Bombyx mori*" OR "*Oxya hyla hyla*" OR "*Zonocerus variegatus*" OR "*Zophobas morio*" OR "*Cirina butyrospermi*" OR "*Grylloides sigillatus*" OR "*Blatta lateralis*" OR "*Oxya fuscovittata*" OR "*Acheta domesticus*" OR "*Gryllus assimilis*" OR "Termite") AND ("fish meal" OR "fishmeal") AND (fish OR shrimp) AND ("growth").

The studies were eligible for meta-analysis database if they met the following criteria: (i) insect meals as an alternative protein source for fishmeal in diets; (ii) focussed on fish and shrimp; (iii) one of the production performance parameters was presented: special growth rate (SGR, %/day), feeding conversion ratio (FCR), protein efficiency ratio (PER, %); (iv) provide sufficient information on investigated parameters, such as mean, sample size, treatment error (standard deviation [SD], standard error [SE]). The studies which investigated mixed insect meals or a mixture of insect meals vs. other protein sources as a replacement for fishmeal were not eligible for the database.

### 2.2 | Data analysis

Hedges' *g* effect size (*g*) was calculated as previously described.<sup>29</sup> The influence of explanatory variables (fishmeal level, fish oil level,

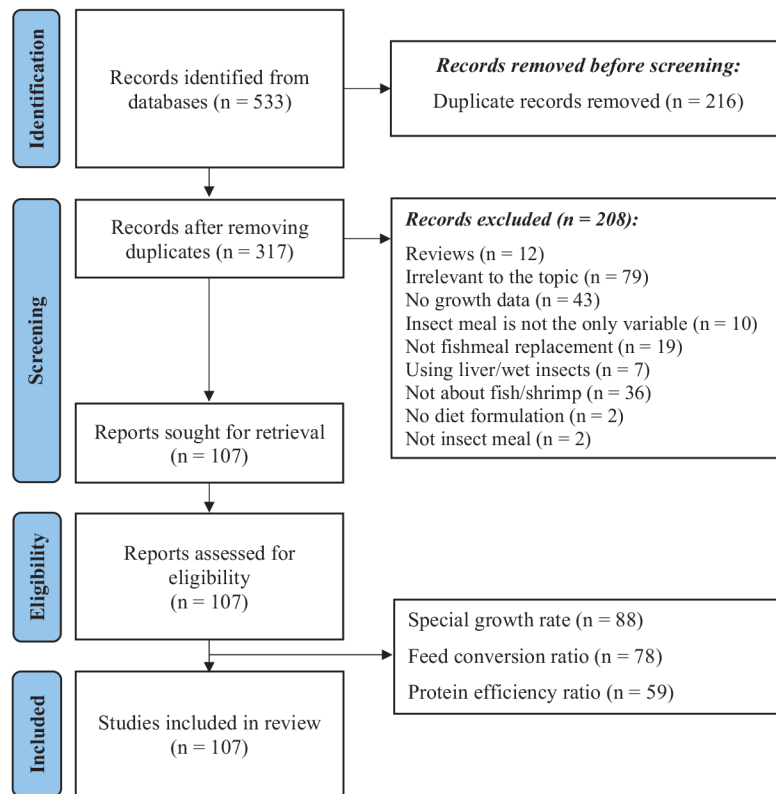


FIGURE 1 PRISMA flow chart for eligible literature for meta-analysis database

trophic level, experimental duration, chitin level, temperature) on Hedges' *g* effect size was performed with multiple linear regression under the ANCOVA framework.

The effect sizes of SGR, FCR and PER were compared across insect meal categories. As data of Hedges' *g* effect sizes were highly skewed, the non-parametric methods, Kruskal–Wallis test and Dunn's post hoc test with Bonferroni correction, were applied for statistical analysis.

### 2.3 | Heterogeneity and publication bias

Among-study heterogeneity was determined via  $I^2$  index. Heterogeneity is considered high and low if  $I^2$  index is  $\leq 50\%$  and  $>50\%$  respectively.<sup>30</sup> To further investigate source of heterogeneity, analysis of covariance (ANCOVA) was conducted to test interactive effects of six potential explanatory variables (fishmeal level, fish oil level, chitin level, trophic level, experimental duration and temperature) on Hedges' *g* effect sizes.

Egger's tests were used to assess publication bias. When publication bias was detected ( $p < 0.05$ ) by Egger's test, we removed outliers and reported results from the outlier-free test according to the previous description.<sup>31</sup> Statistical significance was assumed with  $p < 0.05$ .

All statistical analyses were performed using the R statistical package (Version 1.4.1103, R Development Core Team 2009–2020, available at [www.r-project.org/](http://www.r-project.org/)).

## 3 | RESULTS

### 3.1 | Overview of included database

A total of 107 studies published between 1990 and 2021 that examined the production performance of aquaculture species fed dietary insect meals, were eligible for the meta-analysis database (Figure 2, Table S1). The number of studies was greatest in Italy (15 publications), followed by China (13 publications) (Figure 2a). The aggregation included 17 insect species from 6 orders, with black soldier fly (*Hermetia illucens*) receiving the most interest (44% of total compiled studies), followed by yellow mealworm (*Tenebrio molitor*) (24%); housefly (*Musca domestica*) and silkworm (*Bombyx mori*) each contributed 7% to the dataset. For the first three mentioned insect species, larvae full-fat form was commonly used, whereas prepupal full-fat silkworm was mainly employed in the literature. Most cricket, grasshopper, cockroach and termites in the adult stage with defatted-free process were used in publications (Figure 2b). The majority of study (60% of total fish habitat investigated) converged freshwater fish (23 species, belonging to 12 families), while the remaining data (40%) focussed on marine fish (17 species of 14 families) (Figure 2c). In a recent decade, there was growing attention on the use of insect meals as an alternative for fishmeal in aquaculture fish, as illustrated by the steady increase in the number of publications (Figure 2d).

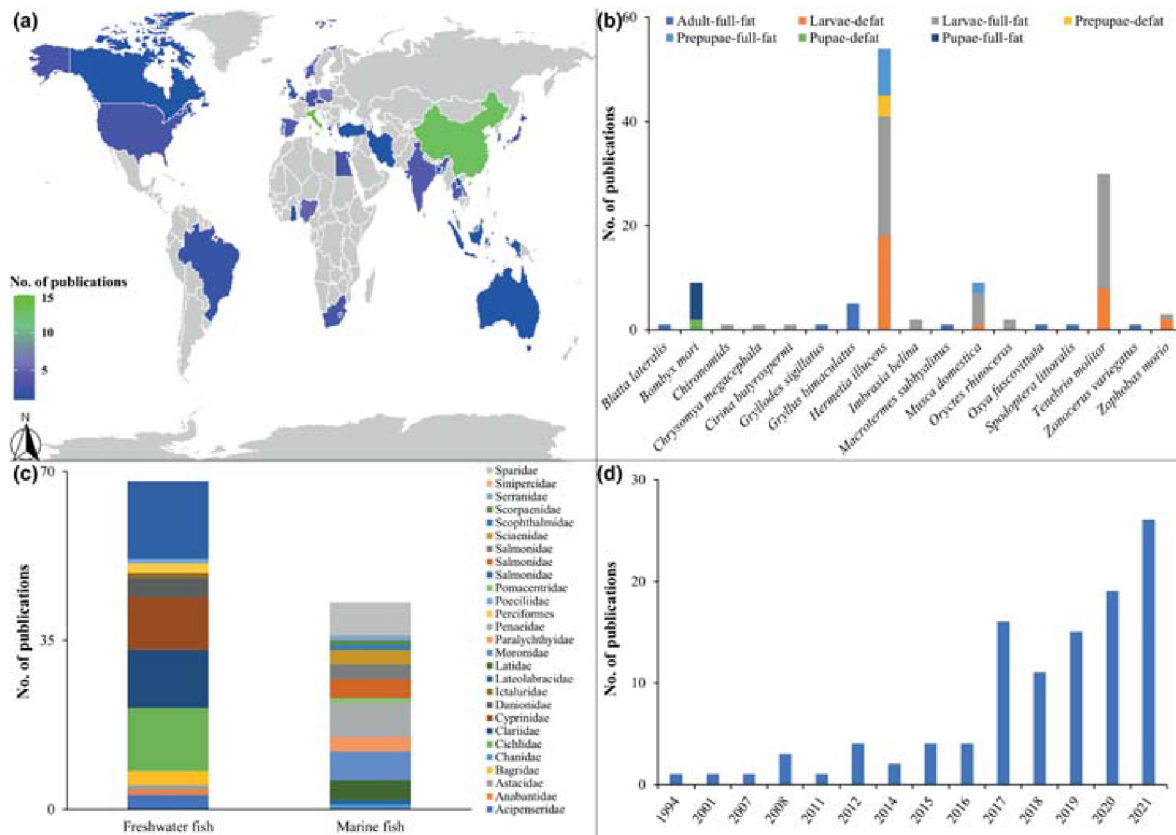


FIGURE 2 Overview of the dataset. (a) distribution of publications by country, (b) distribution of publications by insect life stages and degrees of defatting, (c) distribution of publications by fish habitat and family and (d) cumulative number of publications by year

Given that different life stages and degrees of defatting of insect meals are hypothesized to have significant effects on fed organisms regarding growth indices, a comprehensive assessment of various insect meal forms could thus provide useful information for the insect protein choice for the aquaculture sector.

## 3.2 | Production performance of aquaculture species fed different insect meals

### 3.2.1 | Overall effect size

Inclusion of various insect meals in diet displayed varying impacts on the special growth rate (SGR) of fed organisms (Figure 3). Only two insect meals, *T. molitor* (larvae [L], defat [DF]) and *B. morio* (pupae [P], full-fat [FF]), showed a significant positive effect on SGR ( $p < 0.0001$  and  $p = 0.021$  respectively). In contrast, compared with insect-free groups, most insect meal-containing diets had a negative impact on or did not significantly enhance Hedges'  $g$  value of SGR of aquaculture species (Figure 3, Table S2).

Effect of dietary insect meals on Hedges'  $g$  value for FCR using meta-analysis was illustrated in Figure 4 and Table S3. Among 22 insect meal categories investigated, 6 and 12 groups displayed

significant increase ( $p < 0.05$ ) and non-significant effect ( $p > 0.05$ ) on FCR meta-analysis compared with insect-free diets respectively. In contrast, the inclusion of four insect forms, namely *B. morio* (P-FF), *C. megacephala* (L-FF), *G. bimaculatus* (A) and *T. molitor* (L-DF), significantly reduced FCR Hedges'  $g$  ( $p < 0.05$ ), implying better feed assimilation.

Concerning protein efficiency ratio, our meta-analysis found that aquatic animals received dietary *B. morio* (P-FF) and *G. bimaculatus* (A) had a significant positive effect compared with fishmeal group ( $p < 0.05$ ) (Figure 5 and Table S4).

### 3.2.2 | Effect size comparison among insect meal categories

As the SGR effect sizes were left-skewed (skewness =  $-0.563$ ), Kruskal-Wallis test was performed. Correspondingly, there was a significant difference in SGR effect size of aquaculture species fed different dietary insect meals ( $p < 0.0001$ ). Animals fed with dietary larval defatted mealworm *T. molitor* resulted in significantly higher Hedges'  $g$  value for SGR than larval full-fat worm *Imbrasia belina* ( $p < 0.0001$ ), larval full-fat black soldier fly *H. illucens* ( $p < 0.01$ ), and than prepupal defatted black soldier fly *H. illucens* ( $p < 0.0001$ ).

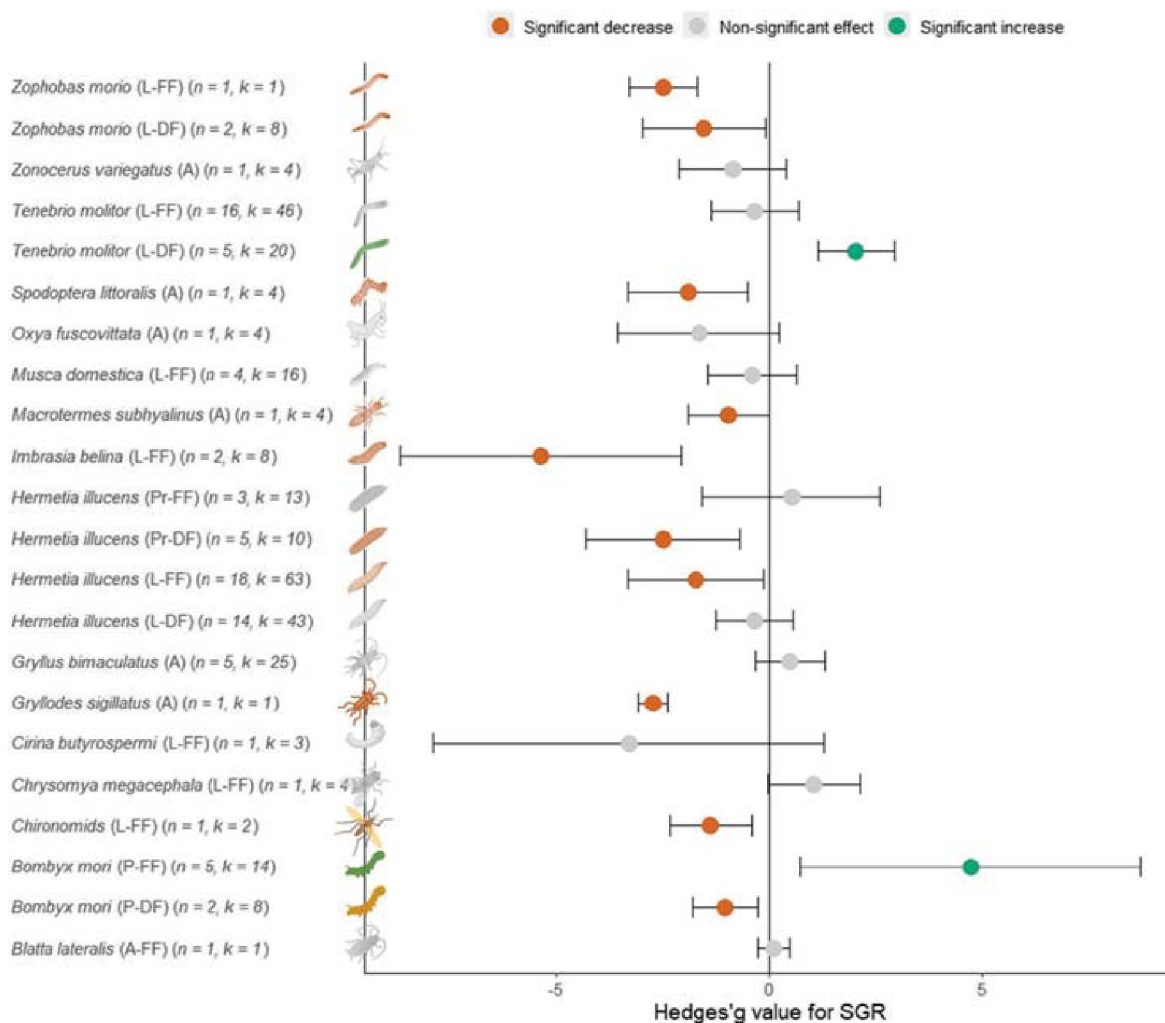


FIGURE 3 Forest plot for Hedges' g effect size (mean and 95% confidential interval) of special growth rate (SGR). n indicates the number of studies; k is the number of comparisons (treatment vs. control group). The letters in the brackets indicate life stage (A, adult; L, larvae; P, pupae; Pr, prepupae) and fat-processed form (DF, defat; FF, full fat) of insect meals

Dietary pupal full-fat silkworm *B. mori* significantly enhanced the growth of the fed organism compared with larval full-fat worm *I. belina* ( $p < 0.01$ ). Different life stages and degrees of defatting within insect species had negligible influence on the SGR Hedges' g effect size of tested fish (Figure 6).

The Kruskal–Wallis test, following Dunn's post hoc, revealed that fish fed dietary *G. sigillatus* (A) significantly improved (lower) FCR effect size than those fed *H. illucens* groups and *I. belina* (L-FF) ( $p < 0.05$ ). A similar pattern was observed for *B. mori* (P-FF), which had significantly lower FCR Hedges' g compared with *H. illucens* (prepupae (Pr)-DF) and *I. belina* (L-FF) ( $p < 0.05$ ) (Figure 7).

Unlike the above-mentioned parameters, the effect size of PER did not significantly differ among insect meal categories ( $p > 0.05$ ) (Figure 8).

Our meta-analysis study found that larval defatted yellow mealworm and pupal full-fat silkworm are important protein sources as an alternative to fishmeal when considering the growth performance

of targeted aquaculture species and, therefore, hinted further focus on practical aquafeeds.

### 3.3 | Meta-regression analysis

#### 3.3.1 | Correlation between insect meal level and effect size

When considering dietary insect meals as continuous variables and SGR as responded ones in the meta-regression models, there was, in most cases, a significant negative linear correlation between insect meal administration and SGR effect size ( $p < 0.05$ ), with the exception of *H. illucens* (prepupae (Pr)-DF) and *B. mori* (P-FF), where significant quadratic ( $p = 0.008$ , adjusted  $R$ -squared = 0.68) and cubic ( $p = 0.043$ , adjusted  $R$ -squared = 0.89) relationship, respectively, were found (Figure 9, Table S5). Accordingly, inclusion of

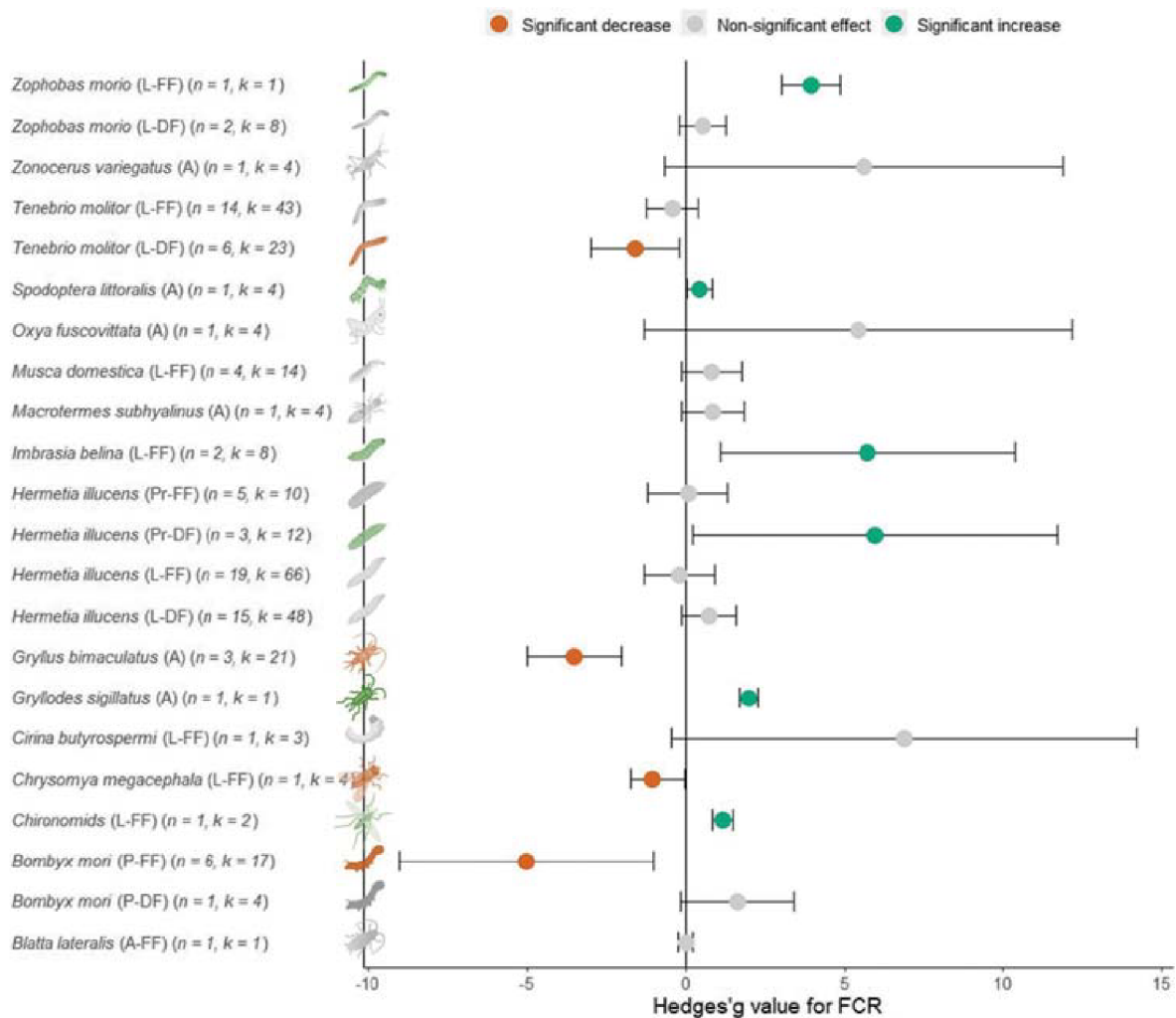


FIGURE 4 Forest plot for Hedges' g effect size (mean and 95% confidential interval) of feed conversion ratio (FCR). n indicates number of studies; k is number of comparisons (treatment vs. control group). The letters in the brackets indicate life stage (A, adult; L, larvae; P, pupae; Pr, prepupae) and fat-processed form (DF, defat; FF, full-defat) of insect meals

*H. illucens* (Pr-DF) of approximately 13.1% could benefit aquaculture species in terms of growth performance compared with the control group. Increasing levels of *B. mori* (P-FF) up to 16.9% resulted in a consistent decline in fish growth, and further inclusion showed fluctuation in SGR of fed species, which requires scrutiny as few data was reported for inclusion of more than 16.9%. The exception was also observed for *C. megacephala* (L-FF), where dietary inclusion significantly enhanced Hedges' g value for SGR ( $p = 0.04$ , adjusted  $R$ -squared = 0.85). It is worth noting that there were thresholds of dietary insect meals to which effect size of SGR remained positive, or in other words, comparable with the reference diets. These insects included adults of *G. bimaculatus* (threshold, 50.3%), *Oxya fuscovittata* (19.2%) and *Zonocerus variegatus* (15.1%) and larval full-fat *Cirina butyrospermi* (32.4%), beyond these plausible levels, an adverse effect was observed (Figure 9, Table S5).

Regarding FCR effect size, a significant positive correlation with dietary *H. illucens* (Pr-DF) ( $p < 0.0001$ , adjusted  $R$ -squared = 0.85),

and significant negative linear with dietary *C. megacephala* (L-FF) ( $p = 0.04$ , adjusted  $R$ -squared = 0.88) was found (Figure 9, Table S6).

Our regression analysis also found that dietary *G. biacutatus* (A) and *C. megacephala* (L-FF) significantly correlated with PER effect size by positive linear models ( $p = 0.019$ , adjusted  $R$ -squared = 0.33 and  $p = 0.04$ , adjusted  $R$ -squared = 0.88 respectively), whereas inclusion of *O. fuscovittata* (A) significantly reduced that value ( $p = 0.015$ , adjusted  $R$ -squared = 0.65) (Figure 9, Table S7).

### 3.3.2 | Correlation between insect-derived chitin levels and effect size

Given that the presence of insect-derived chitin in diet could have varying results on the growth production of fed organisms, a meta-regression analysis was performed to detect the relationship between these variables.

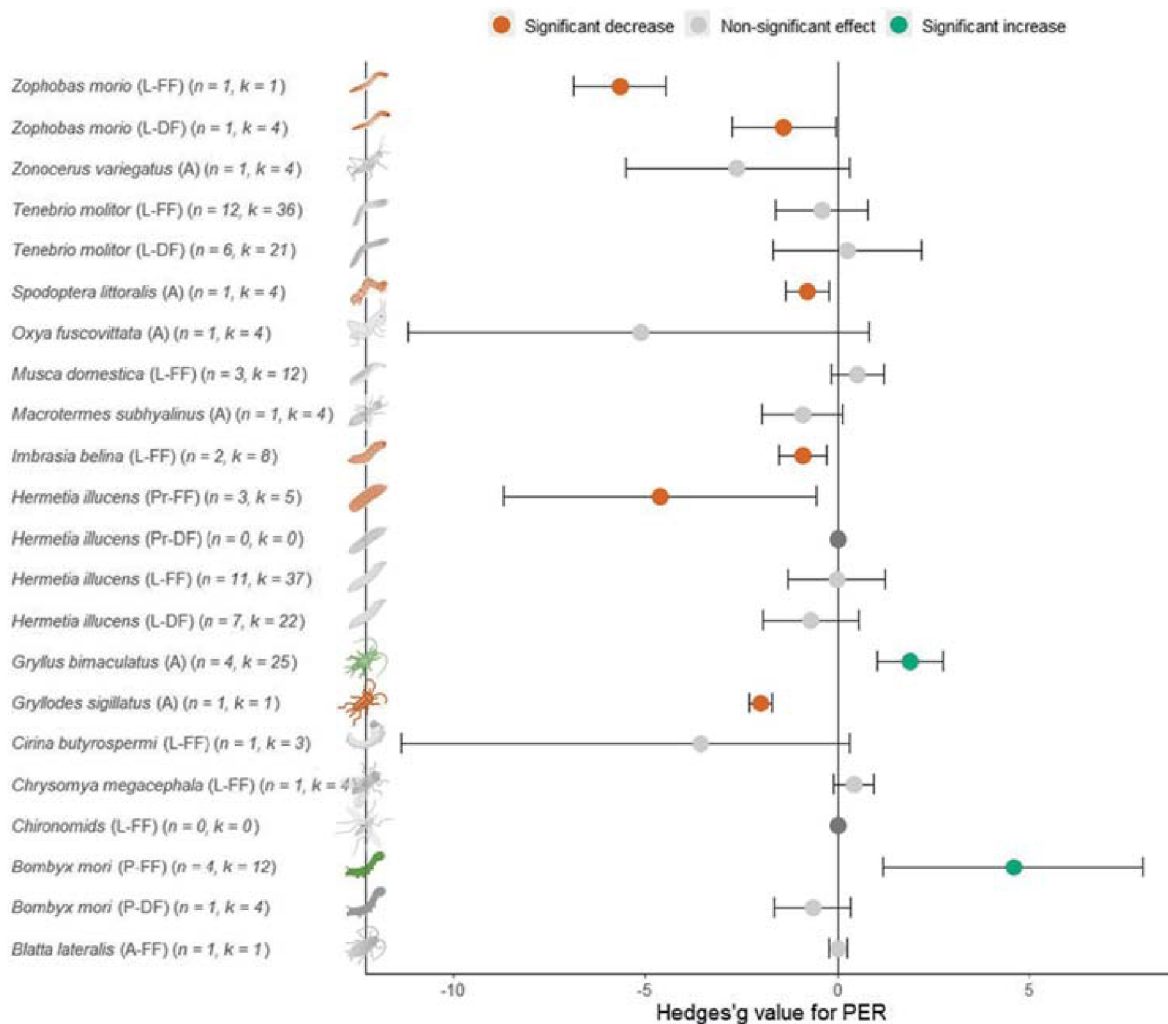


FIGURE 5 Forest plot for Hedges' g effect size (mean and 95% confidential interval) of protein efficiency ratio (PER). n indicates the number of studies; k is the number of comparisons (treatment vs. control group). The letters in the brackets indicate life stage (A, adult; L, larvae; P, pupae; Pr, prepupae) and fat-processed form (DF, defat; FF, full-defat) of insect meals

There was a statistical quadratic correlation between insect-derived chitin and SGR of marine fish species ( $p < 0.0001$ , adjusted R-squared = 0.92) and of turbot *Psetta maxima* ( $p = 0.006$ , adjusted R-squared = 0.99). Dietary insect-derived chitin significantly reduced the effect size of SGR in olive flounder *Paralichthys olivaceus* as demonstrated by a negative linear model ( $p = 0.04$ , adjusted R-squared = 0.86) (Figure 10, Table S8). By this means, it was anticipated that dietary insect-derived chitin of approximately 1.25% and 1.60–1.75% could benefit marine fish and turbot aquaculture, respectively, regarding growth performance.

Dietary chitin was shown to be significantly correlated with FCR effect size for marine fish and turbot, which is well fitted with positive quadratic models ( $p < 0.0001$ , adjusted R-squared = 0.97 and  $p = 0.024$ , adjusted R-squared = 0.95 respectively) (Figure 10, Table S8). Based on these models, the optimum chitin levels, which yielded the lowest FCR, were predicted, respectively, 1.9% and 2.2% for marine fish and turbot (Table S8).

### 3.4 | Heterogeneity and publication bias

Analysis of heterogeneity (as indicated by  $I^2$  values) revealed significant between-study variation for SGR effect size ( $I^2 > 79.22\%$ ), FCR ( $I^2 > 85.28\%$ ) and PER ( $I^2 > 86.53\%$ ). Subgroup analysis indicated significant interaction of explanatory variables on these effect sizes (Tables S2–S4).

Publication bias (Egger's test,  $p < 0.01$ ) was confirmed in most insect meal categories for the effect sizes of SGR, FCR, PER. After removing strong outliers, the outputs of meta-analysis remained valid when compared with the non-outlier-removal results, with the minor exception, *Zophobas morio* (L-DF) for SGR, which changed from significance ( $p = 0.040$ ) to non-significance ( $p = 0.084$ ); *H. illucens* (L-DF) for FCR, changed from non-significance ( $p = 0.100$ ) to significance ( $p < 0.0001$ ); and *M. domestica* (L-FF), changed to significance ( $p < 0.0001$ ) from non-significance ( $p = 0.134$ ) after removing outliers (Tables S2–S4). Therefore, the bias among various

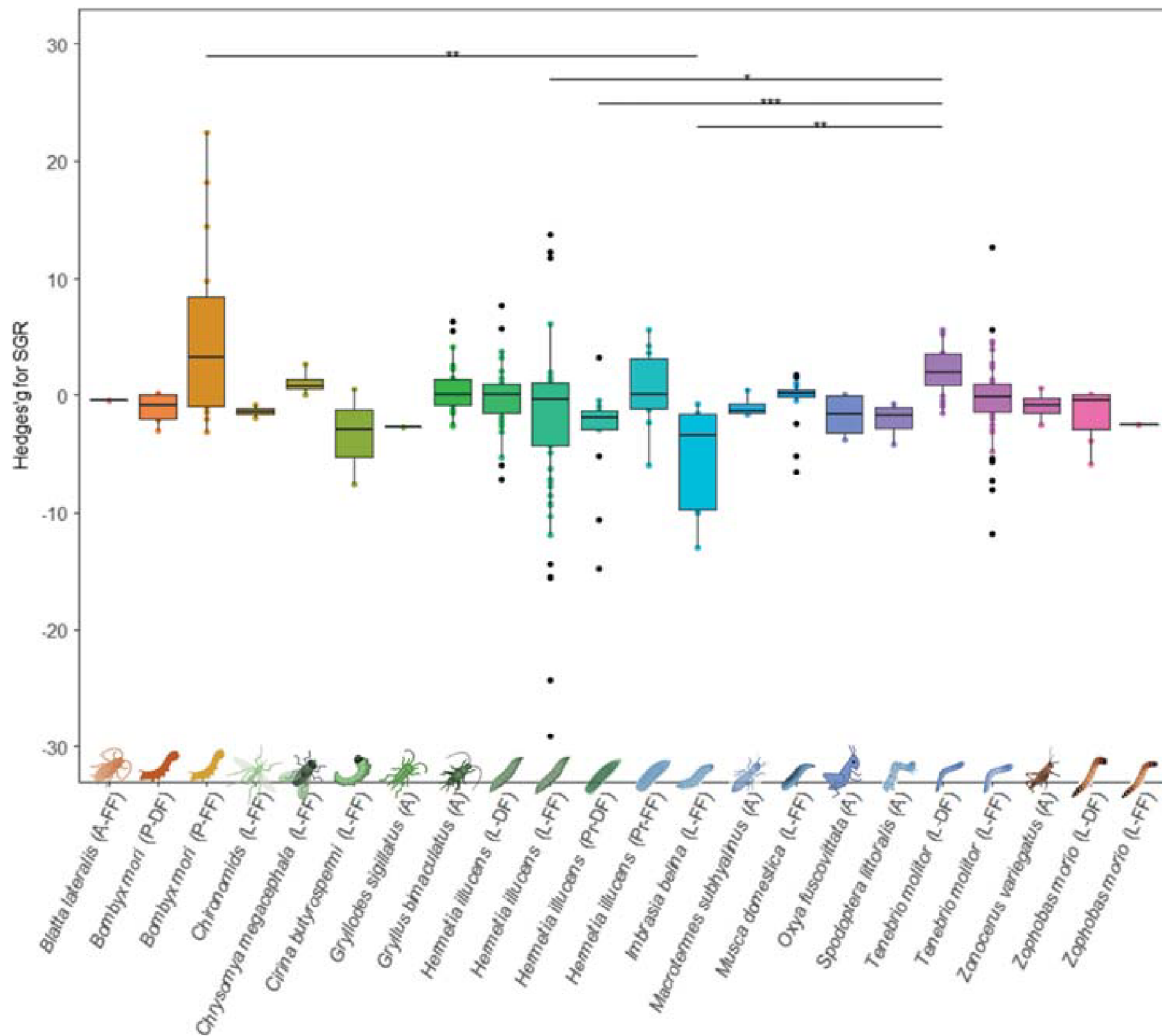


FIGURE 6 Comparison of SGR effect size of different insect meal categories. Coloured filled dots are observed data points. The lower and upper dashed lines represented the first quartile and third quartile. The black filled circles denote outliers. The brackets and "\*" indicate a significant difference between insect meals, with \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

studies did not, in general, interfere with the findings from the present study.

Analysis of covariance (ANCOVA) indicated that investigated effect sizes of SGR, FCR and PER were affected by multiple variables, including fishmeal level, fish oil level, chitin level, trophic level, experimental duration and temperature, rather than dietary insect meals alone (Tables S9–S11).

#### 4 | DISCUSSION

Insect meals have received increasing attention from researchers and practitioners around the globe as the filler for aquafeed protein gap in the coming decades,<sup>6</sup> as evidenced by the rapid rise in the number of publications in recent years (Figure 1d) and by the involvement of insect-produced companies. At a glance, by 2019, more than 40

European enterprises have participated in this fledgling industry, contributing approximately 6000 tonnes of insect meal per year.<sup>32</sup> So far, 17 insect meal species categorized into 22 forms with different life stages and processed modifications have been investigated as an alternative protein source for many aquaculture species (Figure 1b,c). Despite this, recent projects employing a meta-analysis approach have considered insect meals a generic concept while investigating their effects on the production performance of aquatic animal species.<sup>9,13,18</sup> In addition, comparing the effect of various insect meal species or processed forms on aquatic animals remains universally limited.<sup>27,33–37</sup> Therefore, the present study highlighted the importance of breaking down insect meals into individual moderators according to their reported forms from the literature, and of comparing the effect sizes of different insect meal categories. This might significantly impact future aquafeed research and practice in terms of offering appropriate options from numerous available insect meals.



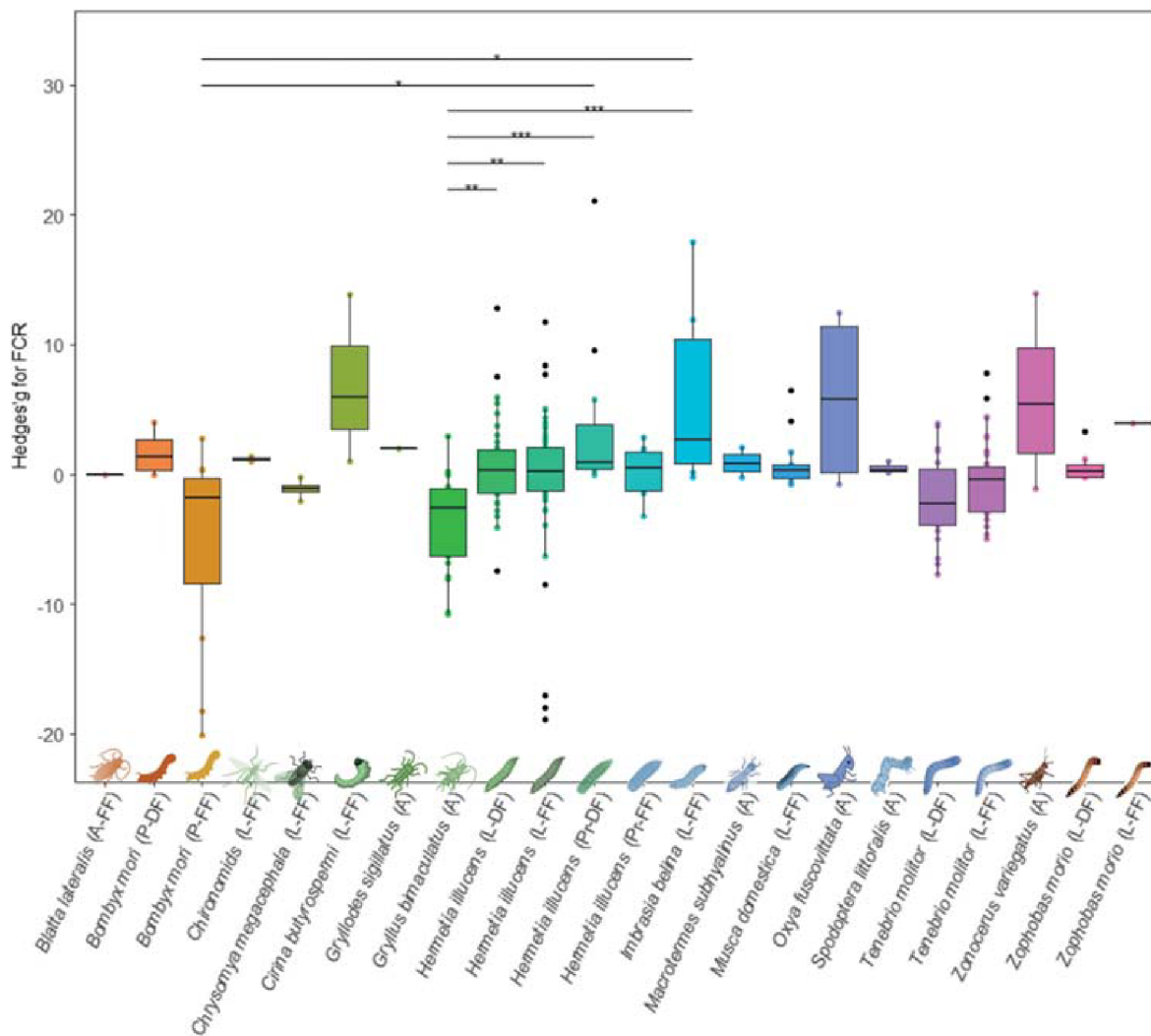


FIGURE 7 Comparison of FCR effect size of different insect meal categories. Coloured filled dots are observed data points. The lower and upper dashed lines represented the first quartile and third quartile. The black filled circles denote outliers. The brackets and ‘\*\*\*’ indicate a significant difference between insect meals, with \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

Our findings demonstrated the advantages of substituting fishmeal with some insect species in aquaculture diets regarding growth production and of insect-derived chitin for marine fish species. Dietary *T. molitor* (L-DF) and *B. mori* (P-FF) improved Hedges’ *g* effect size of special growth rate (SGR), reduced feed conversion ratio (FCR) and to a lesser extent, enhanced protein efficiency ratio (PER). The meta-comparison of effect sizes showed the superiority of these insect meals species in terms of SGR and FCR (Figures 7 and 8). Although cricket (*Gryllus bimaculatus*) (A) did not significantly affect SGR, it significantly reduced FCR and increased PER of fed animals compared with the insect-free group. Given the global increase in the use of insect meals in aquafeeds, most of the research has focussed on *H. illucens*, *T. molitor* and *M. domestica*<sup>6,13,35</sup> (see also Figure 1c). These species have been approved by European Commission (EU-2017/893, 24 May 2017) for use in aquafeeds and *B. mori* is proposed to be on board by the end of 2021. Silkworm

has been received increasing interest from aquaculture nutritionists since its first use in rohu (*Labeo rohita*) in 1994,<sup>38</sup> with a comparable number of publications with *M. domestica* by July 2021(Figure 1c).

Our meta-regression found a threshold to which dietary *H. illucens* (Pr-DF) could yield surpassed growth indices compared with the control group. The plausible inclusion levels while retaining effect size of SGR were also reported for *G. bimaculatus* (A), *O. fuscovittata* (A), *Z. variegatus* (A) and *C. butyrospermi* (L-FF), with dietary *G. bimaculatus* being the most promising, reaching as high as 50%. An increasing level of larval full-fat *C. megacephala*, on the other hand, enhanced the effect size of SGR, PER, and reduced FCR (Figure 9); this relationship, however, should be interpreted carefully due to the small sample size. Given that insect-derived chitin is considered as an influential factor to growth indices of insect-fed animals,<sup>39,40</sup> results from meta-regression revealed that the presence of as low as 2.2% chitin could be beneficial for marine fish species, including

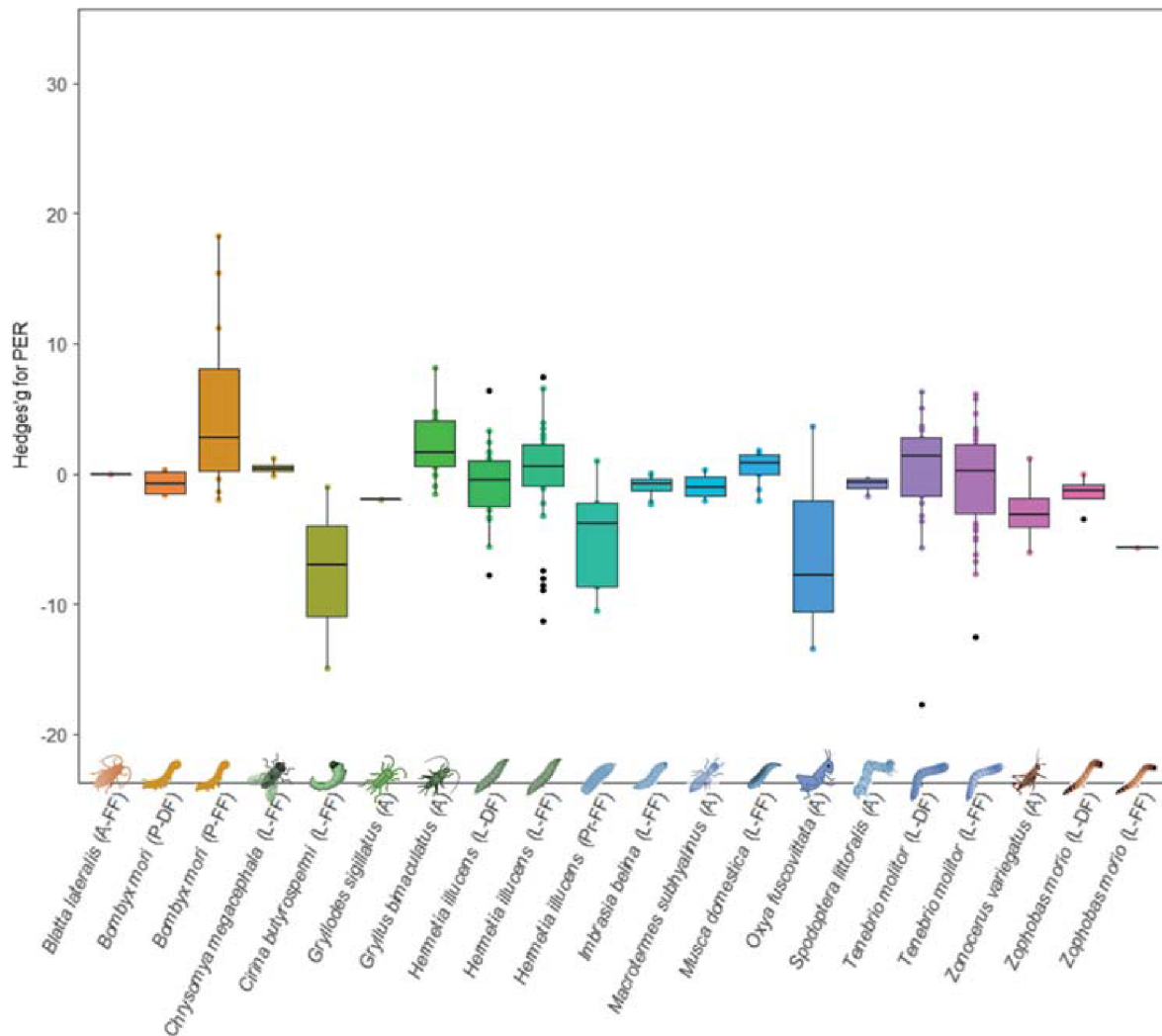


FIGURE 8 Comparison of FCR effect size of different insect meal categories. Coloured filled dots are observed data points. The lower and upper dashed lines represented the first quartile and third quartile. The black filled circles denote outliers. The brackets and '\*' indicate a significant difference between insect meals, with \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

*P. maxima*, seabass *Dicentrarchus labrax*, and *P. olivaceus* with regard to production performance (Figure 10).

Although the present quantitative synthesis discovered the significance of particular insect meals on Hedges'  $g$  effect size of growth performance of aquatic animals, the high heterogeneity and publication bias were detected for subgroup analysis. The high heterogeneity could be attributed to the diversity of examined fish species, fish habitat and fish feeding behaviour among insect categories, as elicited in Tables S2–S4. The covariates, including fishmeal level, fish oil level, chitin level, trophic level, experimental duration and temperature, are important sources of heterogeneity, significantly influencing Hedges'  $g$  effect sizes (Table S9). Furthermore, the discrepancy in feeding protocols, experimental facilities among studies could explain significant heterogeneity in the present study.<sup>41</sup> Regarding publication bias observed in all sub-group analyses, the effect sizes (mean and 95% confidential interval) remained

consistent after removing outliers. Therefore, this inefficiency did not affect the conclusions of the present study.

The efficacy of incorporating larval defatted *T. molitor* in aquatic animal diets regarding growth performance and feed utilization was confirmed in many works.<sup>42–45</sup> In contrast, the inclusion of this insect meal adversely affected SGR of *P. olivaceus*.<sup>46</sup> Overall, the effect size was significantly positive in the present study, thus hinting further study to focus on the use of larval defatted *T. molitor* in aquafeeds. This efficiency could be explained by nutritional composition and nutrient digestibility properties. The defatted process is known to improve protein content, thus, amino acid profile of larval *T. molitor* and, as a result, nutrient digestibility compared with full-fat form in *D. labrax*.<sup>27</sup> The synthesized data showed that protein contents of defatted *T. molitor* meal lie within the protein interquartile range of fishmeal (Figure S1) and amino acid profile was consistent with fishmeal, as demonstrated by similarity cluster (Figure S2). Protein

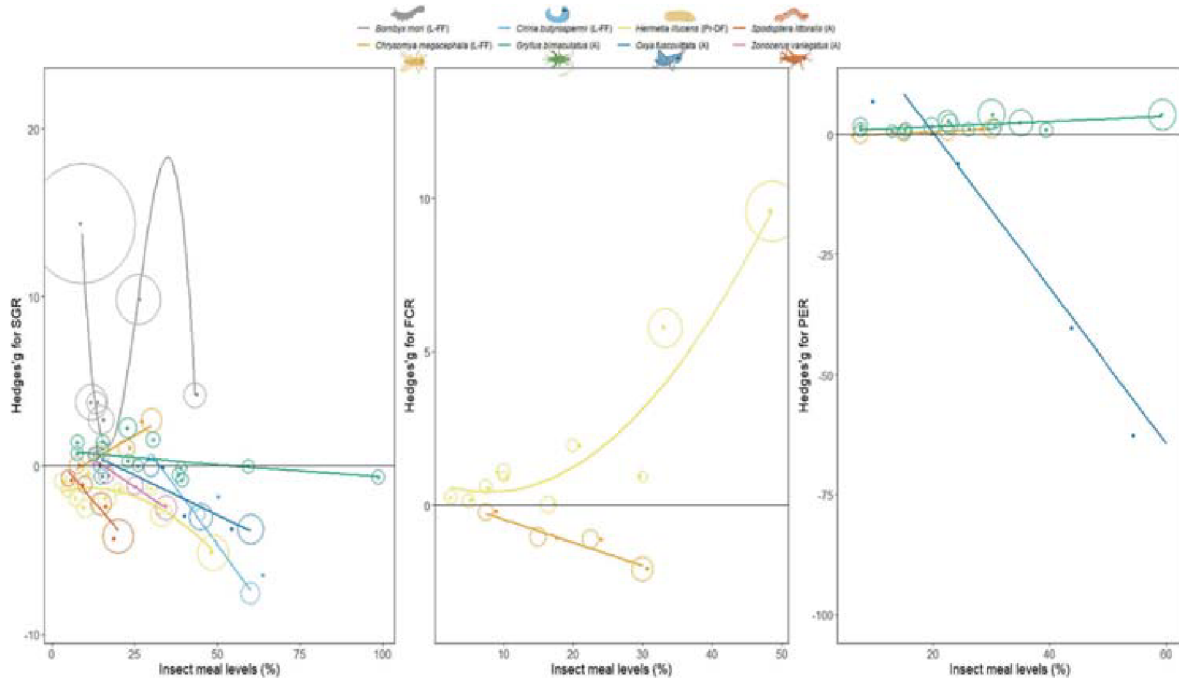


FIGURE 9 Meta-regression analysis for dietary inclusion of various insect meals and the effect size of SGR, FCR and PER. The coloured filled dots and diameters represent mean and 95% CI of the effect size

digestibility of *T. molitor*-containing diets showed mixed results from the literature. Some reported consistent values, while others found a significantly lower, at substantial inclusion levels, compared with fishmeal group for some fish species.<sup>42,44,46</sup> The low diet digestibility could probably lead to a delay in the growth performance of tested fish. The nutrient digestibility of insect-containing diets was, on the other hand, strongly influenced by chitin levels.<sup>17,47</sup> Our meta-regression indicated that increasing insect-derived chitin levels had a detrimental effect on the growth indices of aquatic animals. There was, however, a threshold to which the presence of chitin benefits the organism (Figure 10). Therefore, nutritional balance and chitin levels could be vital factors driving the efficacy of insect meals in animal diets.

Recent consideration of *B. mori* use in aquafeeds by the EU Standing Committee on Plants, Animals, Food and Feed, in combination with the environmental benefits associated with less phosphorus waste output of silkworm-containing diets compared with silkworm-free diets for aquatic animals,<sup>17</sup> and considerably lower price than fishmeal<sup>48,49</sup> could be a spotlight for broader insect protein options in fish diets. Most studies investigated dietary full-fat *B. mori* in aquatic animal diets exhibited surpassed growth performance compared with fishmeal diet.<sup>38,49–51</sup> Likewise, Ji et al.<sup>48</sup> documented that inclusion of 5.7% or 50% replacement fishmeal by silkworm was recommended for juvenile Jian carp *Cyprinus carpio*, whereas higher levels compromised fish growth. Since the majority of studies were conducted on cyprinid fish, it is assumed that pupal silkworm in full-fat form is suitable in diet for these fish. The nutritional composition of pupal silkworm was reported to be similar to

that of fishmeal, in terms of protein content, amino acid profile and a good source of omega-3  $\alpha$ -linolenic acid.<sup>50,52</sup> On the other hand, silkworm contains ecdysteroid, a growth-promoting factor, that improves protein synthesis and tissue formation.<sup>53</sup> This sufficiency could contribute to the significant positive effect of SGR, PER and subsequently reduce FCR of animals fed dietary silkworm meal. The meta-regression indicated an inverse correlation between dietary silkworm and Hedges' g for SGR (Figure 9), which could be attributed to several factors, mainly low feed intake driven by low palatability of silkworm,<sup>48</sup> as well as negative changes in physical properties of extruded silkworm-containing diets.<sup>54</sup>

Comparative studies assessing the effects of various insect meals on growth performance of aquatic animals have piqued the curiosity of researchers. The consistency of growth indices in comparison to insect-free diet of rainbow trout *Oncorhynchus mykiss* fed dietary full-fat insect meals, *H. illucens*, *T. molitor*, *Gryllodes sigillatus*, *Blatta lateralis*, *M. domestica*, as a replacement for fishmeal, was recently documented.<sup>36,55,56</sup> A similar finding was observed for Siberian sturgeon *Acipenser baerii* fed 15% of full-fat *H. illucens*, and *T. molitor* as substitution for fishmeal.<sup>33</sup> These results suggested that at low or moderate inclusion levels, full-fat insect meals had no effect on performance of fed organisms. As far as defatted insect meals are concerned, Mastoraki et al.<sup>35</sup> found no significant difference on SGR of *D. labrax* fed 30% fishmeal replaced by defatted *H. illucens*, full-fat *T. molitor* and *M. domestica*. Of all, previous research supports the current meta-analysis findings that full-fat insect meals as well as defatted *H. illucens* resulted in comparable SGR, FCR and PER when included in diets for fish species (Figures 6–8). Although

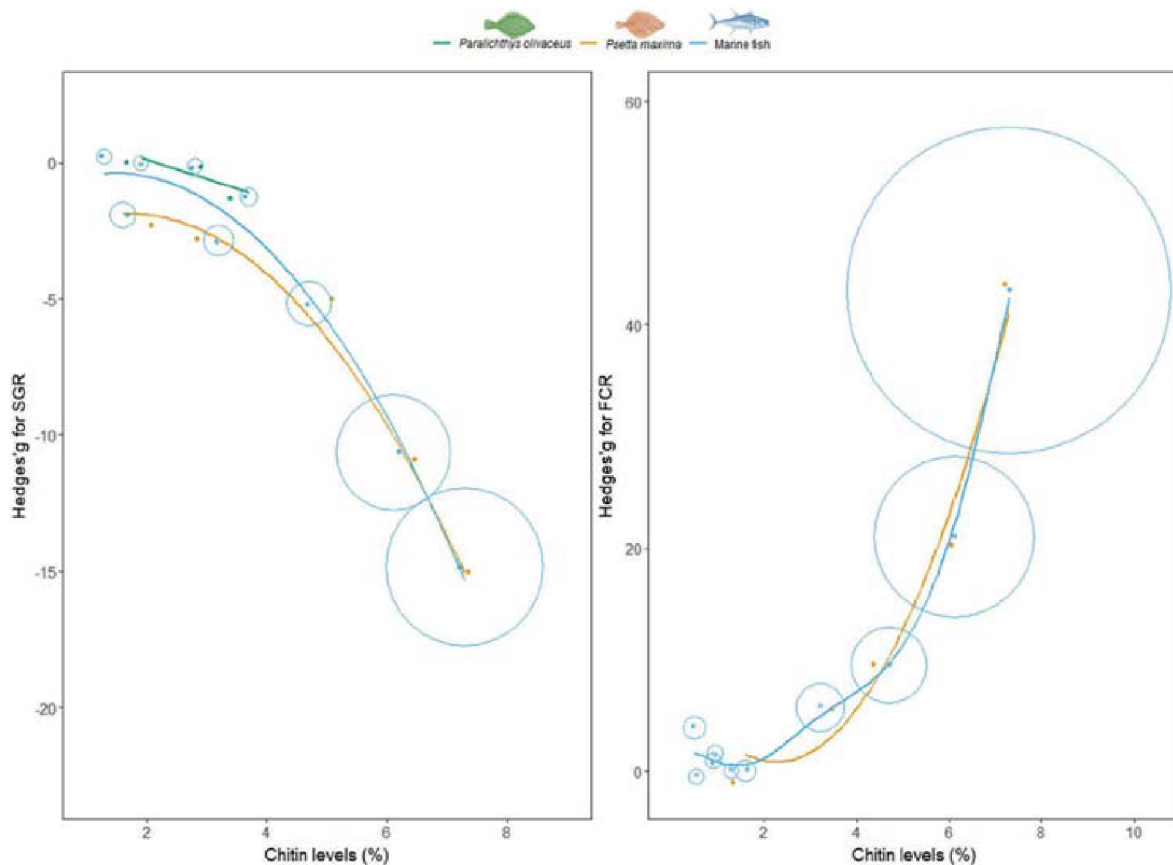


FIGURE 10 Meta-regression analysis for dietary insect-derived chitin and the effect size of SGR, FCR. The coloured filled dots and diameters represent mean and 95% CI of the effect size

the comparison among insect meals, including defatted *T. molitor* in fish performance, is absent from the literature, the superiority of defatted *T. molitor* over full-fat *H. illucens* has been evidenced. For *D. labrax*, Basto et al.<sup>27</sup> reported that larval defatted *T. molitor* had significantly greater nutrient digestibility than larval full-fat *H. illucens*. Given that nutrient digestibility largely affects growth performance of fish,<sup>13</sup> our data, therefore, confirm the magnitude of difference in SGR effect size of fish fed dietary *T. molitor* (L-DF) and *H. illucens* (L-FF) (Figure 6). The Kruskal-Wallis test further demonstrated that fish fed dietary *I. belina* meal had significantly lower SGR Hedges' *g* than either *B. mori* (P-FF) and *T. molitor* (L-DF). This could be explained in part by the fact that both studies evaluating dietary *I. belina* meal in catfish *Clarias gariepinus* and tilapia *Oreochromis mossambicus* showed lower growth than the control group,<sup>57,58</sup> whereas multiple positive changes were observed for dietary *B. mori* (P-FF) and *T. molitor* (L-DF) as above discussion. Consequently, dietary *I. belina* exhibited a significantly higher FCR effect size than *B. mori* (P-FF) (Figure 7).

The capacity of marine fish to digest chitin varies greatly. Cod *Gadus morhua*, yellowtail kingfish *Seriola quinqueradiata*, Japanese eel *Anguilla japonica*, cobia *Rachycentron canadum* and red sea bream *Pagrus major* showed high chitinase activity.<sup>59</sup> While *P. maxima*, Atlantic salmon *Salmon salar* and meagre *Argyrosomus regius* have a poor capacity to

digest chitin.<sup>59-61</sup> Our correlation models revealed that *P. maxima* and marine fish (including *D. labrax*, *P. olivaceus*) (Figure 10) tolerated at low levels of dietary chitin. This phenomenon was also observed in the low chitin digestion group as proven in *S. salar*,<sup>47</sup> where dietary chitin level of as lower as 2% could sustain SGR, the elevated levels caused inverse correlation. In contrast, the high-chitin-digested group could tolerate dietary chitin levels at as high as 10% without affecting growth performance for *A. japonica*, *S. quinqueradiata*,<sup>62</sup> and 5% for *G. morhua*, and halibut *Hippoglossus hippoglossus*.<sup>47</sup> At the low dietary levels, chitin plays an important role in modulating beneficial gut microbiota composition<sup>16</sup> and antioxidative enzyme,<sup>63</sup> thereby improving nutrient digestibility and health of hosts. Their high inclusion levels, however, did not correlate with elevated chitinase activities in some fish species<sup>62</sup> and led to lower nutrient digestibility, as previously demonstrated,<sup>17,47</sup> these authors confirmed that chitin impairs nutrient digestibility of diets in many fish species. This inefficacy of chitin could be ascribed by reducing the digesta transit time, thereby reducing the exposure time of food to digestive enzymes due to viscosity of chitin,<sup>64</sup> by interrupting enzymes in the proximal and middle intestine involved in breaking down peptides into amino acids,<sup>65</sup> and through binding with lipid and bile acids, thereby hampering lipid absorption.<sup>66</sup> The poor nutrient digestibility at the presence of insect-derived chitin might be attributed to the extrusion process. Although insect-containing feeds have been

successfully produced using the extrusion technique,<sup>60,67,68</sup> extruded feeds have some shortcomings compared with insect-free diets, such as reduced expansion, sinking velocity and water stability, which may adversely affect nutrient availability.<sup>60,69</sup>

## 5 | INSECT MEAL SELECTION FOR FUTURE AQUAFEEDS

Among a wide range of insect meals investigated in the present work targeting growth performance of various aquaculture species, larval defatted *T. molitor* and pupal full-fat *B. mori* showed great potential as an alternative to fishmeal in aquafeeds. In addition, the interest should be drawn to prepupal defatted *H. illucens*, larval full-fat *C. megacephala*, adults of *G. bimaculatus*, *O. fuscovittata*, *Z. variegatus* and larval full-fat *C. butyrospermi* as their dietary inclusion supported the growth of fed organisms similar to fishmeal diet at plausible thresholds, ranging from 13.1% to 50.3%. Of these, *H. illucens*, *T. molitor*, *B. mori* and *G. bimaculatus*, in descending order, have received the most attention from worldwide. In contrast, study on *C. megacephala*, *O. fuscovittata*, *Z. variegatus* and *C. butyrospermi* in diet for aquaculture species remained universally limited. Therefore, scrutiny is warranted to explore their potential use in aquafeeds. We also acknowledge that the present meta-analysis study has limitations regarding the degrees of nutritional balance across experimental diets, the magnitude of defatting process and sample sizes among insect meal categories, due to information scarce reported in the literature. For instance, when employing larval full-fat *H. illucens* as a replacement for fishmeal, only four of 18 publications supplied amino acids, while the remainder did not consider this amino acid balance. Our dataset revealed that lysine and methionine were the most common amino acids supplemented in insect-containing diets, followed by tryptophan, arginine, taurine and threonine, which are the limited nutrients found in insect meals compared with fishmeal.<sup>15,70</sup> The publication bias in our meta-analysis encompassed these inefficiencies, as previously stated. Therefore, insect meal options and their dietary threshold for aquatic animal diets identified in the present study remain valid.

## 6 | TACKLING THE CHALLENGES

The successful use of potential insect meals in aquafeeds should be aligned with socioeconomic and environmental factors, the most important of which are economic aspects, environmental consequences and consumer acceptance.

Currently, most insect meals were more expensive than conventional protein sources in animal feed, thus eliciting less economic efficacy while including in aquafeeds.<sup>71-74</sup> The recent review outlined the economic aspects of insect farming has suggested that the interventions on upscaling mass-rearing facilities and low-value feed substrate utilization could substantially reduce operational cost.<sup>71,75</sup> The former has been witnessed worldwide with the establishment of industrial operators in Europe<sup>76</sup> and other parts.<sup>77</sup> The targeted

production by 2030 is expected to be 50 times as high as the current level, reaching 500,000 tonnes, with 40% or 200,000 tonnes destined to aquafeed.<sup>78</sup> The sector's expansion is also driven by increasing interest in using insect meal as functional properties in food and feed<sup>79</sup> (e.g. feed palatability enhancement,<sup>80</sup> gut health effect<sup>81</sup>), by the growth of niche markets (free-range animals),<sup>20</sup> among others. Once global insect protein production reaches half a million tonnes by 2030, the time required to double volume will be significantly shortened, thus one million tonnes is likely achieved.<sup>82</sup> Since the cost of rearing substrates is deemed a vital issue in large-scale insect farming, utilization of side stream substrates and by-products could bring cost- and environmental-effectiveness for insect protein products<sup>83-85</sup> and elicit circular economy perspective.<sup>86-88</sup> Given that the European Commission only authorizes vegetation origin as the substrate for insect farming, diversification of substrates, such as food waste, by-products, could further facilitate large-scale farms and price reduction.<sup>20</sup> For instance, the substrate for *H. illucens* accounted for 81%-90% of total farming costs and increasing use of by-products from sawdust in combination with brewery spent grain considerably reduced the cost of rearing, thus more income generation.<sup>89</sup> Rearing crickets for animal feeds is only profitable when weeds or organic side streams are employed.<sup>90</sup> Production of *H. illucens* fed by-products was more environmentally effective than plant- and animal-based substrates.<sup>91</sup> Therefore, efforts in upscaling rearing facilities and developing suitable substrates could turn insect protein more price-competitive with fishmeal and soybean meal in the coming years<sup>20</sup> (2023<sup>6</sup> or 2030<sup>82</sup>).

Importantly, developing insect rearing facilities based on locally available substrates, adjacent to aquaculture operations, deems a strategic move to produce cost-effective, import-independent and readily available aquafeed ingredients. This may require further considerations into investment, logistics, legislation, among others, but seems viable in specific geographical spectrum.<sup>92-94</sup> For instance, Asia is the leading aquaculture producer, accounting for 92% of global production in 2017,<sup>5</sup> and a large consumer of aquafeeds.<sup>95</sup> Agriculture and human activities have generated a vast volume of waste and by-products.<sup>96,97</sup> Therefore, insect meal protein, based on organic waste valorization, as an aquafeed ingredient appears feasible for the region. Such bioconversion and biotransformation yielded promising results in Asia and worldwide.<sup>92,98,99</sup> Insect farming for animal feed is rising in this region, with some operators established in India, Indonesia, Malaysia, Singapore and Vietnam, primarily focusing on *H. illucens*,<sup>77,93</sup> *B. mori*, *G. bimaculatus* and *T. molitor* farmings have existed for pet and food purposes.<sup>100</sup> This application might secure the sector's long-term development.

Insect meals in aquaculture diets significantly reduced forage fish from marine ecology but entailed enormous nitrogen waste outputs.<sup>17</sup> Therefore, the further development of aquafeed formulas with the inclusion of potential insects, *H. illucens*, *T. molitor* and *Z. variegatus* suggested by the present review, should consider an additional cost for chitin degradation as this non-protein nitrogen compound indirectly causes such inefficacy.<sup>60,101</sup> Recirculating aquaculture systems could be a suitable option for farming fish fed

insect-containing diets as solid wastes are effectively removed, thus maintaining water quality.<sup>5</sup> A more sustainable approach to tackle nitrogen waste output at the addition of insect ingredients could be combining multiple trophic species in the farming system, such as integrated multi-trophic aquaculture systems, polyculture-based aquaculture and aquaponic system. Thereby, nitrogenous loss from targeted-fed species is retained by extractive organisms.<sup>102,103</sup> This approach could enhance consumer perception about fish-fed insect meals' sustainability. Compared with the above-mentioned insect species, *B. mori* appeared to be more environment-effective by reducing nitrogenous and phosphorus waste output when included in aquafeeds at the expense of fishmeal.<sup>17</sup> In this context, *B. mori* could be promoted as an environmentally friendly ingredient in aquafeed and used in offshore aquaculture to minimize solid waste.

Gaining consumers' acceptance of insect-fed fish products will be the critical gateway for successfully adopting insect-based feed for aquaculture, thus mass production expansion. A recent survey, on a worldwide scale targeted more than 2400 consumers from 71 countries, has revealed a promising result, with 66% of respondents willing to eat animals/fish fed insect-based diets.<sup>104</sup> Several works focusing on Western consumers also reported positive consumer perception with insect-fed seafood products.<sup>105,106</sup> On the other hand, the consumers' preference for this innovative food is driven by price and environmental perception.<sup>107-109</sup> Once the upscaling mass production is in place, the cost of insect-based feed will be more price-competitive with conventional feed, thus that of insect-fed seafood.<sup>105,110</sup> The campaigns and marketing focusing on the environmental benefits of insect-fed fish could potentially shift the consumer's awareness in a positive direction.<sup>105</sup> Thereby, biotransformation and bioremediation using insects for seafood products in a circular economy concept, sustainability of insect-fed fish in the multi-trophic fish culture system, among others, are deemed valuable information for promoting insect-fed seafood.

## 7 | OUTLOOKS

The present meta-analysis suggests eight potential insect meals for future aquafeeds concerning the growth performance of fed fish. However, further consideration is needed to determine the extent to which such insect meals might indeed be viable protein sources for aquafeeds in the coming years.

First, the combination of insect meals and other protein sources, rather than the former alone, could potentially fill the protein gap and compensate for limited nutrients. The targeted production of aggregate insect meal by 2030 (200,000 tonnes destined for aquafeed<sup>78</sup>) is much lower than convenient ingredients currently used in aquafeeds.<sup>5</sup> In contrast, novel protein sources for aquafeeds have been recently developed with high nutritional values, sustainability and scalability.<sup>6,14</sup> Such blend has been promoted for *H. illucens*, a tryptophan deficient ingredient, and *B. mori*, a tryptophan-rich one

to preserve complementing nutrients in fish feed.<sup>40</sup> Several studies investigating a mixture of insect meals with terrestrial/aquatic materials to replace fishmeal in aquatic animal diets achieved comparable growth performance but a higher degree of sustainability.<sup>111-114</sup> The most popular combining material in aquafeeds with insect meal is poultry by-products,<sup>112-115</sup> which are well accepted by a wide range of fish species, considerably cheap, and widely available at a large supply volume (17 million tonnes per year<sup>116</sup>).<sup>117</sup> Fishery and aquaculture by-products are potential candidates to supply protein sources for aquafeed.<sup>6</sup> In 2017, approximately 9 million tonnes of these trimming products were used as fishmeal and fish oil in aquafeed worldwide.<sup>5</sup> The share of these materials in the global production of fishmeal and fish oil is expected to reach 34% by 2030.<sup>2</sup> In addition to terrestrial plant ingredients widely used in aquafeeds,<sup>118</sup> poultry and fishery by-products are likely to become co-combination with insect meal regarding protein fillers, complementary nutrients, and the environmental benefit of aquafeeds. Unlike those commercially available materials, the combination of macro-, microalgae and single-cell protein with insect meals in aquaculture diets showed an unfavourable growth rate compared with fishmeal-based diets.<sup>119</sup> In addition, factors associated with low production volume, high cost and unrealistic scalability may hamper their wide adoption in aquafeeds.<sup>6,9</sup>

It is also crucial to acquire comprehensive information on the quality of seafood fed insect-containing diet, including sensory evaluation, nutritional values, and on gut microbiota modification through meta-analysis to explore the effect of potential insect meals on end-products and their benefits to the hosts.

## 8 | CONCLUSIONS

Employing a meta-analysis approach, we investigated the overall effects of specific types of insect meal on Hedges' *g* effect sizes of growth performance, feed utilization and protein efficiency of aquaculture species. The findings highlighted the superiority of two insect meal forms, namely larval defatted *T. molitor* and pupal full-fat *B. mori*, over others in terms of production performance indices. Although larval full-fat *C. megacephala* did not exhibit a significant favourable influence on SGR effect size, increasing dietary inclusion improved the growth performance of fed species. The results from our meta-analysis revealed that some insect meals could support the growth performance of aquaculture species at plausible inclusion levels, such as prepupal defatted *H. illucens* (threshold, 13.1%), adults of *G. bimaculatus* (50.3%), *O. fuscovittata* (19.2%) and *Z. variegatus* (15.1%) and larval full-fat *C. butyrospermi* (32.4%). The results also suggested the importance of considering insect-derived chitin levels in diet formulation for marine fish species, especially those with limited ability to digest chitin. Accordingly, a low dietary supplement could benefit the hosts regarding production performance and health status. Despite the fact that heterogeneity and publication bias were observed in the present study, the findings from our study remained robust after taking into account a wide range of fish

species included in the subgroup analysis, the multiple covariances influencing effect size and removing strong outliers.

It is necessary to move beyond growth performance evaluation by exploring additional benefits of dietary insect meal for aquaculture species, such as gut health, immunological status, diversity of gut microbiota, among others, which can arouse the attention of aquafeed manufacturers and practitioners. Simultaneously, campaigns and marketing strategies focusing on the environmental sustainability of insect meal production and insect-fed aquaculture can bring positive perception from consumers, potentially leading to a shift in their preference towards seafood fed insect meal. These targeted users are key in triggering increased demand for insect meal protein, resulting in upscaling production facilities, and eventually producing cost-competitive products, which currently is a significant obstacle impeding the widespread adoption of this innovative feed ingredient. In this context, it is worthwhile to investigate more information on the physiological responses and meat quality of aquaculture species fed insect meals on a meta-analysis basis. In addition, the environmental benefits of insect meal and their use in aquaculture should be explored, including the lower natural resource use of insect production compared with conventional plant ingredients, bioremediation application for fish fed insect meal in multi-trophic aquaculture systems, and valorization of low-value substrates via insect biomass as a protein source for aquafeed in the circular economy context. Once such scrutiny is warranted, the findings from the present study could provide informative and credible recommendations for various stakeholders in selecting insect meals for specific applications.

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#### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

#### AUTHOR CONTRIBUTIONS

**Hung Quang Tran:** Data curation; Formal analysis; Methodology; Writing – original draft; Writing – review & editing. **Tram Thi Nguyen:** Data curation; Formal analysis. **Markéta Prokešová:** Data curation; Formal analysis; Methodology. **Tatyana Gebauer:** Data curation; Formal analysis; Methodology. **Hien Van Doan:** Data curation; Formal analysis; Investigation; Methodology; Project administration; Writing – original draft. **Vlastimil Stejskal:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Writing – original draft; Writing – review & editing.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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## **CHAPTER 3**

### **ENVIRONMENTAL CONSEQUENCES OF USING INSECT MEAL AS AN INGREDIENT IN AQUAFEEDS: A SYSTEMATIC VIEW**

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REVIEW

# Environmental consequences of using insect meal as an ingredient in aquafeeds: A systematic view

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## Abstract

We retrieved data from various studies to investigate the consequences of insect meal production and insect meal-based diets with respect to their environmental impact, including global warming potential, energy use, land use, water use, acidification, eutrophication as well as to economic fish-in fish-out ratio and solid waste output production. Analysis indicated that insect meals' production exerted positive effects on land use but was associated with greater energy use and a larger carbon footprint compared to conventional protein sources. Substitution of silkworm meal (*Bombyx mori*) meals for fishmeal in aquatic animal diets significantly reduced solid phosphorus waste compared to insect-free diets. In contrast, the inclusion of black soldier fly (*Hermetia illucens*), housefly (*Musca domestica*), mealworm (*Tenebrio molitor*) and grasshopper (*Zonocerus variegatus*) has led, in comparison to insect-free diet, to greater solid nitrogen waste. Reducing the proportion of fishmeal and, to a lesser extent fish oil, by various insect meals in aquatic diet formulations significantly reduces economic fish-in fish-out, indicating less marine forage fish required per unit fish yield. The simulated data showed environmental benefit associated with land use of insect-containing aquafeeds compared to insect meal-free feeds, especially insect species of *M. domestica* and *T. molitor*. In all, this study suggested a trade-off of using insect meal as an aquafeed ingredient regarding environmental consequence. Since insect meal has excellent potential to supply protein for aquafeeds in the coming years, improvement in insect meal production systems and nutritional composition will be essential to make insect meal a sustainable aquafeed ingredient.

## KEYWORDS

alternative protein, aquafeed, economic fish-in fish-out, environmental sustainability, insect meal, waste output

## 1 | INTRODUCTION

The contribution of aquaculture to seafood production has increased continuously over the past two decades, reaching 46% in 2016–2018, up from 25.7% in 2000, with an annual growth rate of 5.3% from 2001 to 2018, surpassing that of any other major food production system.<sup>1</sup> The trend is expected to continue in response

to increasing world population and higher demand for seafood protein.<sup>2</sup> The rapid growth of the sector raises significant concerns regarding forage fish stock, natural resources, environmental issues and waste generation.<sup>3–5</sup> Aquafeed is the major factor driving these challenges.<sup>2,6–10</sup> Typically, aquafeeds rely largely on fishmeal/oil derived from marine forage fish and, to a lesser extent, from fishery/aquaculture by-products, as protein and lipid sources.<sup>1</sup> Aquaculture

has become the largest consumer of global fishmeal and fish oil production, accounting for 68% and 89% respectively.<sup>11</sup> Worldwide, wild fish production decreased by 26.5% from 2000 to 2018,<sup>12</sup> after peaking in 1994,<sup>13</sup> and will reach ecological limits in 2037.<sup>14</sup> The use of by-products from fisheries and aquaculture in aquafeeds has increased but will be insufficient for projected aquafeed demands by 2050.<sup>14</sup> As a result, the growing aquaculture industry will face a severe issue of limited fishmeal/fish oil supply, and fishmeal/fish oil replacement in aquafeeds is inevitable.

Efforts to reduce proportions of fishmeal and fish oil in aquafeeds over the past two decades<sup>15-17</sup> have led to the increasing inclusion of plant-derived ingredients.<sup>18</sup> However, inclusion of these ingredients in aquafeeds, with respect to the environment, places greater pressure on water and land resources,<sup>18-21</sup> and generates more waste<sup>3,22</sup> compared to fishmeal-based diets. Multiple alternative ingredients for aquafeeds have been investigated, among which insect meal and fisheries by-products show the greatest potential to meet protein required for aquafeeds in the coming decades.<sup>11</sup>

Insect meal draws increasing interest as an alternative to fishmeal in terrestrial and aquatic animal diets because of its favourable nutrition profile,<sup>23,24</sup> the feasibility of commercial-scale production and consumer acceptance.<sup>11,25</sup> Successful inclusion of insect meal in the preference to fishmeal in feed has been well reviewed for many aquatic species.<sup>26-30</sup> Partial replacement of fishmeal by black soldier fly (*Hermetia illucens*) in European perch (*Perca fluviatilis*) diet resulted in significant improvement of forage fish input relative to farmed fish production (fish-in fish-out ratio).<sup>31-33</sup>

Production of insect meal has been shown to consume less land and water resources than does soybean meal.<sup>34,35</sup> Meal of the common housefly (*Musca domestica*) as partial replacement for fishmeal in tilapia (*Oreochromis niloticus*) diets exhibited a positive effect on water environmental parameters compared to insect meal-free diets.<sup>36</sup> Reports of the environmental consequences of insect meal-based diets compared to those of fishmeal-based diets for aquatic animals are scarce.<sup>37,38</sup> Le Féon et al.<sup>37</sup> reported that inclusion of yellow mealworm (*Tenebrio molitor*) in rainbow trout (*Oncorhynchus mykiss*) feed reduced net primary production use (in kg C, quantifying the biotic resource that is not available for other systems anymore), and available water remaining (in m<sup>3</sup>, considering the water availability in the studied area minus the water required by humans and aquatic ecosystems), but did not decrease land use, acidification, eutrophication, global warming potential and energy use compared to an insect meal-free diet. Conversely, an *H. illucens*-based diet in arctic char (*Salvelinus alpinus*) resulted in reduced environmental impacts, including abiotic depletion, acidification, eutrophication, global warming potential, human toxicity and marine ecotoxicity compared to an insect meal-free diet.<sup>38</sup> Dietary *H. illucens* in *P. fluviatilis* was reported to require considerably less water than a fishmeal-based diet, while increasing global warming potential, land demand and energy use.<sup>33</sup> A broader understanding of the environmental impact of insect meal and insect meal-based feeds in combination with their effects on fish production (e.g. nutritional properties, growth, meat

quality) can inform the choice of insect meal as a protein source for the sustainability of future aquafeeds.

This review aimed to characterize the environmental consequences of insect meals as a nutrition source for aquatic animals. We retrieved life cycle assessment studies addressing the environmental impact of various insect meals and compare with conventional aquafeed ingredients. Peer-reviewed publications assessing insect meal as replacement for fishmeal in aquatic animal diets were synthesized to calculate economic FIFO ratio, solid waste output and environmental impact categories of insect-containing feed relative to fishmeal (insect-free) feed. We suggest areas to enhance the efficiency and sustainability of insect meals in aquafeeds.

## 2 | METHODS

### 2.1 | Database search and criteria

The relevant literature was searched using online databases Scopus, Web of Science and Google scholar in December 2020.

### 2.2 | Environmental impact of insect meal and other feed ingredients

Life cycle assessment analysis evaluates environmental impacts of products and systems throughout their life cycle.<sup>39</sup> This tool has been increasingly used in assessing the environmental sustainability of aquaculture systems,<sup>8,39-41</sup> aquafeeds,<sup>6,9,37,38,42-44</sup> aquafeed ingredients.<sup>45</sup> The targeted literature reported for life cycle assessment of insect meal production was searched, using keywords such as *insect meal*, *LCA* or *Life Cycle Assessment*, *global warming potential*, *energy use*. A total of 13 published articles and one PhD thesis (from 2014 to 2020) were compiled (Table S1). Environmental impact categories based on life cycle assessment studies included global warming potential (kg CO<sub>2</sub> equivalent (eq.)), energy use (MJ); land use (m<sup>2</sup>a (arable land)), water use (m<sup>3</sup>), acidification (g SO<sub>2</sub> eq.) and eutrophication (g PO<sub>4</sub> eq.).

### 2.3 | Total solid waste and nitrogen and phosphorus waste

The combined keywords, for example *insect meal*, *fish diets* and *digestibility* were used to search publications relevant to insect meal as replacement for fishmeal in aquatic animal diets and apparent digestibility of dry matter, crude protein and phosphorus. The literature also contained information on feed utilization to calculate the following:

$$\text{Total solid waste (TSW)} = [\text{feed (DM) consumed} \times (1 - \text{ADC DM})] + \text{waste feed (DM)}$$



Solid nitrogen waste (SNW) or solid phosphorus waste (SPW) = [N or P consumed  $\times$  (1 – ADC of N or P)] + (N or P of waste feed), where DM is dry matter, N, P are nitrogen and phosphorus, respectively, and ADC is apparent digestibility coefficient.

Data derived from 27 peer-reviewed publications (from 1990 to 2020) were compiled to investigate solid waste output trends corresponding to insect meal inclusion in aquafeeds (Table S2). Insect meal replacement levels for fishmeal ranged 3.52%–50.80% (IQR) with 45% experiments using *H. illucens*, followed by *T. molitor* (22%), silkworm (*Bombyx mori*), *M. domestica* (11%) and other insects (7%). Most insect meals were full-fat processing (68% of total observed insect meals), while defatted and partial defatted forms accounted for 21 and 11% respectively. The calculated solid waste output values were converted to response ratio  $r$ , representing the ratio of measured indices in experimental and control groups,<sup>46</sup> which was employed in the meta-analysis of insect meal inclusion on fish growth performance.<sup>30</sup> Our analyses were constrained to solid waste assessment only because of the insufficient number of studies reported dissolved waste.

## 2.4 | Economic fish-in fish-out ratio and environmental impact categories

To be included in these analyses, experimental studies needed to (i) perform on aquatic animals; (ii) include at least one insect meal level as partial or total replacement for fishmeal; (iii) provide sufficient information on feed formulation, the proportion of each constitution, feed conversion ratio. Studies that assessed the mixture of insect meals or insect meal with other components as replacement for fishmeal were not considered. Keywords such as *insect meal*, *fishmeal*, *replacement*, *fish*, *growth* were used in different combinations to get matches. Altogether 84 peer-reviewed articles (from 1990 to 2020) were compiled (Table S3).

### 2.4.1 | Economic fish-in fish-out

The ratio of forage fish input to farmed fish production (fish-in fish-out) is considered a measure of sustainability.<sup>31</sup> We adopted the term 'economic fish-in fish-out ratio' (eFIFO) from Kok et al.,<sup>19</sup> based on economic outcome and is commonly used in life cycle assessments. The calculation included data on the use of fish by-products, currently reported to comprise 25%–35% of global fishmeal and fish oil production,<sup>1</sup> a useful measure when establishing industry policy. The eFIFO differs from conventional fish-in fish-out,<sup>31,32,47</sup> which did not align with life cycle assessment, omitted fisheries by-products from the calculation,<sup>19</sup> and was recognized as an overestimation of wild fish used,<sup>47</sup> and a flawed concept.<sup>17</sup>

The eFIFO ratio was calculated by the formula:  $eFIFO = FCR \times \sum (F_{ij} \times EF_{ij})$

where FCR is feed conversion ratio,  $i$  is fishmeal or fish oil,  $j$  is source of ingredient,  $F_{ij}$  is proportion of fishmeal or fish oil in the diet (%).

$EF_{ij}$  is embodied fish in fishmeal or fish oil, which is dependent on raw fish used, that is: fish species, size and capture season. eFIFO calculation in our study was based on  $EF_{ij}$  database of Kok et al.<sup>19</sup> during 1995 and 2020.

Seven taxonomic groups contributing to eFIFO data were categorized according to Tacon and Metian<sup>32</sup> (Table S3). To consider whether the eFIFO of each taxon can meet global projections, we calculated eFIFO values predicted for 2025 and estimated the feasible fishmeal substitution level at which the predicted eFIFO is obtained. The projected eFIFO for 2025 of each taxon was calculated based on the above-mentioned formula, in which embodied fish in fishmeal and fish oil ( $EF_{ij}$ ) for 2025 was 3.54 and 4.06, respectively,<sup>19</sup> and FCR and the proportion of fishmeal, fish oil in the diet for each taxon by 2025 were retrieved from Tacon and Metian.<sup>16,32</sup>

### 2.4.2 | Environmental impact categories of insect meal-based and insect meal-free diets

Experimental diets extracted from publications (Table S3) were used to evaluate the environmental impacts of a set of six impact categories, including global warming potential (kg CO<sub>2</sub> eq.), acidification (g SO<sub>2</sub> eq.), eutrophication (g P eq.), land use (m<sup>2</sup>a eq.), energy use (MJ) and water use (m<sup>3</sup>) per kg of feed based on the environmental impact at the plant gate database of feed ingredients generated by the Global Feed Lifecycle Institute.<sup>48</sup> We limited our data to publications focusing on *H. illucens*, *T. molitor* and *M. domestica* because of unavailable life cycle assessment studies on other insect species. Since the environmental impact of ingredients in the GFLI database varies with location, average global values were used. The minerals, additives and vitamins used are classified as 'Total minerals, additives, vitamins, at plant/RER Mass S' in the GFLI database. Environmental impact values for each of the three insect meals were expressed as mean values for each insect group (Table S1). Due to unavailable data on water use for the production of one kg of *T. molitor* meal, we used the value of 4.3 m<sup>3</sup> required for one kg fresh mealworm<sup>49</sup> with an assumption that the drying process of mealworm did not require additional water.<sup>50</sup> The environmental impact categories were also converted to response ratio, as mentioned previously.

## 2.5 | Data analysis

The raw data on the environmental impact of insect meals were calculated for the interquartile range (IQR), from the first (Q1) to the third (Q3) quartile, using the 'summary' function. The relationship between fishmeal replacement with insect meal and waste output was tested using the generalized additive model ('gam') and the linear model ('lm') functions. Analyses of covariance (ANCOVA) were used to determine the variation in waste output parameters. Strong outlier values (Q1 > 3  $\times$  IQR or Q3 < 3  $\times$  IQR) were excluded from

the dataset to mitigate heterogeneity. The significant correlations of eFIFO relative to replacement levels of fishmeal with insect meal were tested with linear and gam models, and the 'ANOVA' function was used to compare regression models. All analyses were performed using the R statistical package (R Development Core Team 2009–2020, available at [www.r-project.org/](http://www.r-project.org/)).

### 3 | RESULTS AND DISCUSSION

#### 3.1 | Environmental impact of insect meal production

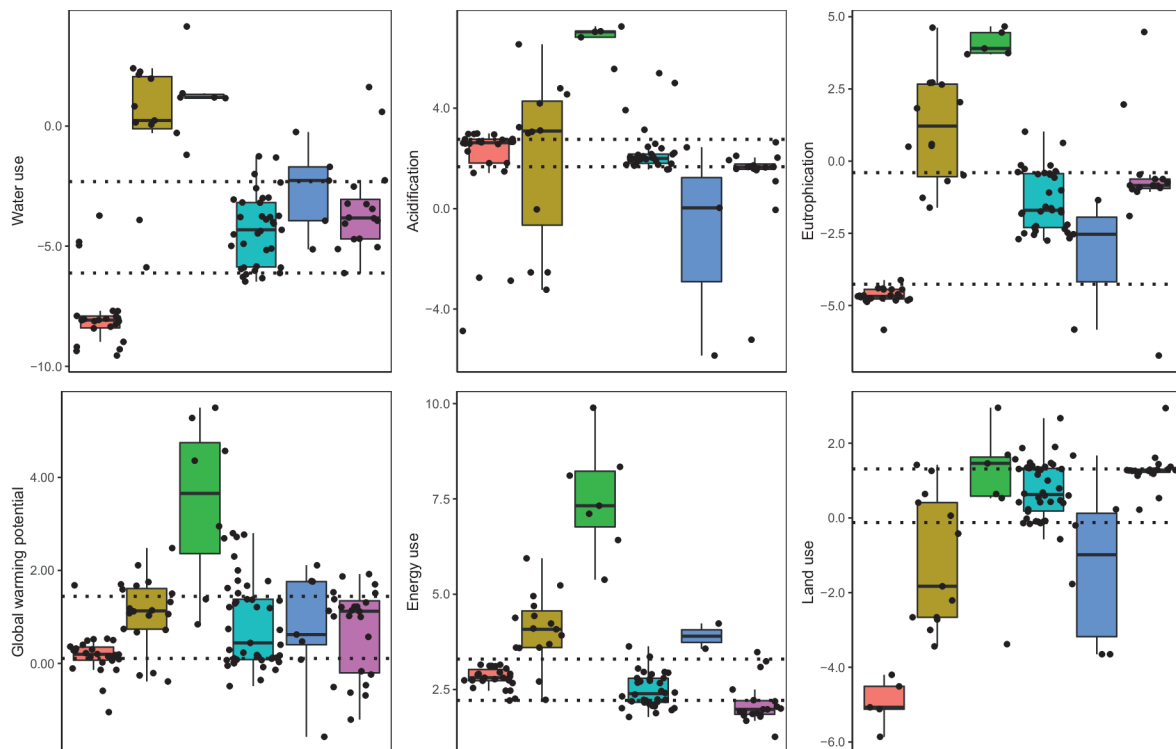
The environmental impact of three insect meals—*H. illucens*, *T. molitor* and *M. domestica*—which were extracted from the literature, and the interquartile range (IQR), mean values were summarized (Table S1). Regarding global warming potential and energy use, the production of the investigated insects was comparable, while *M. domestica* required less land use than did *T. molitor*.

Environmental impact categories of insect meals and other conventional and novel feed ingredients used in aquafeeds are depicted in Figure 1. The IQR baselines of bulked ingredients are also presented. Insect meal production, along with fishmeal and single-cell protein, appeared to be efficient in terms of land use. Land use of

these ingredients was found to be lower than the bulked Q1. Recent research confirmed better land use efficiency of insect meals (e.g. *M. domestica*,<sup>51</sup> *H. illucens*,<sup>34,35</sup> and, to a lesser extent, *T. molitor*<sup>52</sup>) compared to soybean meal. This suggests that the preferable use of alternative aquafeed ingredients (e.g. insect meals, single-cell protein) to terrestrial crops concerning natural resource conservation.

Fishmeal appeared to have the lowest impact in all categories, except energy use, whereas soybean meal and plant protein were instead closed to the bulked Q1 of energy use (Figure 1). Silva et al.<sup>45</sup> also reported that soybean meal had lower energy use (fossil fuel) than fishmeal, which confirmed the reliability of the present compilation.

Six ingredients exhibited a good fit within the bulked IQR concerning greenhouse emissions, the exception was microalgae. Regarding energy use, insect meals, microalgae and single-cell protein ingredients showed an immense impact, falling beyond the bulked Q3. Several studies confirmed the negative impact of global warming potential and energy use associated with insect meal production versus that of fishmeal and soybean meals.<sup>34,35,51,52</sup> A similar pattern was also observed for insect meals in terms of water use and eutrophication. Water use of *H. illucens* meal was comparable with that of fishmeal and less than that of plant ingredients and microalgae, as reviewed by Smetana et al.<sup>35</sup> However, compiled data showed a contradictory pattern (Figure 1), which could be attributed



**FIGURE 1** Log values of environmental impact categories of insect meals and other feed ingredients per kg. Black dots are observed data points. The lower and upper dashed lines represented the first quartile and third quartile of bulked ingredient data. Data sources: insect meals (Table S1), fishmeal<sup>6,45,48,52,59,127–129</sup> (number of observation,  $n = 27$ ), soybean meal<sup>34,45,48,50,52,126,129–135</sup> ( $n = 27$ ) and plant concentrate,<sup>6,48,50,59,136–138</sup> microalgae<sup>50,134,139–141</sup> ( $n = 9$ ), single-cell protein<sup>50,59,126,133,142–144</sup> ( $n = 9$ )

to the high water use required by housefly production (Table S1). Roffeis et al.<sup>53</sup> reported the need for extra water for mixing substrates and killing flies (*M. domestica*). The high impact of housefly production could be explained by the geographical context of their study<sup>54</sup> because a high percentage of water was consumed to maintain facility hygiene.<sup>55</sup> Feed for insects was the most significant driver for water use,<sup>56</sup> especially for those derived from crop products<sup>49</sup> commonly used for insect rearing (Table S1). This could further explain the high impact of insect meal production. Given that most studies compiled in our review were performed under small-scale facilities, system improvement could improve water use and other environmental categories of insect meal products.<sup>55-57</sup> Insect meal production associated with acidification was highly heterogeneous and partly deemed in the bulked IQR. We also found excellent environmental performance associated with acidification in single-cell protein production (Figure 1).

Our review highlighted the high environmental impact of novel aquafeed protein sources, including insect meals, microalgae and single-cell protein, compared to conventional aquafeed ingredients, especially for global warming potential, energy and water use. This could be ascribed to the insufficiency of production technology and production scalability.<sup>35,58,59</sup> Insect production upscaling could reduce environmental impact and consequently compete with conventional ingredients.<sup>35,57</sup> Among those novel alternative aquafeed ingredients, insect meal has been suggested to be the best potential candidates for improving processing techniques, costs and scalability.<sup>11</sup> Moreover, feed for insect rearing was the largest contributor to environmental impact categories (*T. molitor*,<sup>37,52</sup> *M. domestica*<sup>51,53</sup> and *H. illucens*<sup>34,35,60,61</sup>). Therefore, sourcing suitable substrates to feed insects and expanding the efficiency of facilities will be critical to improving the environmental benefit of insect meals.

### 3.2 | Total solid waste, nitrogen and phosphorus waste

The total solid waste and phosphorus and nitrogen waste from aquaculture are considered primary eutrophication agents of aquatic ecosystems. Minimizing these outputs through diet formulation has been proposed as a long-term strategy to ensure environmentally friendly and sustainable aquaculture.<sup>3,5</sup>

There was no significant relationship between the fishmeal replacement level and total solid waste for all insect meals ( $p = 0.597$ ) as well as for individual insect meals ( $p > 0.05$ ). Dietary insect meals comprising *B. mori*, *H. illucens*, *M. domestica* and *T. molitor* significantly increased solid nitrogen waste ( $p < 0.05$ ). A significant negative relationship was found between dietary *B. mori* and solid phosphorus waste ( $p < 0.05$ ; Figure 2, Table S4). ANCOVA analysis showed a significant association between nutrient digestibility (dry matter,  $F$ -value = 24.75,  $p < 0.0001$ ; protein,  $F$ -value = 4.80,  $p = 0.032$ ; phosphorus,  $F$ -value = 7.19,  $p = 0.01$ ) and chitin ( $F$ -value = 5.98,  $p = 0.017$ ) with total solid waste. Chitin ( $F$ -value = 6.78,  $p = 0.02$ ), protein digestibility ( $F$ -value = 13.57,  $p = 0.002$ ), fish habitat ( $F$ -value = 13.57,

$p = 0.002$ ) and insect species ( $F$ -value = 5.46,  $p = 0.033$ ) imparted significant variations in nitrogen waste. Solid phosphorus waste was found to be significantly influenced by the phosphorus digestibility ( $F$ -value = 11.56,  $p = 0.011$ ), dietary phosphorus ( $F$ -value = 8.49,  $p = 0.002$ ) and insect species ( $F$ -value = 4.71,  $p = 0.045$ ).

A recent study by Weththasinghe et al.<sup>62</sup> on *O. mykiss* fed full-fat *H. illucens* meal confirmed our finding that there was a positive correlation between dietary insect meal and the faecal excretion of nitrogen. Nitrogen waste load is directly linked to the apparent protein digestibility of the diet. Digestibility of dietary protein was reported to be affected by the presence of chitin in insect meal.<sup>63-67</sup> Chitin is not digestible by monogastric animals, and it exhibits a high protein-binding capacity, which could impair protein digestion.<sup>64</sup> Chitin may interfere with leucine aminopeptidase activity—a brush border enzyme that breaks down peptides into amino acids in the proximal and middle intestine, where the majority of proteins are digested and absorbed.<sup>68</sup> Gasco et al.<sup>65</sup> reported that the assumed 1.7% chitin content of 36% fishmeal replacement by *T. molitor* meal improved protein digestibility compared to an insect-free diet in European sea bass. This could be associated with the chitinase that is present in some marine species that degrade chitin, consequently reducing the digestibility constraints of including insect meal.<sup>69-72</sup> A ratio of non-essential-to-essential amino acids of  $\leq 1.0$  in most insect meals<sup>23</sup> was known to negatively affect protein digestibility.

Weththasinghe et al.<sup>62</sup> evidenced reduction of faecal phosphorus output with increasing insect meal inclusion in diets of *O. mykiss*, which was confirmed in our compilation. This could result in a significantly lower phosphate concentration ( $P - PO_4^{3-}$ ) in the rearing water of fish fed insect-based diets compared to insect-free diets, which was reported earlier for *O. niloticus*.<sup>36</sup> The ANCOVA analysis in the present study showed that the solid phosphorus load is mainly attributed to feed digestibility and phosphorus content, which is in agreement with previous work.<sup>3</sup> Some studies have confirmed a positive relationship between dietary insect meals and phosphorus digestibility in aquatic animals. For example Rahimnejad et al.<sup>73</sup> reported that the phosphorus digestibility of *L. vannamei* was significantly enhanced by increasing the inclusion level of *B. mori*. A similar finding was reported for bullfrogs (*Rana catesbeiana*) fed dietary *M. domestica* meal.<sup>66</sup> It is likely that a higher calcium-to-phosphorus ratio (Ca:P) and a higher phosphorus content in fishmeal compared to insect meal could impact phosphorus digestibility.<sup>66,73</sup> For instance *M. domestica* meal has a lower Ca:P (0.29) and phosphorus content (1.60%) than fishmeal (1.56% and 2.79% respectively).<sup>24</sup> In contrast, the major phosphorus forms, hydroxyapatite and tricalcium phosphate, in fishmeal are not well utilized by aquatic animals.<sup>74</sup> Our findings suggest that the use of silkworm (*B. mori*) in aquatic animal feeds could reduce the phosphorus load from aquaculture.

Accessed studies employed different techniques for producing experimental diets, for example meat grinder,<sup>75</sup> extruder,<sup>66</sup> resulting in variation in physicochemical composition, digestibility. The extrusion technique can significantly improve aquafeed nutrient digestibility and feed stability<sup>17,76</sup> and is an environmentally

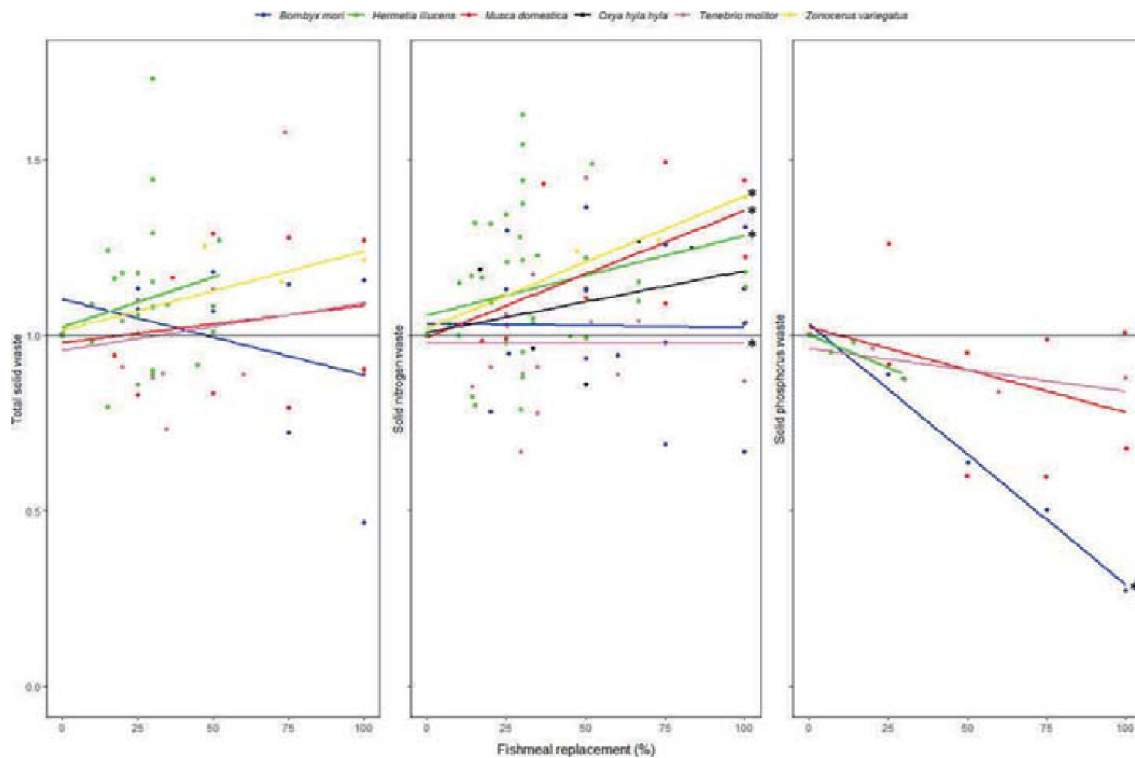


FIGURE 2 Effect of insect meals inclusion in aquatic animal diets on total solid waste, nitrogen waste and phosphorus waste compared to insect meal-free diets. Asterisks indicate a significant linear relationship between insect meal and waste output indices ( $p < 0.05$ )

friendly process.<sup>77</sup> The broader application in aquafeed production may reduce waste output and enhance the performance of the cultured animal. Further study is needed to confirm the benefits of dietary insect meals in aquafeeds on water quality. Manipulation of insect meal composition, for example chitin and nutrient imbalance, and improvement of feed manufacturing technology, feeding strategy could further benefit waste output from insect-based aquafeeds.

### 3.3 | Economic fish-in fish-out

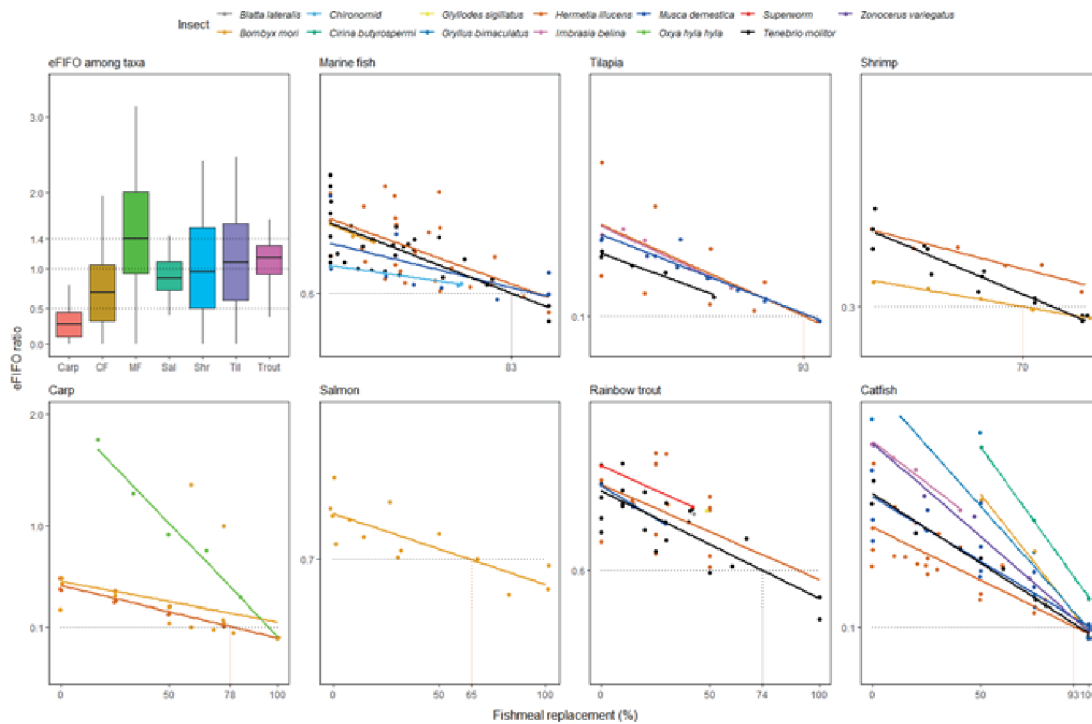
Globally, approximately 70% of forage fish from capture fisheries is used in the production of animal feed, a large proportion of which goes into aquafeeds in the form of rendered fish oil and fishmeal.<sup>1,14</sup> Under the current aquaculture scenario, this finite resource will be close to its ecological limits by 2037,<sup>14</sup> which, in combination with the continuous rise in the market price of the rendered products, poses a challenge to the ever-growing aquaculture industry.<sup>78</sup> Reducing forage fish-derived fishmeal and fish oil in aquafeeds is an essential strategy for the long-term sustainability of fishery resources and aquaculture operations.<sup>79</sup> The eFIFO has been considered a novel and suitable proxy for quantifying fish demand for aquaculture production based on the economic allocation principle and suggests the

importance of finite wild marine resources to meet marine aquafeed ingredients.<sup>19</sup>

Our compiled data comprised 13 insect species and found that replacing fishmeal with these insect meals in aquatic animal feeds steadily decreases eFIFO in all taxa (Figure 3). Comparing regression models using ANOVA indicated that linear regression models were the best description of the relationship between insect meal replacement and the eFIFO. The overall eFIFO of carp taxon felt lower than the Q1 bulked eFIFO of all taxa and remained low compared to that of other taxa (Figure 3).

Substantial or total replacement of fishmeal with insect meals could reduce eFIFO to  $<1.0$  in all taxa, suggesting the potential for insect meals to turn the aquaculture industry from a net consumer to a net producer of fish. This is consistent with the current trend in forage fish use in aquaculture, which produces three or four times the number of fish it consumes.<sup>19</sup>

Reduction in the proportion of fishmeal and fish oil in aquafeeds by increasing insect meal to meet predicted eFIFO by 2025 seems feasible in all taxa. This requires a substitution of insect meals for fishmeal, from 65% (for salmon) to 93% (for shrimp and tilapia) (Figure 3). Shrimp, marine fish and salmon are reported to be the highest consumers of fishmeal and fish oil.<sup>32</sup> The predicted eFIFO in 2025 is 0.3, 0.6 and 0.7 for shrimp, marine fish and salmon, respectively, necessitating 93%, 83% and 65% fishmeal replacement by insect meals (Figure 3). Panini et al.<sup>80</sup> and Motte et al.<sup>81</sup>



**FIGURE 3** Relationship between eFIFO and fishmeal substitution by various insect meals in cultured fish species. The solid lines and dots represent the mean values and observed data points respectively. Horizontal dotted lines represent predicted eFIFO by 2025, and vertical dotted lines indicate the threshold of fishmeal substitution by insect meal at which predicted eFIFO would be reached. In the ‘eFIFO among taxa’ boxplot, the horizontal dashed line represents the mean of bulked eFIFO separating the interquartile range (the first and third quartile) of the bulked eFIFO. CF, catfish; MF, marine fish, Sal, salmon; Shr, shrimp; Til, tilapia

reported 100% fishmeal replacement by *T. molitor* without impairing the growth and feed utilization of shrimp. Similar patterns were confirmed in the marine fish, *P. major*<sup>82</sup> and *S. salar*.<sup>83,84</sup> The eFIFO in 2025 of carp, catfish and tilapia groups was predicted to be as low as 0.1 (Figure 3), meaning that diets for those species would include low levels of fishmeal/fish oil or no fish-derived components. Growth rate and feed conversion efficiency were not negatively affected with fishmeal/fish oil-free diets in common carp (*C. carpio*),<sup>85,86</sup> tilapia (*O. niloticus*)<sup>87,88</sup> and catfish (*C. gariepinus*).<sup>89</sup> Tacon and Metian<sup>32</sup> stated that fish oil has not been included in feeds for carp since 1995 or for tilapia since 2007, which will continue to 2030 as projected by Cottrell et al..<sup>90</sup> Herbivorous and/or omnivorous fish, such as carp and tilapia, are less sensitive to dietary fishmeal/fish oil reduction than carnivorous species.<sup>91</sup> Carp was the largest aquafeed consumer in 2017, which is a trend that is likely to continue in the coming years,<sup>13</sup> therefore, the simulation model<sup>14</sup> suggests that this sector has the highest potential to reduce forage fish use by 2050.

Globally, reducing forage fish demand for aquafeeds would be more effective by limiting fish oil than fishmeal<sup>90</sup> due to the low conversion rate from whole fish to fish oil (5%) than to fishmeal (22.5%).<sup>32</sup> The majority of global FO production goes into feed for salmonids, marine fish and shrimp.<sup>16,92</sup> In addition to providing protein, insect meal represents a potential source of fat for aquaculture feeds. Lipid content varies among *T. molitor* (16.6%–40.3%),

*H. illucens* (11.3%–40.7%), *M. illucens* (7.1%–25.3%),<sup>23</sup> and is highly dependent on rearing substrate and processing.<sup>30</sup> Investigation of insect oil as a lipid source for aquatic animals is in its infancy and currently limited to carp, trout and salmon.<sup>68,93–96</sup> Insect fatty acid profiles comprise large proportions of saturated and monounsaturated FAs, oleic acid and negligible levels of long-chain polyunsaturated FAs.<sup>23,24,97</sup> The lack of long-chain polyunsaturated FAs could limit use of insect oil as lipid source or replacement for fish oil in nutrition of aquatic animals, especially high-value species.

Insect meals and oils could play an essential role in conserving finite forage fish resources while meeting the increasing demand for aquafeed protein/lipid sources. This requires nutritional fortification.<sup>98</sup> Blending insect meal or oil with other materials<sup>96,99–101</sup> or compensating for deficient components in insect-based diets, for example amino acids,<sup>84,102,103</sup> essential FAs from microalgae and supplementing digestive enzymes<sup>65</sup> could allow the inclusion of higher proportions of insect ingredients, thus reducing dependence on marine fish resources.

### 3.4 | Environmental impact of insect meal-based aquafeeds

Feed production is a key driver of the environmental impact of aquaculture,<sup>10</sup> and modification in feed ingredients is considered critical

to reducing that burden.<sup>43</sup> Since insect meal is recognized as a potential protein source in aquafeed<sup>11,104</sup> and aquafeed protein sources differ in environmental impact,<sup>42</sup> understanding the environmental consequences of insect meal- and fishmeal-based feeds is essential to increasing sustainability of aquafeeds.

The relationship between fishmeal replacement level with insect meal and environmental impact in aquafeeds is depicted in Figure 4 and Table S6. The generalized additive model demonstrated a significant increase in fishmeal replacement level with the increased environmental impact of global warming potential, energy use, water use, acidification and eutrophication. A similar pattern was observed for land use with linear models (Table S6), except for *M. domestica*-contained diets. The results suggested that the dietary housefly (*M. domestica*) linearly reduced the environmental impact associated with land use. Our synthesis also evidenced an eligible impact concerning land use of a *T. molitor*-based diet compared to a fishmeal diet, as illustrated by a relatively low model slope (0.002; Table S6). Further scrutiny of these insect meals in aquafeeds with respect to mitigating environmental consequences hints towards promising outcomes.

This finding primarily reflects the environmental properties of insect meal versus fishmeal, as presented in Figure 1, because most impacts were influenced by modifying the proportion of insect meal over fishmeal. In addition, the experimental diets in the compiled studies (Table S3) were formulated on an isonutrient basis, in which changes in the proportion of other ingredients were also made. Therefore, to some extent, the differences in environmental impacts between insect meal-containing diets and fishmeal diets could also be influenced by other components, such as plant ingredients, which were considered to have a similar environmental performance to fishmeal/fish oil.<sup>45</sup>

Reported environmental impacts associated with insect meal in aquafeeds are scarce, but include feed for perch (*P. fluviatilis*),<sup>33</sup> rainbow trout (*O. mykiss*)<sup>37</sup> and arctic char (*S. alpinus*).<sup>38</sup> Stejskal et al.<sup>33</sup> confirmed the reduction of water use associated with *H. illucens*- compared to fishmeal-based feed, while global warming potential, land use, energy use increased. However, Smáráson et al.<sup>38</sup> compared *H. illucens*- and fishmeal-based feed associated with seven impact categories and reported benefits of *H. illucens* inclusion on abiotic depletion, acidification, eutrophication, global warming potential, human toxicity potential and marine aquatic ecotoxicity potential, but with a negative impact on energy use. Le Féon et al.<sup>37</sup> confirmed more enormous impacts of acidification, eutrophication, global warming potential, land use and energy use associated with *T. molitor*-compared to fishmeal-based feed. The discrepancy in those results could be attributed to data source and diet formula modification with a various share of insect meal, fishmeal and other ingredients. We synthesized environmental impact data for insect meals from up-to-date life cycle assessment studies (Table S1), which varied with respect to numerous factors, for example growth substrate, location and size of facility. Our study suggested environmental

benefit associated with land use of *M. domestica*-, and to a lesser extent *T. molitor*-contained aquafeeds. Notably, aquafeeds are formulated from multiple components, and thus with regard to reducing environmental impacts, insect meal protein is probably not a holistic option for this purpose. Diet modification by combining insect meal and other environmentally efficient ingredients and further improving environmental performance associated with insect meal production could lower environmental impacts.

### 3.5 | Increasing environmental benefits of insect meals and insect meal-based diets

#### 3.5.1 | Improving nutritional value

As mentioned, insect meal possesses properties associated with solid waste output and eFIFO that hinder its inclusion in aquafeeds. Limitations related to chitin content and inadequate nutritional profiles could be addressed by manipulating substrates and processing.<sup>11,26,30</sup>

Chitin, one of non-protein nitrogen compound found in the cuticle of most insects, is reported to exert a negative effect on diet digestibility and growth performance of fed organisms,<sup>105</sup> while a low proportion of chitin can induce immunological effects and microbiota modulation.<sup>75,106-108</sup> Manipulation of chitin content in insect meal products to a threshold level that ensures a positive response in fed species requires further research. Chitin can be easily removed by alkaline extraction,<sup>109</sup> but this may result in high cost as well as issues of chemical residue and pollutants.<sup>110</sup> Supplementation with chitinase/chitinolytic-producing bacteria could be a feasible approach to ensure cost-effective feed, reduce the environmental impact of chitin waste and induce an immune response to fed fish.<sup>110</sup>

Reduction of eFIFO in aquafeeds by inclusion of insect meal requires substantial fishmeal replacement without compromising fish growth, which is chiefly influenced by nutritional-balance of experimental diets.<sup>30</sup> Deficiency of amino acids and fatty acids of insect meal relative to fishmeal has been reported,<sup>23,24</sup> and manipulation of those components via rearing substrates remains a challenge.<sup>111,112</sup> Defatting could be an efficient means of improving amino acid content,<sup>113</sup> but this involves intensive energy use, which in turn increases environmental impact and costs.<sup>26,34,52,114</sup> It is more efficient to combine IM with complementary raw materials<sup>11,30,115</sup> or supplement IM-based diets with amino acids.<sup>83</sup>

Manufacturing techniques such as extrusion could be an effective means of enhancing nutrition utilization of aquafeeds by the fed organism.<sup>17</sup> The suitability of extruded insect-contained aquafeeds has recently been reported.<sup>62,77,95</sup> Feeding practices should also address minimizing feed waste and improving feed conversion ratio,<sup>3,5</sup> thereby improve environmental impacts.

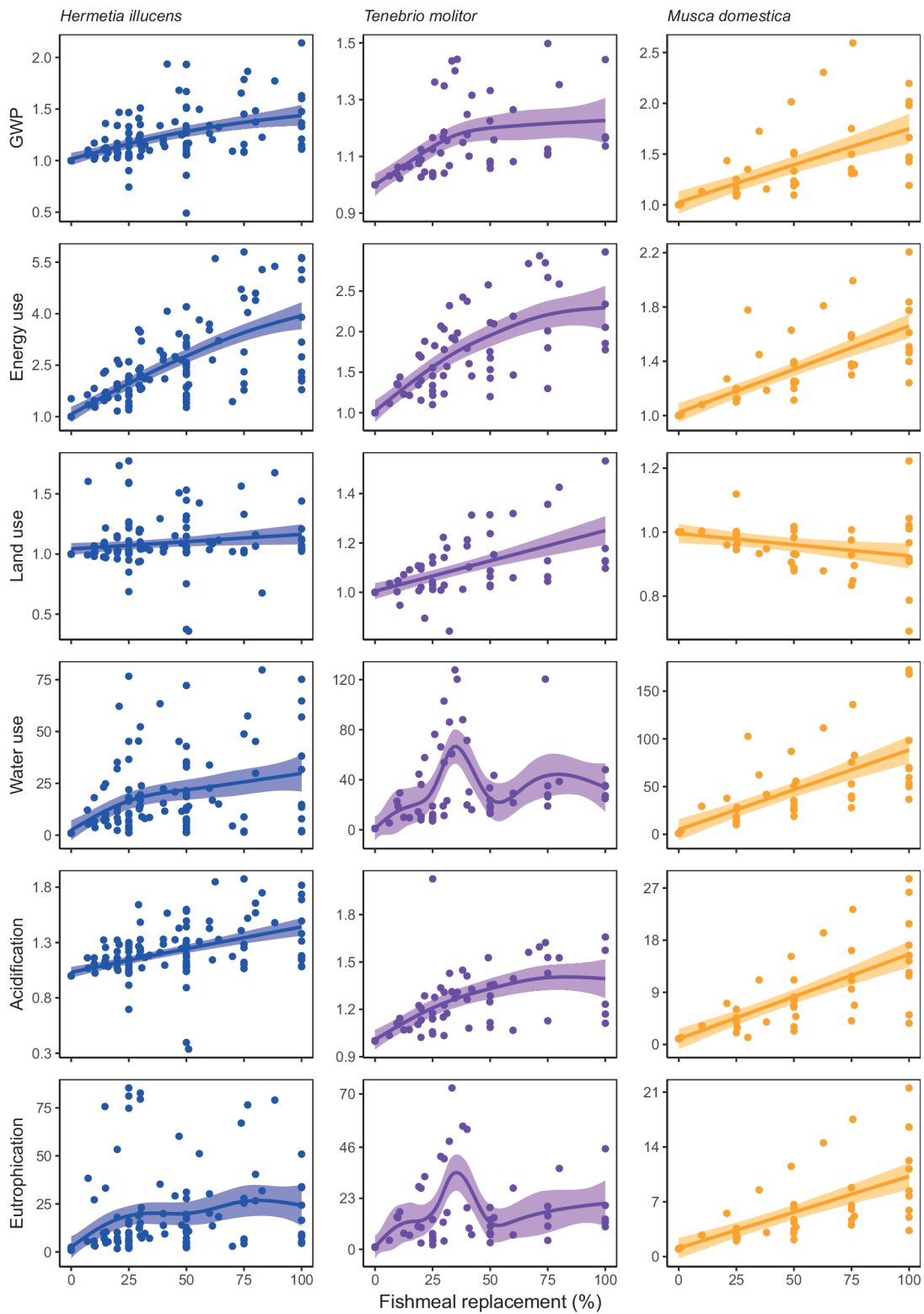


FIGURE 4 The relationship between response ratio of fishmeal replacement level and environmental impacts in aquafeeds. Coloured solid lines and shaded areas represent fitted models with the mean and 95% confidence interval respectively. Dots are observed data points. GWP, global warming potential

### 3.6 | Reducing the environmental impact of insect meals

Insect rearing facilities, including production of insect feed, rearing area and processing have been reviewed,<sup>58,116</sup> in this section, we focussed on reducing inefficiency of insect production with respect to environmental issues.

#### 3.6.1 | Insect feeds

Feed production for insect is the most critical aspect of insect meal production with respect to the environment.<sup>37,56</sup> Investigating environmental aspects of substrates for insect rearing could enhance the benefits of insect meal use in aquafeeds. Some insect species have effectively transformed organic waste and manure into biomass,<sup>117</sup> which could partially address global waste concerns. From an environmental perspective, culture of *H. illucens* on cattle manure and municipal waste is superior to using traditional media such as chicken manure and beet pulp, but comparative to distiller's grains with solubles (DDGS).<sup>118</sup> Bava et al.<sup>61</sup> reported lower environmental impact of farming *H. illucens* on maize distiller compared to hen diet substrates. It was recommended that brewer spent substrates generated from sorghum and barley with supplementation by brewer's yeast or brewer's yeast plus molasses are more suitable for *H. illucens* rearing with respect to improving protein content and minerals.<sup>119</sup> Housefly (*M. domestica*) was found to thrive on manure,<sup>56</sup> with chicken manure more environmentally efficient than sheep manure.<sup>53</sup> Spent grain substrates (fermented or heat-dried) offer superior greenhouse gas emission levels compared to crop-derived feed for *H. illucens*.<sup>120</sup> At insect rearing on an industrial scale, Scala et al.<sup>121</sup> reported superior output of *H. illucens* reared on spent grains than with fruit substrates, suggesting potential of spent grain for more sustainable insect productive systems.

Feeding insects on waste food should be done with caution, as it may compete with the bioenergy industry in sourcing material, increasing the environmental impact.<sup>51</sup> The most efficient solution is to source surplus organic substrate and to use waste material from insect rearing as fertilizer or in the bioenergy sector.<sup>122</sup> Organic waste and manure substrates are not favourable for *T. molitor*<sup>37,123</sup> in term of growth production (Table S1), which could be attributed to low nutrient values and high starch content of these substrates.<sup>123,124</sup> However, *T. molitor* thrives on mixtures such as dried brewing by-products, derivatives of potato processing, DDGS, by-products of the biofuel industry, livestock feeds and plant-derived products.<sup>37,56</sup> Distiller's dried grains with solubles are not shown to be an ideal substrate for *T. molitor* culture and resulted in a higher environmental burden compared to others tested, while a mixture of wheat bran and animal feeds is preferable.<sup>37</sup>

In Europe, substrates used in insect culture are regulated by European Commission regulation 767/2009 and 999/2001, with non-authorized substrates including separated digestive tract content, manure, catering waste and processed animal protein, except

fishmeal. Regulations 1069/2009, 142/2011 and 767/2009 list authorized plant-based substrates. Effort should focus on identifying the most suitable authorized materials to limit the environmental footprint of insect products. Distiller's dried grain with solubles appears to be the most promising candidate for both *H. illucens* and *M. domestica* production, while livestock feed is optimal for *T. molitor*. To confirm this, more research is needed to explore potential of those substrates on production output, nutritional composition of the resulting insect meal, economic feasibility and environmental impact.

It should be taken into consideration that these substrates are currently used as feed in the livestock sector, and their demand for insect production could lead to elevate the global price<sup>57</sup> and/or increase demand for alternative sources to fill the protein gap for other animals.<sup>35</sup> Therefore, criteria for insect diets should comply with current regulations and prioritize local sources to reduce cost of transport,<sup>53</sup> utilizing surplus production/side streams or ingredients that are not competitive with other farming sector.<sup>116,121,125</sup>

#### 3.6.2 | Insect rearing facilities

It is necessary to design space-efficient insect rearing production facilities to optimize land use, which is species-specific. For instance the use of three-dimensional crawling space design for vertical crawling, jumping, flying insect is preferable to two-dimensional flat spaces which could yield higher productivity per unit area.<sup>116</sup> Expanding production vertically or increasing use of multilevel shelves or stackable boxes in insect construction facilities can further optimize land use.<sup>37,51,116</sup>

The largest portion of energy use for insect meal production is associated with providing heat for insect rearing<sup>34,51,52</sup> and drying.<sup>126</sup> It may be energy efficient to install insect production facilities in an equatorial climate.<sup>34</sup> It has been suggested that renewable energy sources could be a promising solution, potentially reducing the burden by approximately 25%.<sup>35</sup> The same finding was reported by Samuel-Fitwi et al.<sup>42</sup> who stated that aquafeed production showed lower environmental impact when using wind power compared to fossil fuel sources. Use of by-products of insect meal production for anaerobic digestion and fertilizer could contribute to environmental conservation.<sup>51</sup> The use of photovoltaic energy as an energy source for insect meal production is also a potential option. This was applied in Italy, resulting in a decrease of 14.2% in global warming potential, 19.2% in energy use and 1.8% in land use.<sup>34</sup> Adaptation to utilize residual heat from nearby facilities could considerably reduce impact, saving 1247 kg CO<sub>2</sub> eq. global warming potential, 23,949 MJ energy use and 1 m<sup>2</sup>a land use per ton of insect meal.<sup>51</sup>

Insect farming is undergoing increasing production,<sup>11,104</sup> which could offer considerable environmental benefits in general<sup>125</sup> and energy use in particular<sup>51</sup> associated with insect meal products and insect meal-based aquafeeds. Further life cycle assessment studies should focus on broader perspectives of production facilities and location in combination with optimal insect rearing substrates.



## 4 | CONCLUSIONS

Our results provide insights into the environmental impact of insect meal production and its use in aquafeeds. Insect meals of *H. illucen*, *T. molitor* and *M. domestica* are the least land use among conventional and novel aquafeed ingredients, while together with alternative protein sources, exert an enormous impact on global warming potential, energy use, water use, acidification and eutrophication. Dietary silkworm (*B. mori*) significantly reduced faecal phosphorus waste, whereas inclusion of *H. illucens*, *M. domestica*, *T. molitor* and *Z. variegatus* elevated faecal nitrogen waste in comparison with insect-free diets. Substitution of fishmeal by insect meal also significantly reduced economic fish-in fish-out, the marine fish and whole fish demand for one unit of aquaculture fish produced. In addition, from a life cycle assessment perspective, insect meal shows promising in terms of mitigating the environmental impact of aquafeeds associated with land use, especially *T. molitor* and *M. domestica* insect species. Therefore, our study suggested the potential of insect meal for an aquaculture industry to thrive on the limited natural resources—agriculture land, and to grow with less phosphorus load. Intensifying industrial insect farming with standard and energy-efficient facilities and developing suitable insect-specific substrates to address nutritional composition and environmental aspects will be essential for insect meal as a future protein source supply for aquafeeds. We also suggest that insect meal is not the sole solution for lowering the environmental impacts of future aquafeeds. The combination of multiple alternative protein and lipid sources in aquafeeds will be strategic approach for environmental sustainability of aquafeeds, thus aquaculture sector.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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## CHAPTER 4

### **PRODUCTION PERFORMANCE, NUTRIENT DIGESTIBILITY, SERUM BIOCHEMISTRY, FILLET COMPOSITION, INTESTINAL MICROBIOTA AND ENVIRONMENTAL IMPACTS OF EUROPEAN PERCH (*Perca fluviatilis*) FED DEFATTED MEALWORM (*Tenebrio molitor*)**

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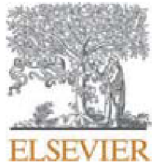
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## Production performance, nutrient digestibility, serum biochemistry, fillet composition, intestinal microbiota and environmental impacts of European perch (*Perca fluviatilis*) fed defatted mealworm (*Tenebrio molitor*)

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Solid waste

### ABSTRACT

Yellow mealworm (*Tenebrio molitor*) larvae meal (TM), one of the seven insect species approved for use in aquafeed in Europe, is a frequently investigated candidate for fish diets. This study aimed to investigate the effects of dietary defatted TM on production performance, serum biochemistry, nutrient digestibility, fillet traits, intestinal microbiota, and environmental impacts of perch (*Perca fluviatilis*). Four experimental diets, characterized by defatted TM inclusion levels of 0, 6.8, 13.5 and 20.3%, respectively, or 0, 25, 50, and 75% replacement for fishmeal on a w/w basis (TM0, TM25, TM50, and TM75, respectively), were fed to juvenile perch (body-weight  $20.81 \pm 3.36$  g, total length  $117.7 \pm 7.2$  mm) (quadruplicated per diet) for 105 days. Inclusion levels of 6.8% or 25% fishmeal replacement by defatted TM did not show a significant effect on specific growth rate and feed conversion ratio, while further levels of 13.5 and 20.3%, or 50 and 75% fishmeal replaced by defatted TM, respectively, displayed a significant delay in the former ( $P = 0.01$ ), but increase in the latter indices ( $P = 0.04$ ) compared to TM0 diet. The aspartate aminotransferase activities in perch's serum increased with increasing dietary TM ( $P = 0.02$ ). Protein digestibility of perch exhibited a negative correlation with dietary TM ( $P = 0.03$ ). Dietary defatted TM did not, alter fillet composition of perch ( $P > 0.05$ ) and modify diversity of fish gut microbiota (Chao1 index,  $P = 0.742$ ; Shannon index,  $P = 0.557$ ; and observed species,  $P = 0.522$ ), but significantly reduced abundance of *Lactobacillus* ( $P = 0.04$ ) and *Streptococcus* ( $P = 0.01$ ). Diets containing more than 6.8% TM generated a comparable amount of total solid waste and solid phosphorus waste as the TM0, whereas solid nitrogen waste significantly increased with elevated TM levels ( $P < 0.001$ ). The estimated environmental impacts of perch fed TM25 were comparable to TM0 for global warming potential, acidification, and land use ( $P > 0.05$ ), whereas TM50 and TM75 exerted heavier burdens on energy use, eutrophication, and water use than TM0 ( $P < 0.001$ ). Fishmeal replacement by TM significantly reduced the economic fish-in fish-out ratio ( $P < 0.001$ ). Results of this multidisciplinary study revealed important considerations for formulating diets with insect meals and their potential effects on fish performance and environmental impacts.

### 1. Introduction

The increasing use of alternative aquafeed ingredients for fishmeal (FM) and fish oil is necessary to ensure the sustainability of the aquaculture sector (Cottrell et al., 2020). Terrestrial plants have become the most common alternative for aquafeeds (Tacon et al., 2011; Tacon and Metian, 2015). However, the limitations associated with specific unfavorable nutritional components (Gatlin et al., 2007), and the

environmental consequences of product intensification, especially the increasing demand for arable land - the immense pressures on the planet (Foley et al., 2005; Foley et al., 2011), could hamper the expansion of terrestrial plant ingredients used in aquafeeds. Fishery and aquaculture by-products, together with insect meal, represent the most excellent protein sources to satisfy the aquafeed demand in the coming years (Hua et al., 2019; Gasco et al., 2020a). The share of fish by-products in the global production of FM has increased over the last few years and is

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expected to reach 34% by 2030 (FAO, 2018). The supply of insect protein to humans and feed use has been forecasted to reach approximately 1.2 million tonnes by 2025 (Gasco et al., 2020a). Insect meals have become sustainable protein sources for livestock and aquaculture production due to their favorable nutritional profiles (Nogales-Mérida et al., 2019), health benefits for the fed organisms (Gasco et al., 2020c), lower environmental impacts associated with land and water resource demand than that of plant proteins (Smetana et al., 2019; Gasco et al., 2020b), and positive effects on the aquatic environment than an FM-based diet (Wang et al., 2017).

Yellow mealworm (*T. molitor*) larvae meal, one of the seven insect species approved for use in aquafeed in Europe (European Commission, Regulation 2017/893), is a frequent candidate for use in fish diets (Gasco et al., 2016; Basto et al., 2020; Hua, 2021). The success of TM inclusion as a replacement for FM in aquatic animal diets without any detrimental impact on growth performance and feed efficiency, has been documented for the top FM consumers among aquaculture species. For example, 20.5–30.5% inclusion, or 100% FM replacement in shrimp, *Litopenaeus vannamei* (Panini et al., 2017; Motte et al., 2019), 20–25% inclusion, or 67–100% FM replacement in rainbow trout, *Oncorhynchus mykiss* (Belforti et al., 2015; Rema et al., 2019; Chemello et al., 2020). Dietary TM has been reported to affect the nutrient digestibility and meat quality of fed organisms to a great extent (Gasco et al., 2019); however, it did not modulate the bacterial community in the intestine of rainbow trout, *Oncorhynchus mykiss* (Terova et al., 2021), or gilthead seabream, *Sparus aurata* and European sea bass, *Dicentrarchus labrax* (Antonopoulou et al., 2019).

Although aquafeed is a key contributor to environmental burdens, such as carbon footprint and waste output (e.g., total solid waste, solid nitrogen waste) of aquaculture system (Amirkolaie, 2011; Bohnes et al., 2019), the investigation on the environmental impacts of TM inclusion in aquafeeds remains fragmentary (Le Féon et al., 2019). Moreover, aquaculture has become a dominant consumer of FM and fish oil derived from forage fish since the 2000s (Shepherd and Jackson, 2013). Investigation into forage fish use to produce one unit of farmed fish, the fish-in fish-out ratio (FIFO) (Tacon and Marc, 2008), could be considered an essential measure of sustainability (Naylor et al., 2009), especially for the alternative ingredients that are increasingly being used in aquafeeds, such as insect meal (Stejskal et al., 2020). Therefore, it is necessary to consider a broad spectrum of indicators whenever new aquafeed ingredients are introduced.

European perch (*Perca fluviatilis*) is a promising candidate for intensive aquaculture (Stejskal et al., 2011a). Globally, the aquaculture production of perch is on the rise, reaching 700 t in 2018 (FAO, 2020), and will become an established aquaculture sector in Europe, together with other percid fish species (Polícar et al., 2019). In nature, aquatic insects are essential food sources for the ontogeny of *Perca fluviatilis* (Rezsú and Specziár, 2006). Therefore, the inclusion of TM into aquafeeds for European perch, with minimal adverse effects on growth performance and physiology traits, is expected. This study aimed at investigating the effects of dietary *T. molitor* larvae meal, as a substitute for FM, on production performance, serum biochemistry, nutrient digestibility, meat quality, and intestinal microbiota of juvenile European perch. Moreover, the environmental impact indicators associated with dietary TM were also estimated.

## 2. Materials and methods

### 2.1. Ethics statement

The experimental procedures were conformed with the European Community Directive (No. 2010/63/EU) and were authorized by the Czech Ministry of Health (No. MSMT-6744/2018-2) regarding the protection of animals used for scientific purposes.

### 2.2. Experimental facilities and procedures

The experimental facilities and procedures were described elsewhere (Tran et al., 2021). Briefly, defatted mealworm *T. molitor* was obtained from a commercial source (NovoProtein, Fishag Edelhof GmbH, Wien, Austria). Four experimental diets, including one control insect-free diet (TM0), and three diets with defatted TM inclusion levels of 6.8, 13.5 and 20.3%, were formulated as replacements of FM on a w/w basis at 25, 50, and 75% (diets abbreviated as TM25, TM50, TM75, respectively). Yttrium oxide (Y<sub>2</sub>O<sub>3</sub>) was added (0.5%) as an inert marker for nutrient digestibility evaluation. All the diets were produced, using a twin-screw extruder, by a commercial aquafeed manufacturer (Exot Hobby s.r.o, Černá v Pošumaví, Czech Republic). The main ingredients, proximate composition, and amino acid profiles of the defatted TM and the experimental diets are presented in Tables 1, 2, respectively. The experimental diets were formulated to meet the nutritional requirements of juvenile perch (Fiogbé et al., 2001; Xu et al., 2001; Fiogbé, 2009).

Perch juveniles were obtained from a commercial hatchery (Anapartner s.r.o, Prague, Czech Republic) and transported, in oxygenated 1 m<sup>3</sup> tanks, to the Research Institute of Fish Culture and Hydrobiology. Fish were acclimated to the experimental facility for two weeks and were fed a commercial diet (Aller Aqua, Christiansfeld, Denmark).

At the start of the experiment, eighty-two fish (bodyweight 20.81 ± 3.36 g, total length 117.7 ± 7.2 mm; mean ± SD) were randomly assigned to each of sixteen black circular 180-L tanks (quadruplicated per diet) connected to a recirculating system (total volume 11,400 L). A

**Table 1**  
Ingredients (% as it) and proximate composition of defatted *Tenebrio molitor* larvae meal (TM) and experimental diets (Tran et al., 2021).

Ingredients (%)	Fishmeal	TM	Experimental diets			
			TM0	TM25	TM50	TM75
Soybean concentrate			29.0	29.0	29.0	29.0
Fishmeal	27.1	20.3	13.5	6.8		
<i>T. molitor</i>	0.0	6.8	13.5	20.3		
Soybean meal	14.5	14.5	14.5	14.5		
Corn flour	9.7	9.7	9.7	9.7		
Fish oil	7.7	7.7	7.7	7.7		
Rapeseed oil	5.8	5.8	5.8	5.8		
Methionine <sup>a</sup>	0.8	0.8	0.8	0.8		
Lysine <sup>b</sup>	0.5	0.5	0.5	0.5		
Valine <sup>c</sup>	0.2	0.2	0.2	0.2		
L-Threonine <sup>d</sup>	0.05	0.05	0.05	0.05		
Vitamins & minerals <sup>e</sup>	0.8	0.8	0.8	0.8		
Additives <sup>f</sup>	3.5	3.5	3.5	3.5		
Yttrium oxide (Y <sub>2</sub> O <sub>3</sub> )	0.5	0.5	0.5	0.5		
<b>Proximate composition (as it)</b>						
Dry matter (%)	96.5	95.0	94.8	95.7	95.6	95.6
Crude protein (%)	71.2	71.1	47.5	48.7	47.4	47.2
Crude lipid (%)	7.9	8.5	16.3	13.9	15.6	17.0
Ash (%)	14.0	7.1	8.9	9.0	8.3	7.6
Fibre (%)	1.24	2.8	2.0	2.0	2.2	2.3
Nitrogen-free extract (%) <sup>g</sup>	1.3	5.5	19.5	21.8	22.3	21.6
Gross energy (MJ/kg) <sup>h</sup>	20.1	21.1	21.0	20.8	21.2	21.5
Chitin <sup>i</sup>	–	4.8	–	0.33	0.65	0.97

<sup>a</sup> Adisseo, China.

<sup>b</sup> Inner Mongolia Eppen Biotech Co., Ltd.

<sup>c</sup> Ajinomoto Animal Nutrition Europe.

<sup>d</sup> Ningxia Eppen Biotech, China.

<sup>e</sup> Aminovitan Sak, Trouw Nutrition Biofaktory s.r.o, Czech Republic.

<sup>f</sup> Feed limestone (0.5%); Pentasodium triphosphate (Fosfa a.s, Czech Republic). (0.5%) and binder (NutriBind, Adisseo, China) (2.5%).

<sup>g</sup> Nitrogen-free extracts (NFE) = dry matter – (crude protein + crude lipid + ash + fibre).

<sup>h</sup> Gross energy (MJ/kg) as gross energy content of protein (23.6 MJ/kg), lipid. (39.5 MJ/kg) and NFE (17.2 MJ/kg).

<sup>i</sup> Estimated from Basto et al., 2020 for defatted TM.

*Production performance, nutrient digestibility, serum biochemistry, fillet composition, intestinal microbiota and environmental impacts of European perch (Perca fluviatilis) fed defatted mealworm (Tenebrio molitor)*

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**Table 2**  
Amino acid profile (g/100 g of total amino acids) of yellow mealworm *Tenebrio molitor* larvae meal (TM) and experimental diets.

	TM	Experimental diets				Perch muscle	% difference		
		TM0	TM25	TM50	TM75		TM25	TM50	TM75
<b>Indispensable amino acids</b>									
Methionine	2.20	3.89	3.80	4.03	3.22	3.11	-2.38	3.50	-17.16
Threonine	4.60	4.26	4.47	4.34	4.85	4.94	4.84	1.85	13.70
Valine	5.04	5.06	5.05	5.07	4.81	6.30	-0.26	0.06	-4.99
Isoleucine	3.69	4.26	4.21	4.14	4.07	5.13	-1.25	-2.75	-4.44
Leucine	9.09	8.05	8.11	8.28	8.29	8.06	0.68	2.78	2.99
Phenylalanine	3.94	4.50	4.58	4.59	4.43	4.49	1.87	2.15	-1.54
Histidine	1.87	3.29	2.98	2.83	2.99	2.88	-9.52	-14.06	-9.21
Lysine	7.18	8.26	8.11	7.35	7.82	8.83	-1.91	-11.02	-5.39
Arginine	7.91	7.12	7.21	7.37	7.43	6.18	1.16	3.51	4.33
Tryptophan	0.59	0.47	0.53	0.59	0.67	NA	13.17	25.30	42.97
<b>Dispensable amino acids</b>									
Cysteine	2.61	1.21	1.47	1.73	1.67	0.58	21.50	42.91	37.57
Aspartic acid	8.26	12.10	11.06	11.04	10.68	7.63	-8.59	-8.71	-11.76
Serine	7.30	5.00	5.49	5.63	5.79	4.81	9.84	12.75	15.81
Glutamic acid	18.24	17.88	18.22	18.28	18.65	14.10	1.91	2.23	4.30
Proline	0.71	0.40	0.50	0.52	0.63	4.79	22.94	29.24	55.57
Glycine	6.40	5.18	5.10	4.97	5.12	7.97	-1.59	-4.10	-1.21
Alanine	5.83	5.41	5.24	5.19	5.10	6.56	-3.13	-4.02	-5.80
Tyrosine	4.54	3.64	3.88	4.03	3.79	3.64	6.70	10.78	4.19
<b>Cumulative difference (%)</b>							<b>56.00</b>	<b>92.41</b>	<b>119.93</b>

% difference calculated as  $(AA_{TM\ diet} - AA_{TM0})/AA_{TM0} * 100$ . Amino acids for perch muscle were retrieved from [Fiogbé \(2009\)](#).

water inflow of 6.5 L/min, combined with in-tube stone aeration, created a constant rotational flow of 4.6 cm/s in each tank. The circular tanks contained funnel-like bottoms, which facilitate the collection of feces and any unconsumed feeds to be collected conventionally. The photoperiod (12 h:12 h, Light:Dark) and light intensity (58.6 lx) were held constant throughout the experimental period. Water temperature ( $22.44 \pm 0.66$  °C), pH ( $7.00 \pm 0.29$ ), oxygen saturation ( $80.41 \pm 8.02\%$ ), ammonia-N ( $0.28 \pm 0.16$  mg/L), and nitrite nitrogen ( $<0.45$  mg/L) parameters were maintained during the experiment as optimum levels for perch farming ([Toner and Rougeot, 2008](#); [Policar et al., 2019](#)).

Fish were fed five times daily, with an excessive amount, at 7.00, 9.00, 11.00, 13.00, and 15.00 using automatic feeders (EHEIM Twins, Deizisau, Germany) for 105 days. Any unconsumed feed was removed fifteen minutes after each feeding and dried to determine the feed intake. Fish mortality was recorded daily in each experimental tank.

### 2.3. Sample collection and calculations

#### 2.3.1. Growth performance

The fish were inspected every three weeks and at the end of the experiment, following 24 h of feed deprivation, for the biometry (weight, total length) under a light anesthesia dose (50 mg/L) of tricaine methanesulfonate (MS222) (Sigma-Aldrich Chemicals, Missouri, USA) in order to minimize handling stress. The production performance indices, survival rate, and feed efficiency were calculated as follows:

Survival rate (%) =  $100 \times$  the final number of fish/the initial number of fish

Condition factor (CF) =  $100 \times$  (body weight (g)/total length<sup>3</sup> (cm))

Weight gain (WG, g) =  $(W_f - W_i)$ , where  $W_f$  is the final wet weight, and  $W_i$  is the initial wet weight

Specific growth rate (SGR, %/day) =  $[(\ln W_f - \ln W_i)/t] \times 100$ , where  $t$  is the number of days

Feed conversion ratio (FCR) = total feed fed (g)/WG (g)

Protein efficiency ratio (PER) = WG (g)/dry protein intake (g)

Daily feeding rate (DFR, % body weight/day) =  $[\text{total dry feed intake (g)} \times 100]/[t \times ((W_i + W_f) \times 0.5)]$

#### 2.3.2. Digestibility trial

Feces from each tank were collected from the 42nd day of the experiment to evaluate the apparent nutrient digestibility of the

experimental diets. At that time, the morphometrics of the fish fed the experimental diets were  $42.2 \pm 1.9$  g (weight) and  $146.2 \pm 0.2$  mm (total length) for TM0;  $41.6 \pm 1.4$  g and  $143.6 \pm 0.3$  mm for TM25;  $37.9 \pm 1.3$  g and  $141.2 \pm 0.2$  mm for TM50;  $33.0 \pm 0.7$  g and  $135.6 \pm 0.2$  mm for TM75. The feces were collected after the last feeding time (at 15.00) following unconsumed feed removal, by means of siphoning, and stored at  $-20$  °C until being analyzed. The apparent digestibility coefficients (ADC) of the dry matter, protein, lipid, ash, phosphorus, and fatty acids of the experimental diets were calculated according to the following equation ([Cho and Slinger, 1979](#)):

ADC of nutrient (ADC, %) =  $100 - (100 \times (\%Y_{2O_3} \text{ in diet}/\%Y_{2O_3} \text{ in feces}) \times (\% \text{Nutrient in feces}/\% \text{Nutrient in diet}))$ .

#### 2.3.3. Serum biochemistry

A random sample of 3 fish/tank ( $n = 12$  fish/group) was taken at the end of the feeding trial (105th day), following 24 h of starvation, and sacrificed by an overdose of anesthesia (MS222, 125 mg/L). Blood samples (approximately 1 mL) were collected from the caudal vein and centrifuged at  $3000 \times g$  at  $4$  °C for 10 min. The separated serum was frozen at  $-80$  °C until further analysis.

#### 2.3.4. Organo-somatic indices and proximate fillet composition

The liver, intestines, spleen, and viscera were removed immediately after blood sampling and weighed (and the length of intestine was measured) to calculate the organo-somatic indices. Skin-off fillets were sampled for fillet yield and stored at  $-20$  °C for further proximate composition analysis. The organo-somatic indices were calculated with the following formulae:

Hepatic somatic index (HSI, %) =  $100 \times$  liver weight (g)/body weight (g)

Visceral somatic index (VSI, %) =  $100 \times$  viscera weight (g)/body weight (g)

Spleen somatic index (SSI, %) =  $100 \times$  weight of spleen (g)/body weight (g)

Intestine somatic index (ISI, %) =  $100 \times$  weight of intestine (g)/body weight (g)

Mesenteric fat index (MSI, %) =  $100 \times$  (perivisceral fat weight/body weight)

Relative gut length (RGL) = intestinal length (mm)/fish total length (mm)

Fillet yield (FY, %) =  $100 \times ((\text{right fillet weight (g)} + \text{left fillet weight (g)}) / \text{body weight (g)})$

### 2.3.5. Microbiota

At the end of the feeding trial, two fish per tank ( $n = 8$  fish/diet group) were randomly taken and euthanized by means of overdose anesthesia (MS222, 125 mg/L). To ensure all the sampled fish had digesta throughout the intestinal tract, the fish were deprived of feeds 12 h before the sampling time. The exterior of the fish was wiped with 70% ethanol before opening the abdomen, the whole intestine was subsequently removed from the abdominal cavity from each fish and the digesta from the proximal to distal intestine was squeezed gently into a 1.5 mL sterile Eppendorf and immediately stored at  $-80^\circ\text{C}$  for further analysis.

### 2.3.6. Environmental impact indices

The total solid waste (TSW), solid nitrogen waste (SNW), and solid phosphorus waste (SPW) were calculated as described by Bureau and Hua (2010).

$\text{TSW} = \text{feed consumed} \times (1 - \text{ADC of dry matter})$

$\text{SNW} = \text{feed consumed} \times \text{Nitrogen content in diet} \times (1 - \text{ADC of protein})$

$\text{SPW} = \text{feed consumed} \times \text{Phosphorus content in diet} \times (1 - \text{ADC of phosphorus})$

The simulated environmental impacts associated with one-kilogram farmed perch production were calculated as environmental impacts of the diet multiplied by the respective FCR. The environmental impacts of one kilogram diet were calculated using the life cycle assessment database generated by the Global Feed Lifecycle Institute (GFLI, 2021) as described by Quang Tran et al. (2021). Given that the environmental impact of ingredients in the GFLI database varies with location, average global values were used. The minerals, vitamins, additives and supplemented amino acids in the present study are classified as 'Total minerals, additives, vitamins, at plant/RER Mass S' in the GFLI database. Due to unavailable data on water use for the production of one kg of *T. molitor* meal, we used the value of  $4.3 \text{ m}^3$  required for one kg fresh mealworm (Miglietta et al., 2015) with an assumption that the drying process of mealworm did not require additional water (Tallentire et al., 2018). The six environmental impact categories comprise global warming potential (GWP, kg CO<sub>2</sub> equivalent (eq.)), energy use (kg oil eq.), acidification (kg SO<sub>2</sub> eq.), eutrophication (kg P eq.), land use (m<sup>2</sup> arable land (a.)) and water use (m<sup>3</sup>). The impacts associated with feed production at the feed mill and fish farming phase were beyond the scope of the present study.

The economic fish-in fish-out ratio (eFIFO), indicating the amount of fish used to produce one unit of farmed fish, was developed by Kok et al. (2020); it is based on an economic allocation commonly used in life cycle assessments. The formula used to calculate the eFIFO ratio is:  $\text{eFIFO} = \text{FCR} \times \sum (\text{Fi}_{i,j} \times \text{EFi}_{i,j})$ , where FCR is feed conversion ratio; Fi is the fraction of ingredient i in the feed (%); EFi is the embodied fish in ingredient i; i, FM or FO; j is the source of the ingredient. The value of embodied fish in ingredient was taken from a 2021 database (Kok et al., 2020).

## 2.4. Analytical methods

### 2.4.1. Chemical composition

The defatted insect meal (*T. molitor*), experimental diets, feces, fillets were well homogenized and analyzed according to AOAC (2000) for dry matter (Method 934.01), crude ash (Method 942.05) and crude fibre (Method 985.29). Crude protein was determined, by means of the Kjeldahl method, using an automatic Kjeldahl System (Buchi, Flawil, Switzerland). The lipid and fatty acid profiles were determined as described by Mráz and Pickova (2009). The phosphorus content in the insect meal, diets and feces was determined using an inductively coupled plasma atomic emission spectrophotometer (ICPOES, Prodigy7, Leeman Labs, USA). Yttrium oxide (Y<sub>2</sub>O<sub>3</sub>) in the diets and feces samples

were analyzed using inductively coupled plasma emission spectrometry (ICPOES) following digestion with nitric acid at  $180^\circ\text{C}$  for 48 h. Amino acid profile of insect meal and diets was analyzed as described by Stejskal et al. (2019).

### 2.4.2. Serum biochemistry

Serum samples were determined using an Architect c4000 automatic analyzer (Abbott Laboratories, Abbott Park, Illinois, USA). The serum biochemical parameters included alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, glucose, cholesterol, triglyceride, and alkaline phosphatase (ALP).

### 2.4.3. Gut microbiota

DNA extraction and 16S rRNA amplicon target sequencing were performed according to the following procedures:

Nucleic acid was extracted from the gut content (500 mg as the starting material). The total DNA was extracted from the samples using an RNeasy Power Microbiome KIT (Qiagen, Milan, Italy) according to the manufacturer's instructions. One microliter of RNase (Illumina Inc., San Diego, CA) was added to digest RNA in the DNA samples and incubated of 1 h at  $37^\circ\text{C}$ . DNA was quantified using the NanoDrop and standardized at  $5 \text{ ng}/\mu\text{L}$ .

DNA extracted directly from digesta samples was used to assess the microbiota by the amplification of the V3-V4 region of the 16S rRNA gene (Klindworth et al., 2013). The PCR products were purified according to the Illumina metagenomic standard procedure (Illumina Inc., San Diego, CA). Sequencing was performed with a MiSeq Illumina instrument with V3 chemistry and 250 bp paired-end reads were generated according to the manufacturer's instructions.

## 2.5. Statistical analyses

The obtained data were checked for normal distribution (Shapiro-Wilks's test) and homogeneity of variances (Levene's test). All the statistical analyses were performed using the R Statistic Package, R Development Core Team 2009–2021.

Regression analyses were performed for all data results, except for nutritional compositions of experimental diets and ingredients, using a linear model ('lm' function) to evaluate the correlation between TM dose and data results. The best fit model was estimated using 'aov' function. One-way ANOVA was used to test the differences in environmental impacts, and body weight, total length over time. Tukey's post-hoc test was used when appropriate. Differences were regarded as significant at  $P < 0.05$ .

Paired-end reads were first joined by means of FLASH software (<http://sourceforge.net/projects/flashpage>) to default parameters for gut microbiota. The reads obtained after quality filtering (at Phred < Q20), using QIIME 2 software (v2018.11) (Caporaso et al., 2010) were analyzed by means of a recently described pipeline (Biasato et al., 2018). Picking the operational taxonomic units (OTUs) was performed at 97% of similarity, and taxonomy assignment was done by using the Greengenes16S rRNA gene database 2017 (<http://greengenes.lbl.gov>). The centroids sequence was then manually blasted to confirm the taxonomic assignment. The OTU table obtained with QIIME was rarefied at the lowest number of sequences and the higher taxonomy resolution genus or family was displayed. The vegan package of R (Dixon, 2003) was used to calculate the alpha diversity. The diversity indices and the OTU table were further analyzed using the pairwise comparisons from the Wilcoxon rank-sum test to assess any differences between the diets. A difference was considered significant at  $P < 0.05$ . Weighted UniFrac distance matrices and OTU tables were used to perform Adonis and Anosim statistical tests in the R environment.

# Production performance, nutrient digestibility, serum biochemistry, fillet composition, intestinal microbiota and environmental impacts of European perch (*Perca fluviatilis*) fed defatted mealworm (*Tenebrio molitor*)

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## 3. Results

### 3.1. Growth performance

The inclusion of defatted *T. molitor* larvae meal had a significant effect ( $P < 0.05$ ) on body weight and total length of juvenile European perch over time (Fig. 1). Feeding perch with dietary TM did not affect survival ( $P = 0.32$ ) and fillet yield ( $P = 0.37$ ) after 105-day trial, but, at substantial inclusion levels (TM50 and TM75), significantly compromised weight gain ( $P = 0.04$ ) and SGR ( $P = 0.01$ ), and increased FCR ( $P = 0.04$ ), DFR ( $P = 0.03$ ) compared with TM0. A negative correlation between TM level and PER ( $P = 0.04$ ) was also detected (Table 3, Table S1).

There was a significant quadratic linear model between TM inclusion levels and fish growth indices (CF, WG, SGR, FCR) ( $P < 0.05$ ). The model illustrated that these indices of perch fed TM25 were comparable to those fed TM0. Organo-somatic indices, VSI, HIS, MFI, ISI, RGL, SSI were TM inclusion independent ( $P > 0.05$ ) (Table 3, Table S1).

Serum biochemistry indices of perch did not differ among diet groups ( $P > 0.05$ ), except AST activity which significantly elevated with increasing inclusion levels of TM ( $P = 0.02$ ; Table 3).

### 3.2. Apparent digestibility of experimental diets

The protein digestibility of European perch was negatively affected by dietary defatted TM ( $P = 0.03$ ), whereas digestibility of other nutrients and fatty acids remained TM-dose independent ( $P > 0.05$ ) (Table 4; Table S2).

### 3.3. Proximate composition fish fillet

Feeding defatted TM levels did not alter the proximate composition and fatty acid profile of the perch fillets ( $P > 0.05$ ). DHA, ranged from 14.95% (TM50) to 18.18% (TM75) of the total fatty acids, was the second-largest constitute in perch fillets following oleic acid, and unaffected by administration of defatted TM ( $P > 0.05$ ). The EPA share was meager (2.47–3.08% of the total fatty acids) and was consistent among diet groups ( $P > 0.05$ ) (Table 5).

### 3.4. Microbiota analysis

After sequencing and quality filtering, 334,095 reads were obtained and used for further analysis with an average value of 12,661 reads/sample. Analysis of the rarefactions and estimated sample coverage indicated a satisfactory coverage of all samples (median coverage value of 98%). No significant differences were observed for alpha diversity indices of Shannon ( $P = 0.557$ ), observed species ( $P = 0.523$ ), and Chao1 ( $P = 0.741$ ) (Fig. 2).

The bacterial community in the perch intestine was mainly dominated by phyla Firmicutes (56–95%), Actinobacteria (4–34%), and Fusobacteria (0.1–22%) (Fig. 3A). The microbiota composition at the genus level (Fig. 3B) was mainly represented by *Clostridium* (52%, 87%, 50 and 63% in TM0, TM25, TM50 and TM75, respectively), *Mycobacterium* (33, 4, 31 and 9%), *Lactobacillus* (4, 3, 1 and 1%) and *Cetobacterium* (1, 0.1, 9 and 22%). A minor OTUs fraction (below 0.2%) was composed of *Enterobacteriaceae*, *Streptococcus*, *Candidatus*, *Chlamydia*, *Clavibacter*, *Bacillus*, *Parachlamydiaceae*, and *Solirubrobacterales* (Fig. 3B).

The principal component analysis based on OTUs abundance showed

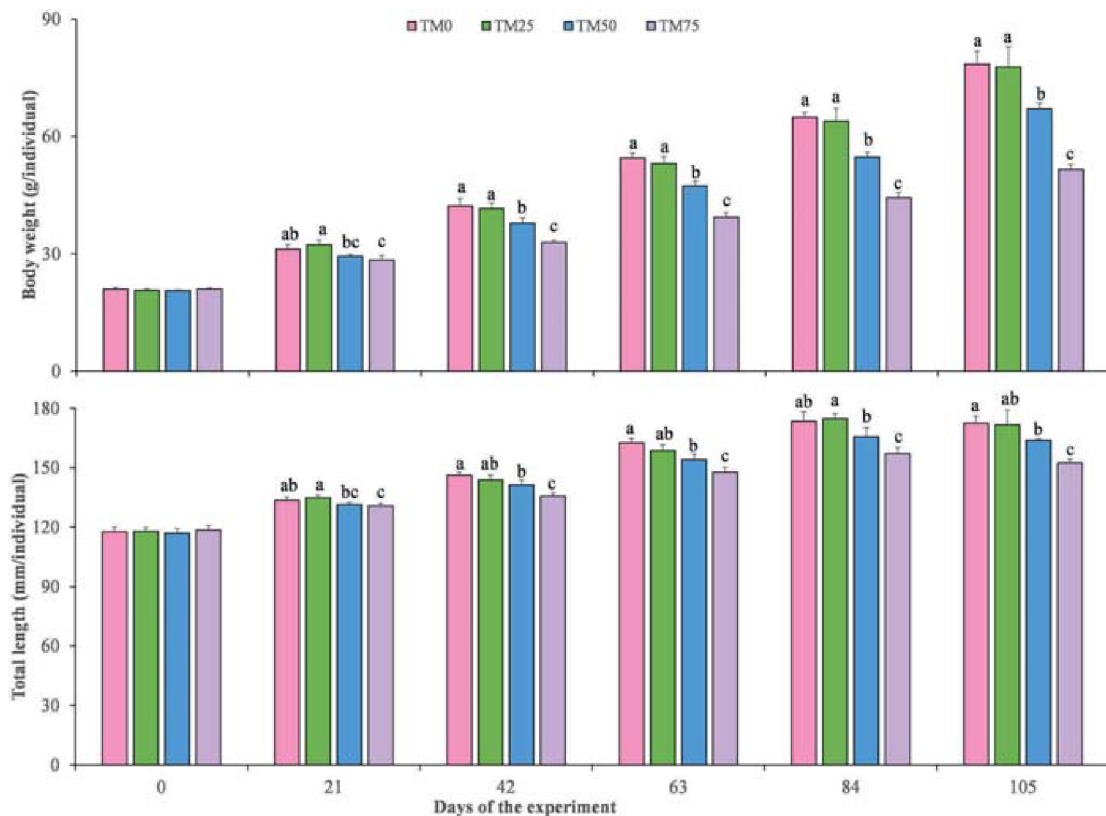


Fig. 1. Body weight and total length of European perch (*Perca fluviatilis*) fed graded levels of yellow mealworm *Tenebrio molitor* larvae meal over time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 3**

Production performance, feed efficiency, organo-somatic indices, and serum biochemistry of European perch (*Perca fluviatilis*) fed graded levels of yellow mealworm *Tenebrio molitor* larvae meal for 105 days.

Item	Experimental diets				SEM	P value
	TM0	TM25	TM50	TM75		
<b>Production parameters</b>						
Survival (%)	99.09	99.09	98.48	98.78	0.21	NS
CF	1.53	1.54	1.52	1.46	0.02	0.03
WG (g)	57.56	57.14	46.48	30.59	2.90	0.04
SGR (%/day)	1.26	1.26	1.12	0.86	0.04	0.01
FCR	1.15	1.19	1.33	1.77	0.07	0.04
PER	1.85	1.72	1.60	1.21	0.06	0.04
DFR (% BW/day)	1.26	1.31	1.33	1.41	0.02	0.03
Fillet yield (%)	38.50	39.79	38.22	37.42	0.40	NS
<b>Somatic indices</b>						
VSI (%)	14.23	13.65	14.14	14.27	0.26	NS
HSI (%)	1.53	1.53	1.65	1.58	0.05	NS
MFI (%)	9.27	8.89	8.87	8.75	0.22	NS
ISI (%)	0.75	0.73	0.84	0.87	0.03	NS
RGL	0.58	0.61	0.60	0.56	0.01	NS
SSI (%)	0.08	0.09	0.09	0.10	0.00	NS
<b>Serum biochemistry</b>						
ALT (ukat/L)	0.29	0.33	0.30	0.36	0.01	NS
AST (ukat/L)	0.62	1.29	1.48	2.04	0.19	0.02
Glucose (mmol/L)	5.90	4.75	4.65	6.94	0.42	NS
Cholesterol (mmol/L)	5.66	4.61	4.55	4.61	0.21	NS
Triglycerides (mmol/L)	9.75	7.79	7.97	9.05	0.44	NS
Total protein (g/L)	40.70	32.64	43.29	42.25	2.54	NS
ALP (ukat/L)	0.43	0.37	0.40	0.49	0.02	NS

SEM = Standard error of the mean (pooled); CF = condition factor; WG = weight gain; SGR = specific growth rate; FCR = feed conversion ratio; PER = protein efficiency ratio; DFR = daily feeding rate; VSI = visceral somatic index; HSI = hepatic somatic index; MFI = mesenteric fat index; ISI = intestine somatic index; RGL = relative gut length; SSI = spleen somatic index; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; NS = Not significant ( $P > 0.05$ ).

**Table 4**

Nutrient digestibility (%) of European perch (*Perca fluviatilis*) fed graded levels of yellow mealworm *Tenebrio molitor* larvae meal for 105 days.

Nutrients	Experimental diets				SEM	P value
	TM0	TM25	TM50	TM75		
Dry matter	78.95	77.01	77.58	76.52	0.28	NS
Crude protein	92.72	90.99	90.42	87.48	0.58	0.03
Crude lipid	92.62	91.96	92.95	89.14	0.49	NS
Phosphorus	45.93	36.26	38.24	37.76	1.46	NS
Ash	41.98	38.14	40.11	38.30	0.61	NS
<b>Fatty acids</b>						
C14:0	98.13	97.91	98.05	97.97	0.04	NS
C16:0	96.81	96.09	96.10	96.33	0.10	NS
C16:1	98.53	98.53	98.62	98.40	0.04	NS
C18:1n9	97.90	97.79	97.93	97.71	0.03	NS
C18:2n6	98.40	98.10	98.21	98.21	0.04	NS
C18:3n3	98.90	98.70	98.84	98.15	0.09	NS
C20:1n9	97.05	96.81	95.68	77.40	2.50	NS
SFA	96.71	95.91	95.89	96.13	0.11	NS
MUFA	97.76	97.77	97.88	97.60	0.03	NS
PUFA	98.34	97.97	98.07	98.08	0.04	NS
Σn3	98.99	98.70	98.83	98.65	0.04	NS
Σn6	98.43	98.14	98.22	98.21	0.04	NS
DHA	98.93	98.49	98.61	98.91	0.06	NS
EPA	99.38	99.16	99.24	99.37	0.03	NS

SEM = Standard error of the mean (pooled); EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; NS = Not significant ( $P > 0.05$ ).

no clear separation across diet groups. There was a significant negative relationship between TM addition and the abundance of *Lactobacillus* (linear model,  $P = 0.04$ , adjusted R-squared = 0.82) and *Streptococcus*

**Table 5**

Fillet composition (% as wet basis) and fatty acid profile (% of total FA) of European perch (*Perca fluviatilis*) fed graded levels of yellow mealworm *Tenebrio molitor* larvae meal.

	Experimental diets				SEM	P value
	TM0	TM25	TM50	TM75		
Moisture	79.04	77.70	77.93	76.99	0.35	NS
Crude protein	22.25	22.74	22.55	22.0	0.25	NS
Crude lipid	1.45	1.30	1.21	1.28	0.04	NS
Ash	1.11	1.11	1.12	1.11	0.01	NS
<b>Fatty acid profile</b>						
C14:0	1.59	1.62	1.37	1.53	0.05	NS
C16:0	16.97	18.35	15.94	17.20	0.55	NS
C16:1	4.54	5.09	4.50	4.87	0.18	NS
C18:0	2.51	2.68	2.37	2.73	0.10	NS
C18:1n9	32.58	31.44	27.39	29.69	1.16	NS
C18:2n6	12.34	12.07	10.58	12.24	0.43	NS
C18:3n3	2.84	2.66	2.15	2.37	0.11	NS
C20:1n9	1.56	1.42	1.18	1.36	0.05	NS
C20:5n3 (EPA)	3.06	3.08	2.47	3.03	0.12	NS
C22:6n3 (DHA)	17.00	16.92	14.95	18.18	0.71	NS
ΣSFA	22.34	23.87	20.76	22.67	0.71	NS
ΣMUFA	39.97	38.92	34.34	38.66	1.33	NS
ΣPUFA	37.61	37.13	44.83	38.47	1.98	NS

SEM = Standard error of the mean (pooled); NS = Not significant ( $P > 0.05$ ). Fatty acids with less than 1% total fatty acids in experimental diets (C10:0, C12:0, C13:0, C14:1, C15:0, C15:1, C17:0, C17:1, C16:3, C18:1n9 trans, C18:1n7, C18:2n6 trans, C18:3n6, C20:0, C21:0, C20:3n6, C20:3n3, C20:4n6, C22:0, C24:0, C24:1n9, C22:5n6) were not presented in the table but included in fatty acids group calculation. EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

(quadratic model,  $P = 0.01$ , adjusted R-squared = 0.99).

### 3.5. Solid waste output and environmental impacts

Dietary defatted TM significantly affected environmental impacts associated with TSW ( $P = 0.0002$ ), SNW ( $P = 0.0001$ ) and SPW ( $P = 0.0008$ ) (Table 6). TM75 showed a significant reduction in TSW ( $P = 0.031$ ), whereas feeding perch with TM25 inverted the pattern ( $P = 0.005$ ) compared with the control diet. SPW was not different among TM0, TM50 and TM75 ( $P > 0.05$ ), but was significantly higher in TM25 ( $P = 0.0008$ ). Dietary defatted TM significantly increased SNW ( $P = 0.0001$ ) (Table 6).

The eFIFO, ranged from 0.99–1.45, was significantly reduced with increasing levels of defatted TM ( $P < 0.001$ ). Among the TM-containing groups, the ratio was less than one for the TM75 diet, whereas those of TM25, and TM50 were greater than 1 (1.19 and 1.07, respectively) (Table 6).

As far as the environmental impacts associated with one kg farmed perch production are concerned, TM25 was comparable with TM0 for the global warming potential, acidification, and land use ( $P > 0.05$ ). TM50 and TM75 exerted heavier burdens than the control diet on all the impact categories ( $P < 0.05$ ).

## 4. Discussion

Insect meal has been considered the most promising raw material for the supply of protein sources in aquafeeds for the coming decades (Hua et al., 2019; Gasco et al., 2020a). A wide range of aquatic animals has been investigated for the possibility of including insect meals in their feeds (Henry et al., 2015; Gasco et al., 2019; Hua, 2021). European perch (*P. fluviatilis*) is a potential candidate for aquaculture diversification in Europe, and the intensive aquaculture of this species is taking off with increasing production volume over the last decades, reaching approximately 700 t in 2018 (FAO, 2020). The potential use of insect meal as an alternative protein source for perch was investigated by



**Table 6**

Solid waste output and environmental impacts associated with 1 kg production of European perch (*Perca fluviatilis*) fed graded levels of yellow mealworm *Tenebrio molitor* larvae meal for 105 days.

	Experimental diets				SEM	P value
	TM0	TM25	TM50	TM75		
<b>Solid waste output</b>						
TSW (g)	1115 <sup>b</sup>	1268 <sup>a</sup>	1104 <sup>b</sup>	1004 <sup>c</sup>	52	0.0002
SNW (g)	385 <sup>e</sup>	497 <sup>ab</sup>	471 <sup>b</sup>	535 <sup>a</sup>	30	0.0001
SPW (g)	2860 <sup>b</sup>	3514 <sup>a</sup>	3038 <sup>b</sup>	2660 <sup>b</sup>	178	0.0008
<b>Environmental impacts associated with 1 kg perch production</b>						
GWP (kg CO <sub>2</sub> eq.)	3.00 <sup>c</sup>	3.24 <sup>c</sup>	4.01 <sup>b</sup>	5.58 <sup>a</sup>	0.27	<0.001
Energy use (kg oil eq.)	0.35 <sup>d</sup>	2.45 <sup>c</sup>	5.29 <sup>b</sup>	10.19 <sup>a</sup>	0.96	<0.001
Acidification (kg SO <sub>2</sub> eq.)	10.71 <sup>c</sup>	11.94 <sup>c</sup>	15.17 <sup>b</sup>	21.65 <sup>a</sup>	1.11	<0.001
Eutrophication (kg P eq.)	0.36 <sup>d</sup>	1.54 <sup>c</sup>	3.14 <sup>b</sup>	5.94 <sup>a</sup>	0.54	<0.001
Land use (m <sup>2</sup> a)	2.40 <sup>c</sup>	2.76 <sup>c</sup>	3.60 <sup>b</sup>	5.23 <sup>a</sup>	0.29	<0.001
Water use (m <sup>3</sup> )	0.03 <sup>d</sup>	0.37 <sup>c</sup>	0.82 <sup>b</sup>	1.61 <sup>a</sup>	0.15	<0.001
<b>Economic fish-in fish-out</b>						
eFIFO	1.45 <sup>a</sup>	1.19 <sup>b</sup>	1.07 <sup>bc</sup>	0.99 <sup>c</sup>	0.05	<0.001

SEM = Standard error of the mean (pooled); TSW = total solid waste; SNW = solid nitrogen waste; SPW = solid phosphorus waste; GWP = global warming potential, eFIFO = economic fish-in fish-out.

Means with the same superscript letter in a same row are not significantly different ( $P > 0.05$ ).

well accepted by European perch as indicated by DFR, which was significantly higher in the TM-based diets than in the control group. Stejskal et al. (2020) also reported a comparable feeding rate for perch fed dietary defatted *H. illucens*. Similar findings were observed for rainbow trout (*O. mykiss*) (Chemello et al., 2020), red seabream (*Pargus major*) (Ido et al., 2019). On the contrary, Gasco et al. (2016) reported a significant reduction in feed intake for European sea bass (*D. labrax*) fed increasing full-fat *T. molitor* levels. These differences could be attributed to the different processing forms of the consumed insect meal, as defatted insect meal has been reported to improve the palatability of catfish (*Clarias gariepinus*) (Fasakin et al., 2003).

In our study, growth performance of perch in the present study (SGR, ranged from 0.86–1.26) was consistent with published data (1.14–1.30) for perch fed dietary insect meal, *H. illucens* (Stejskal et al., 2020). Feeding perch with TM25 showed a consistent growth performance compared to the control diet, whereas higher replacement levels had detrimental effects. This phenomenon could be linked to the presence of chitin, which has been shown to affect the growth rate of fed organisms (Gasco et al., 2016; Weththasinghe et al., 2021). The compromising performance mechanism consists of a lowering of the energy availability and a reduction of the nutrient digestibility of fish (Ringø et al., 2012). Defatted TM contained 4.63% chitin (Basto et al., 2020), and increasing dietary TM, therefore, corresponded to increasing the chitin levels in TM-containing diets (Table 1). Consequently, a reduction in nutrient digestibility of perch fed these diets relative was observed, compared to the control diet (Table 5). The limited ability of fish to utilize chitin as energy hampers fish growth when substantial FM replacements with insect meal (*H. illucens*) are introduced (Weththasinghe et al., 2021). Another nutritional factor that may impair fish performance is linked to a fatty acid deficiency (Henry et al., 2015). The declining EPA and DHA observed in our study as dietary defatted TM increased (Tran et al., 2021) could evidence a growth delay. Although the amino acid of taurine was not measured in our study, it is known, at low availability, to compromise fish growth (Salze and Davis, 2015). Basto et al. (2020) found that defatted TM contains a lower content of this sulfonic acid than the full-fat form. As a result, increasing inclusion levels of defatted TM, accompanied by a reduction in taurine levels, could have hampered the performance of perch fed TM50 and TM75 in our study.

Stejskal et al. (2020) reported that inclusion levels of up to 40% (or

42% FM replacement) of defatted *H. illucens* in diets showed no adverse effect on the growth performance of perch compared to the insect-free diet. It is evidenced that dietary *H. illucens* is preferable to *T. molitor* for perch, in terms of growth rate. A study on European seabass (*D. labrax*) fed a diet with 30% FM replacement with TM and *H. illucens* meal also showed a superior growth and feed efficiency of the latter insect species compared to the former (Mastoraki et al., 2020).

The present study found that organ-somatic indices were not affected by dietary TM, which is in agreement with the previous publications in which dietary TM was fed to blackspot seabream, *Pagellus bogaraveo* (Iaconisi et al., 2017) and mandarin fish, *Siniperca scherzeri* (Sankian et al., 2018). The HSI index, ranged from 1.53–1.65, was consistent with that of Stejskal et al. (2020), who reported an HSI of 1.21–1.76 in perch fed dietary *H. illucens*. However, VSI in our study, which varied between 13.65 and 14.27, was higher than that reported for perch, which was 8.79–10.56 (Xu et al., 2001); 7.30–9.81 (Kestemont et al., 2001) and 2.79–3.06 (Stejskal et al., 2020). This discrepancy could be attributed to differences in fish size, dietary lipid content and mesenteric fat among trials. Our data on MFI (8.75–9.27) were comparable with those reported in perch (Blanchard et al., 2008). The SSI value is also in agreement with one reported earlier (Stejskal et al., 2020). The RGL in the present study ranged from 0.56 to 0.61 and is in agreement with that of carnivores (0.5–2.4) (Kramer and Bryant, 1995). Our RGL data did not differ among the experimental diets, which is in contrast with recent findings that have pointed out the significant effect of dietary insect meal (*T. molitor*) on RGL of trout, *O. mykiss* (Iaconisi et al., 2018) and seabream, *Sparus aurata* (Piccolo et al., 2017). Despite the adaptive plasticity of gut length to different food sources, perch might experience an initial reduction in their body conditions when consumed diet is changed (Olsson et al., 2007). This may be a reasonable explanation for the phenomenon observed in our study whereby the body weight and length of perch significantly reduced with increasing inclusion of TM while maintaining their RGL.

The fillet yield in our study (37.42–39.79%) was slightly higher than the 34.87–36.68% reported for perch fed commercial feeds (Bochert, 2020), but unaffected by dietary defatted TM, a result that is consistent with other publications, reporting the independence of fillet yield and insect meal dose (Piccolo et al., 2017; Iaconisi et al., 2018; Moutinho et al., 2020).

Our study revealed that the serum biochemistry was unaffected by dietary insect meal (*T. molitor*), except for AST. The AST activities could be a proxy for stress-induced tissue damage (Velíšek et al., 2009). Song et al. (2018) reported that a low level of FM replacement by TM could induce liver damage as indicated by a significant increase in AST activities compared to the control group, thereby indirectly impairing the growth performance of gentian grouper (*Epinephelus lanceolatus* × *Epinephelus fuscoguttatus*). In our study, perch fed TM75 showed a significantly lower growth rate than the other groups, which could be linked to stressors. Similarly, Iaconisi et al. (2017) evidenced a stress status of sea bream (*P. bogaraveo*) fed dietary TM. This pattern seemed to be too mild in the present study to induce severe mortality.

#### 4.2. Nutrient digestibility

In this study, dietary defatted TM significantly affected nutrient digestibility of European perch, except for ash. All experimental diets resulted in high digestibility values for protein and lipid, whereas lower results were observed for ash and phosphorus (Table 5). In general, chitin in TM-containing diets could be responsible for the different levels of digestibility of perch. This substance could hinder nutrient digestibility by interfering with the digestive enzyme activities of other nutrients (Weththasinghe et al., 2021). Although many fish can produce the chitin-degrading enzyme, chitinase (Ringø et al., 2012), it seems null in other fish species (Kroeckel et al., 2012; Guerreiro et al., 2021). The chitinase enzyme is presented in perch and is mainly excreted from the pancreas and, to a lesser extent, produced by intestinal bacteria (Craig,



# Production performance, nutrient digestibility, serum biochemistry, fillet composition, intestinal microbiota and environmental impacts of European perch (*Perca fluviatilis*) fed defatted mealworm (*Tenebrio molitor*)

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2000). However, the animals' capacity to digest chitin remains particularly low and tends to decline with increasing chitin levels (Olsen et al., 2006; Khempaka et al., 2011). High fibre and chitin contents in TM-containing diets (Table 1) could reduce the digesta transit time in the gastrointestinal tract, as confirmed in humans, chickens (Razdan and Pettersson, 1994) and fish (Olsen et al., 2006), thereby reducing the exposure time of food to digestive enzymes.

In the present study, the observed declining lipid digestibility due to dietary insect meal (*T. molitor*) was consistent with previous findings (Piccolo et al., 2017; Belghit et al., 2018; Belghit et al., 2019; Weththasinghe et al., 2021). Chitin has been reported to bind with lipid and bile in fish and mammals (Tharanathan and Kittur, 2003; Weththasinghe et al., 2021), thereby reducing lipid digestibility. Feeding chitin-containing diet has been reported to numerically lower lipase activities in meager (*Argyrosomus regius*) compared to the chitin-free diet (Guerreiro et al., 2021). These effects seem to be too mild to impair digestibility of TM25 and TM50 but does for TM75 in our study. The high fatty acid digestibility observed in our study is in agreement with those reported for Atlantic salmon (*Salmo salar*) (Lock et al., 2016; Belghit et al., 2019).

Wang et al. (2017) reported phosphorus digestibility of tilapia (*Oreochromis niloticus*) fed dietary insect meal (*Musca domestica*) ranged from 89.21 to 92.41% and found the independence to *Musca domestica* inclusion. The phosphorus digestibility of whiteleg shrimp (*L. vannamei*), ranged from 31.4 to 76.4%, was insect meal (*Bombyx mori*)-dose-dependent (Rahimnejad et al., 2019). Basto et al. (2020) reported that the phosphorus digestibility of seabass (*D. labrax*) fed diets, in which 20% protein diets were replaced by *T. molitor*, *H. illucens* and locust meal, were in the 57.2–63.9% range. Our results (36.26–45.93%) were affected to a great extent by the dietary defatted TM. The stomach pH (Zhang et al., 2015) and dietary calcium to phosphorous ratio (Rahimnejad et al., 2019) are known to influence phosphorus digestibility of fish. Moreover, the capacity of fish to utilize phosphorous or bind phytate-phosphorous has been shown to vary a great deal from carnivores to omnivores (Kumar et al., 2012).

### 4.3. Fillet composition

The composition of perch fillets in the present study was similar to that of published data on perch fed commercial feeds (Bochert, 2020). The TM-dose independence of fillet traits was also in agreement with previous studies on seabream, *P. bogaraveo* (Iaconisi et al., 2017) and rainbow trout, *O. mykiss* (Iaconisi et al., 2018). The particularly low lipid content of fillet (1.20–1.45%) was similar to previous studies on wild and farmed perch (Kestemont et al., 2001; Xu and Kestemont, 2002; Mairesse et al., 2006; Orban et al., 2007; Stejskal et al., 2011b).

Fillets of perch farmed in an intensive aquaculture system have been considered a valuable source of fatty acids, such as linoleic acid (LA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Stejskal et al., 2011b). Our DHA and LA values were relatively higher than those found for wild and farmed perch in earlier studies, whereas EPA remained lower (Jankowska et al., 2010; Stejskal et al., 2011b). Greater bio-conversion of DHA than EPA, and peroxisomal  $\beta$ -oxidation synthesis of the former from the latter through intermediate product C24:6n – 3 (Kestemont et al., 2001) could explain the discrepancies in the proportion of those FAs in the fillets in our study. We also observed a high LA in the fillets, which was consistent with the result of Xu and Kestemont (2002), who reported a ready accumulation of this fatty acid into fish tissues as a stored lipid. The low proportion of linolenic acid (C18:3n3) in the fillets as well as high proportions of DHA and EPA, relative to the respective diets, indicated the high capacity of elongation and desaturation of C18:3n3 in perch, which was confirmed the previous findings (Xu and Kestemont, 2002; Blanchard et al., 2008). Our study indicated that palmitic acid (15.94–18.35%) and oleic acid (27.39–32.58%) were the predominant saturated and monounsaturated FAs, respectively, in fish fillets. A similar finding was reported for perch (Jankowska et al.,

2010; Stejskal et al., 2011b) and other farmed fish (Testi et al., 2006).

### 4.4. Gut microbiota

In accordance with previous works on trout, *Salmo trutta* (Mikolajczak et al., 2020), rainbow trout, *O. mykiss* (Terova et al., 2021), seabream, *S. aurata* and European sea bass, *D. labrax* (Antonopoulou et al., 2019) fed dietary TM, our results showed the consistency of the bacterial diversity and richness of perch regardless of the diet groups. On the other hand, Antonopoulou et al. (2019) reported that administration of TM significantly altered the microbiota community of trout, *O. mykiss*. (Ghanbari et al., 2015). Therefore, the discrepancies among studies could be linked to fish physiology and nutritional availability of fish gut, which could be responsible for the dietary treatment effects on the microbiota population (Bolnick et al., 2014a).

The most prevalent bacteria in intestine of perch fed experimental diets belonged to Firmicutes, Actinobacteria, and Fusobacteria phyla. The first two phyla were found abundant in rainbow trout, *O. mykiss* fed dietary insect meal, *H. illucens* (Huyben et al., 2019) and also in mealworm larvae, *T. molitor* (Stoops et al., 2016). Our sequencing data were aligned with the intestinal microbiota composition of perch and freshwater fish, which are dominated by *Clostridium* genus (Bolnick et al., 2014b; Romero et al., 2014). This shows that *Clostridium* spp. is a core species in the intestine of European perch.

Contradictory results on the effect of dietary TM on the intestinal population of *Lactobacillus* spp. are present in the literature. Mikolajczak et al. (2020) highlighted a significant decrease in the concentration when fed *S. trutta* with 10% hydrolyzed TM administration compared with a FM-based diet. An opposite result was reported for *O. mykiss* (Terova et al., 2021), where an 0.2-fold increase was observed in a TM-diet, compared to a FM-based one (Józefiak et al., 2019). Józefiak et al. (2019) found TM-dose independence pertaining to the count of this lactic acid-produced bacteria in Siberian sturgeon (*Acipenser baerii*). Such discrepancy could be ascribed to the nutrition status in the intestine of tested fish, as it has been well known that *Lactobacillus* group requires nutritious substrates to thrive (Havenaar and Huis In't Veld, 1992). A reduction in the *Lactobacillus* genus in intestine of perch fed TM75 in present study could be linked to unfavorable status of perch intestine associated with the deficiency of certain amino acids, fatty acid (DHA, EPA), and with the presence of chitin.

The *Clostridium* and *Lactobacillus* genera, which are among the prevalent species in this study, have been used as probiotics for fish (Ringø, 2020). Therefore, our results suggest the potential application of beneficial microorganisms isolated from the intestine of perch fed insect meal (*T. molitor*).

Bacteria from the *Mycobacterium* genus were also found predominant in perch fed diet treatments. Moutinho et al. (2017) conducted a feeding trial on seabream (*S. aurata*) fed dietary meat bone meal as a replacement for FM and reported the existence of these bacteria in the intestine of specimens. *Mycobacterium* spp. are commonly known as the causative agent of mycobacteriosis syndromes in aquaculture species (Yanong et al., 2010) and have a zoonotic potential (Mrlik et al., 2012). Although many of *Mycobacteria* spp. were found to be present in the aquaculture systems in Czech Republic, the clinical pathogen, *Mycobacterium marinum*, for humans and fish was absent (Beran et al., 2006). The high survival rate and absence of pathogenic syndromes of perch during the 105-day feeding trial could confirm the benignity of these microorganisms in our systems.

Previous studies on Perciformes fish, pikeperch (*Sander lucioperca*), largemouth bass (*Micropterus salmoides*) and bluegill (*Lepomis macrochirus*), reported the relatively high abundance of *Cetobacterium* genus and suggested the critical role of this genus in fish digestion (Larsen et al., 2014; Dulski et al., 2018). The sequencing results detected a prevalence of this genus across four diet treatments and, although the absence of any statistical difference, perch fed TM50 and TM75 tended to proliferate *Cetobacteria* relative to control and TM25 diets. The

replacement of 30% of FM by soybean meal was reported to significantly increased the abundance of *Cetobacterium* spp. in largemouth bass (He et al., 2020). These authors also suggested that the inclusion of plant ingredients in the diets of carnivorous fish could enhance the *Cetobacterium* genus's community, which is responsible for the production of cobalamin, fermented proteins and carbohydrates.

Although established at a low relative abundance (<0.2%), we found a significant reduction in the population of *Streptococcus* genus in TM75 compared to TM0 diets. These bacteria were found to be present at a low abundance in the digestive tract of *S. salar* (0.6% of the culturable bacterial community) (Ringø et al., 2000), *O. mykiss* (<0.01%) (Desai et al., 2012), and to be affected by dietary treatment (Merrifield et al., 2014). Dietary fatty acids were confirmed to alter growth of intestinal bacteria, and linoleic acid, in particular, was shown to inhibit the growth of *Lactobacillus* spp. in the intestine of Arctic charr (*Salvelinus alpinus*) (Ringø, 1993). Gram-positive bacteria species were sensitive to dietary fatty acids, and a decrease in *Streptococcus* and *Lactobacillus* communities in TM75 group could be attributed to a significantly higher linoleic acid content in this diet than in the control group (Tran et al., 2021).

#### 4.5. Environmental impacts

In the present study, we investigated the environmental consequence of dietary insect meal (*T. molitor*) in perch aquafeeds, concerning solid waste output, environmental impact associated with one kg of perch produced, and eFIFO, which has been considered as an important proxy for environmental sustainability of aquaculture sector (Amirkolaie, 2011; Bohnes et al., 2019; Kok et al., 2020).

The dietary defatted TM in the present study did generally not affect solid waste outputs associated with phosphorus waste, except for TM25, although increased nitrogen waste was observed, compared to the FM diet. Therefore, replacement of FM with defatted TM in perch diets could be an essential way of ensuring environmental benefit associated with the waste output, which has remained a critical concern for the public (Cho and Bureau, 2001). The digestibility of diet has been considered the most critical issue driving the waste output of aquaculture practices (Amirkolaie, 2011). As previously mentioned, the presence of chitin is the factor that impairs nutrient digestibility of perch the most. Removing chitin components from insect meals (Henry et al., 2015), and supplementing enzymes (Gasco et al., 2016) and probiotics containing chitinase-producing bacteria could be an effective way of improving digestibility of insect-containing diets for fed fish. Developing a suitable processing technique for aquafeeds could be considered for digestibility efficiency (Turchini et al., 2019), which has recently been achieved for extruding feeds containing insect meals (Irungu et al., 2018; Weththasinghe et al., 2021).

As far as the environmental impacts associated with 1 kg of farmed perch production is concerned, our simulated data indicated consistency of TM25, compared to the control diet, regarding global warming potential, acidification, and land use, but increased impacts pertaining to energy use, eutrophication, and water use. These environmental consequences were mostly influenced by the proportion of insect meal (*T. molitor*) vs. fish meal and FCR in/of experimental diets. The higher environmental impact associated with insect meal production than FM (Salomone et al., 2017; Smetana et al., 2019; Quang Tran et al., 2021) and higher FCR in TM diets and FM diet (Table 3) in our study could be responsible for the aforementioned findings. Le Féon et al. (2019) confirmed greater impacts of acidification, eutrophication, GWP, land use, and energy use of TM-containing than insect-free feed for one kg trout produced. Stejskal et al. (2020) documented a reduction of water use associated with insect meal (*H. illucens*)- compared to FM feeds for perch, whereas GWP, land use and energy use increased. However, Smárason et al. (2017) compared *H. illucens*- and FM-based feed associated with seven impact categories, and reported benefits of insect meal inclusion on abiotic depletion, acidification, eutrophication, GWP,

human toxicity potential, and marine aquatic ecotoxicity potential, but a negative impact on energy use.

The present study revealed that increasing inclusion levels of insect meal (*T. molitor*) in perch feeds significantly reduced eFIFO, indicating fewer marine forage fish required per unit fish produced. A substantial replacement of FM with defatted TM at 75% reduced eFIFO to as low as 1, whereby the production of perch is a net producer of fish that is aligned with the current trends of most aquaculture species (Kok et al., 2020). The observed reduction in eFIFO is consistent with data of Stejskal et al. (2020), reporting a significant decrease in the FIFO ratio when dietary *H. illucens* increased. Our eFIFO data could be important information for percid aquaculture in order to move towards an established aquaculture sector in Europe (Polcar et al., 2019).

#### 5. Conclusion

The present study highlighted the possibility of using defatted insect meal (*T. molitor*) in the diets of European perch (*P. fluviatilis*), an emerging, potential aquaculture candidate in Europe. It is recommended, for future aquaculture of this species, including as low as 6.8% or 25% FM replacement by defatted yellow mealworm, which could benefit the sector with respect to growth performance and environmental consequences. Although a substantial replacement of FM by defatted TM did not show promising outcomes for all the aspects considered in the present study, in particular concerning the waste output perspective, this replacement could reduce the total solid load, phosphorus waste, and economic fish-in fish-out in the aquaculture of European perch. Our study also underlined the major bottleneck of a substantial inclusion of defatted insect meal (*T. molitor*) in fish diets, as nitrogen waste and environmental consequences associated with one unit of farmed perch produced.

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#### Declaration of Competing Interest

The authors declare that they have no competing interests.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2021.737499>.

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## CHAPTER 5

### EUROPEAN PERCH (*Perca fluviatilis*) FED DIETARY INSECT MEAL (*Tenebrio molitor*): FROM A STABLE ISOTOPE PERSPECTIVE

Tran, H.Q., Kiljunen, M., Van Doan, H., Stejskal, V., 2021. European perch (*Perca fluviatilis*) fed dietary insect meal (*Tenebrio molitor*): From a stable isotope perspective. *Aquaculture* 545, 737265.

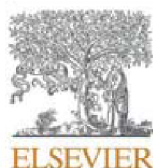
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## European perch (*Perca fluviatilis*) fed dietary insect meal (*Tenebrio molitor*): From a stable isotope perspective

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### ABSTRACT

Stable isotope analysis was conducted to investigate stable isotope ratios of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ), diet-tissue discrimination factors of carbon ( $\Delta^{13}\text{C}$ ) and nitrogen ( $\Delta^{15}\text{N}$ ). Bayesian mixing models were performed to assess relative contribution of insect meal and other ingredients to the development of tissues of European perch (*Perca fluviatilis*). Accordingly, four experimental formulations, characterized by the increasing inclusion levels of yellow mealworm (*Tenebrio molitor*) larvae meal (TM) at 0, 6.8, 13.5 and 20.3% as replacement for fishmeal at 0 (TM0), 25 (TM25), 50 (TM50) and 75% (TM75), respectively, were fed to juvenile perch (initial bodyweight,  $20.81 \pm 3.36$  g) in a recirculated aquaculture system for 105 days.

$\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of TM were  $-16.75$  and  $3.53\text{‰}$  and significantly distinguished from other terrestrial and marine feed components ( $P < 0.05$ ). Inclusion of dietary TM did not affect  $\Delta^{13}\text{C}$  value in blood and liver ( $P > 0.05$ ) but did reduce in muscle ( $P < 0.05$ ), whereas  $\Delta^{15}\text{N}$  was significantly increased with the increasing inclusion level of TM in all tissues ( $P < 0.05$ ). The growth of perch had a significant negative relationship with diet-muscle  $\Delta^{15}\text{N}$ . The contribution of TM to muscle ( $7.7 \pm 3.8\%$ ) was comparable to its dietary inclusion (6.8%) in TM25 but double in the blood ( $13 \pm 6\%$ ). TM appeared to be an essential ingredient incorporated into liver, as its contribution was consistent or higher than dietary inclusion (TM25:  $25.4 \pm 12.1$  vs. 6.8%; TM50:  $31.1 \pm 14.9$  vs. 13.5%; and TM75:  $29.4 \pm 14.4$  vs. 20.3%). The higher inclusion levels of TM (more than 6.8%) did not elevate its contribution to muscle, blood, and liver (probability,  $P_{\text{BIC}} < 0.95$ ) but significantly decreased that of fishmeal in all tissues ( $P_{\text{BIC}} > 0.95$ ). Soy-derived ingredients, soybean meal and soy protein, were an important ingredient in the development of all tissues regardless of dietary TM.

The present study provided insightful information on the role of various diet components in perch tissues, which could underlie further development of aquafeed formulations for emerging perch farming in Europe.

### 1. Introduction

European perch (*Perca fluviatilis*) has been identified as the promising candidate for intensive aquaculture with excellent nutritional value, especially beneficial fatty acids and increasing market demand (Stejskal et al., 2011; Toner, 2015; Stejskal et al., 2020b). Production of perch is mainly relied on re-circulating aquaculture systems and followed an upward trend, reaching approximately 700 t in, 2018 (FAO, 2020). Together with other percid fishes, perch farming moves towards an established aquaculture sector in Europe (Polcar et al., 2019). In perch farming practice, salmonid aquafeeds and commercial perch feeds are

commonly used (Bochert, 2020). These feeds become critical for the success of perch aquaculture with respect to profitability, meat quality traits and health status of farmed perch (Polcar et al., 2019). Aquafeeds for carnivorous fish, including *P. fluviatilis*, require high protein sources, fishmeal traditionally derived from marine fish resources (Langeland et al., 2016). However, limited supply, continuous rise in price, and unsustainability of this marine-derived ingredient (Naylor et al., 2000; Foley et al., 2011; Tacon et al., 2011; Froehlich et al., 2018; Kok et al., 2020) have challenged the expansion of ever-growing aquaculture sector. Consequently, a number of alternatives aquafeed materials have been investigated. Among them, insect meals and by-products from

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fishery and aquaculture represent the most promising candidates to meet aquafeed demand over the next decades (Hua et al., 2019; Gasco et al., 2020). Along with the rise in production of by-products which will share approximately 35% global fishmeal production by 2030 (FAO, 2018), the output of insect meal production is on the global rise and forecasted to be price-competitive with fishmeal by 2023 (Hua et al., 2019).

Many insect meals have been investigated as replacement for fishmeal in aquatic animal diets, in which the black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), and yellow mealworm (*Tenebrio molitor*) has drawn the most attention in the research (Hua et al., 2019; Fabrikov et al., 2020; Mastoraki et al., 2020). Gasco et al. (2019), in their review, reported that dietary insect meals significantly influenced growth performance, digestibility, and meat quality, especially the fatty acid profile of fed fish compared with insect-free diets. A meta-analysis indicated that a moderate inclusion level of insect meal was comparable with fishmeal diet in terms of growth performance, while effects of higher level on the growth rate of fed fish were insect-species-specific (Hua, 2021). Feeding European perch with 40% insect meal (*H. illucens*) inclusion levels did not affect growth performance, feed efficiency and hematological indices, but modified body fatty acid profile and hepatic somatic index (Stejskal et al., 2020). Those investigated indices mainly reflect integrated impacts of formulated diet, which consist of different ingredients, whereas the critical role of individual dietary components incorporated into fish and their tissues remained fragmentary (Yu et al., 2015; Cyrus et al., 2020). Understanding the importance of each ingredient in aquafeed formula to the development of particular tissues of fed organism could be informative for diet improvement, especially for new aquaculture species or raw materials. Stable isotope analysis could be a suitable way to address the importance of these individual ingredients.

Stable isotope techniques have become a valuable tool to investigate the diet proportion of various aquatic species in ecological studies (Post, 2002). Recently, the use of nitrogen and carbon stable isotope ratios ( $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ , respectively) and the Bayesian isotope mixing models (Parnell et al., 2013) has been employed in aquaculture nutrition research to explore further insights into the contribution of each ingredient in diet formula to the construction of particular tissues (Gamboa-Delgado and Le Vay, 2009; Enyidi et al., 2013; Gamboa-Delgado et al., 2013; Yu et al., 2015; Gamboa-Delgado et al., 2016; Cyrus et al., 2020; Nahon et al., 2020).

This study aimed to investigate the effects of *T. molitor* larvae meal as a replacement for fishmeal using stable isotopic values of different tissues by assessing diet-tissue isotopic discrimination factors, and modeling the contribution of a particular ingredient to the growth of three tissues, blood, liver and muscle, of juvenile perch.

## 2. Materials and methods

### 2.1. Ethics statement

The experimental procedures were conformed to the European Communities Directive (No. 2010/63/EU) and authorized by the Czech Ministry of Health (No. MSMT-6744/2018-2) regarding the protection of animals used for scientific purposes.

### 2.2. Experimental facilities and procedures

Experimental facilities and procedures of the present study were reported elsewhere (Tran et al., 2021). Briefly, four experimental diets, including a control diet (TM0), and three diets with TM replacement for fishmeal at 25, 50, and 75% (abbreviated diets as TM25, TM50, TM75). The main ingredients of experimental diets are presented in Table 1.

Each diet was fed to quadruplicate 180-L tank groups held juvenile European perch (bodyweight:  $20.81 \pm 3.36$  g) (82 fish/tank), connected in a recirculating system. Parameters included photoperiod (12 h:12 h L:

Table 1

Ingredients and proximate composition of fishmeal, *Tenebrio molitor* larvae meal, and experimental diets (Tran et al., 2021).

	Fishmeal	Experimental diets				
		TM	TM0	TM25	TM50	TM75
<b>Ingredients (g/kg)</b>						
Soybean concentrate			290	290	290	290
Fishmeal			271	203	135	68
<i>Tenebrio molitor</i>			–	68	135	203
Soybean meal			145	145	145	145
Corn flour			97	97	97	97
Fish oil			77	77	77	77
Rapeseed oil			58	58	58	58
Methionine <sup>a</sup>			8	8	8	8
Lysine <sup>b</sup>			5	5	5	5
Valine <sup>c</sup>			2	2	2	2
L-Threonine <sup>d</sup>			0.5	0.5	0.5	0.5
Vitamins & minerals <sup>e</sup>			8	8	8	8
Additives <sup>f</sup>			40	40	40	40
<b>Proximate composition (dry basis)</b>						
Dry matter (%)	96.5	95.0	94.8	95.7	95.6	95.6
Crude protein (%)	71.2	71.1	47.5	48.7	47.4	47.2
Crude lipid (%)	7.9	8.5	16.3	13.9	15.6	17.0
Ash (%)	14.0	7.1	8.9	9.0	8.3	7.6
Fibre (%)	1.24	2.8	2.0	2.0	2.2	2.3
Nitrogen-free extract (%) <sup>g</sup>	1.3	5.5	19.5	21.8	22.3	21.6
Gross energy (MJ/kg) <sup>h</sup>	20.1	21.1	21.0	20.8	21.2	21.5
Chitin <sup>i</sup>	–	4.8	–	0.33	0.65	0.97

<sup>a</sup> Adisseo, China.

<sup>b</sup> Inner Mongolia Eppen Biotech Co., Ltd.

<sup>c</sup> Ajinomoto Animal Nutrition Europe.

<sup>d</sup> Ningxia Eppen Biotech, China.

<sup>e</sup> Aminovitak Sak, Trouw Nutrition Biofaktory s.r.o, Czech Republic.

<sup>f</sup> Feed limestone (0.5%); Pentasodium triphosphate (Fosfa a.s, Czech Republic) (0.5%) and binder (NutriBind, Adisseo, China) (3.0%).

<sup>g</sup> Nitrogen-free extracts (NFE) = dry matter - (crude protein + crude lipid + ash + fibre).

<sup>h</sup> Gross energy (MJ/kg) as gross energy content of protein (23.6 MJ/kg), lipid (39.5 MJ/kg) and NFE (17.2 MJ/kg).

<sup>i</sup> Estimated from Basto et al. (2020) for defatted TM.

D), light intensity 58.6 lx, water temperature  $22.44 \pm 0.66$  °C, pH 7.00  $\pm 0.29$ , oxygen saturation  $80.41 \pm 8.02\%$ , ammonia-N  $0.28 \pm 0.16$  mg/L, and nitrite nitrogen  $<0.45$  mg/L were maintained throughout experimental period.

Fish were fed five times daily at 7.00, 9.00, 11.00, 13.00, and 15.00 with an excessive amount, using automatic feeders (EHEIM Twins, Deizisau, Germany). Fifteen minutes following each feeding, unconsumed feed was flushed from the funnel-like tank bottom and dried to determine feed intake. Fish mortality in each tank was recorded daily.

### 2.3. Sample collection and calculations

After 105 days, following 24 h starvation, fish from each tank were inspected for biometry, and thermal growth coefficient (TGC) was calculated with the formula:  $TGC = ((W_f^{1/3} - W_i^{1/3}) / (T \times t)) \times 1000$ , where  $W_f$  and  $W_i$  are final and initial weight (g), respectively, and T and t are water temperature (°C) and experimental duration (105 days), respectively.

Subsequently, three fish from each tank (12 fish/diet group) were randomly selected and sacrificed with an overdose (125 mg/L) Tricaine methanesulfonate (MS222, Sigma-Aldrich Chemicals, St Louis, MO, USA). Approximately 1 mL blood, and small piece of liver and dorsal muscle were sampled.

Experimental diets, ingredients, and perch's tissues were freeze-dried (Alpha 2-4 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and ground to a fine powder using an agate mortar and pestle. The samples were subsequently analyzed for

stable isotope ratios according to procedures described by Kiljunen et al. (2020). Briefly, approximately 0.5 mg sample was prepared in tin cups (D4057 Elemental Microanalysis, Okehampton, UK) and analyzed at the University of Jyväskylä (Jyväskylä, Finland) using a Thermo Finnigan DELTA<sup>plus</sup> Advantage mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA) connected to a FlashEA 1112 Elemental Analyzer. Northern pike (*Esox lucius*) tissue and birch leaves (*Betula pendula*) were used as internal standards. The results were presented as standard  $\delta$  notation ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ) as parts per thousand (‰) differences from the international standard. The percentage of carbon (%C), nitrogen content (%N), and C:N ratio (by weight) of samples were also derived from analysis.

Stable isotopes of carbon in animal tissue are  $^{13}\text{C}$  depleted with the presence of lipid content in the samples (Post et al., 2007). Therefore,  $\delta^{13}\text{C}$  values of fish tissues (except for muscle) were corrected according to Kiljunen et al. (2006) when the C:N ratio of specimens is greater than 3.5 (Skinner et al., 2016), and  $\delta^{13}\text{C}$  values of ingredients and diets were corrected as described previously (Post et al., 2007), diet to tissue discrimination factors ( $\Delta^{15}\text{N}$  and  $\Delta^{13}\text{C}$ ) were calculated as follows:  $\Delta X = \delta X_{\text{tissue}} - \delta X_{\text{diet}}$ , where  $X = \delta^{13}\text{C}$  or  $\delta^{15}\text{N}$ .

Bayesian mixing model framework (Parnell et al., 2013) in the *simmr* package of R environment (Parnell, 2016) was employed to estimate the contribution of feed ingredients to tissues of European perch. The mixing model employing *simmr* package has recently been used to estimate the proportional contribution of feed ingredients to rainbow trout fry tissue (Nahon et al., 2020). We performed Markov Chain Monte Carlo (MCMC) methods in the *simmr* package (Parnell et al., 2010) by running for 100,000 iterations, 10,000 burn-in rate, 100 thinning, and 4 chains. The model convergence was confirmed using Gelman–Rubin diagnostics (Gelman and Rubin, 1992). Due to the comparable isotopic ratios of soy protein and soybean meal (Table 2), we combined them as one source as previously recommended (Phillips et al., 2005). Other sources in the model consisted of fishmeal, TM and corn meal. The contribution of ingredients within diet groups and each ingredient across diet groups was compared, using the “compare\_groups” and “compare\_sources” function, respectively, in *simmr* package. The probability value ( $P_{\text{BIC}}$ ) from comparison functions is considered a significant difference with  $P_{\text{BIC}} > 0.95$  (Masson, 2011; Santana et al., 2020).

The mixing model assumes that the isotopic equilibrium of consumer tissue and its diets is reached (Gamboa-Delgado and Le Vay, 2009). Recent publications on aquatic species have reported that the muscle was in isotopic equilibrium with its feed ingredients within 10–90 days (Table 3). Blood and liver were reported to be faster than muscle in reflecting isotopic ratios of diet (Phillips and Eldridge, 2006). Therefore, our 105-days feeding trial of perch was long enough for feed ingredients to isotopically equilibrate with perch tissues.

The estimation of isotopically distinct feed components to the fish tissues using the isotopic mixing models requires corrected ingredient to tissue discrimination factors (Gamboa-Delgado and Le Vay, 2009; Parnell et al., 2010). The values for perch’s tissue ( $\Delta^{13}\text{C}$  (‰) and  $\Delta^{15}\text{N}$  (‰))

was calculated according to Caut et al. (2009):  $\Delta^{13}\text{C} = -0.248 \times \delta^{13}\text{C}_{\text{ingredient}} - 3.477$ ;  $\Delta^{15}\text{N} = -0.281 \times \delta^{15}\text{N}_{\text{ingredient}} + 5.879$  (for muscle);  $\Delta^{13}\text{C} = 0.77 \pm 0.30$  and  $\Delta^{15}\text{N} = 1.61 \pm 0.34$  (for liver), and  $\Delta^{13}\text{C} = 1.0 \pm 0.1$  and  $\Delta^{15}\text{N} = 1.3 \pm 0.2$  for blood according to Matley et al. (2016), reporting for leopard coral grouper (*Plectropomus leopardus*), the same Perciformes order as perch in present study. The ingredient to tissue discrimination factors used in the mixing model are presented in Table 4.

#### 2.4. Statistical analyses

Data were checked for normal distribution (Shapiro-Wilks’s test) and homogeneity of variances (Levene’s test). The correlation between explanatory variables (diet-muscle discrimination of nitrogen and carbon) and TGC of fish were tested using *lm* function. ANOVA was used to test the differences, followed by Tukey’s post-hoc test, when appropriate. All statistical analyses were performed using the R Statistic Package, R Development Core Team 2009–2021. Differences were regarded as significant at  $P < 0.05$ .

### 3. Results

The TGC of European perch exhibited a significant difference among diet treatments after a 105-day feeding trial. Fish fed TM0 and TM25 showed highest TGC ( $0.65 \pm 0.02$  and  $0.65 \pm 0.03$ , mean  $\pm$  standard deviation, respectively), which is significantly higher than that of TM50 ( $0.56 \pm 0.01$ ) and TM75 ( $0.41 \pm 0.01$ ) ( $P < 0.05$ ).

Isotope ratios of main feed ingredients used in experimental formulations were significantly different ( $P < 0.05$ ), except soy protein and soy meal (Table 2). Carbon isotope  $\delta^{13}\text{C}$  did not differ among experimental diets ( $P > 0.05$ ), while increasing replacement levels of fishmeal by TM significantly reduce  $\delta^{15}\text{N}$  value ( $P < 0.05$ ) (Fig. 1).

Fishmeal replacement by TM significantly reduced  $\delta^{15}\text{N}$  values ( $P < 0.05$ ) in all tissues but increased  $\delta^{13}\text{C}$  values in muscle and blood ( $P < 0.05$ ).  $\delta^{13}\text{C}$  in liver remained insect-dose independent ( $P > 0.05$ ) (Fig. 1). Muscle was more enriched in  $^{15}\text{N}$  than in blood and liver. The enrichment of  $^{13}\text{C}$  was more pronounced in liver than blood and fillet (Fig. 1).

Diet-tissue discrimination factor,  $\Delta^{13}\text{C}$  in blood (0.54–0.65‰) and liver (2.65–3.35‰) of European perch were not significantly affected by dietary treatments ( $P > 0.05$ ). However,  $\Delta^{13}\text{C}$  in muscle was significantly lower in TM-containing diets than TM0 ( $P < 0.05$ ). European perch fed with each of the four experimental diets showed higher diet-tissue discriminations in liver than other tissues ( $P < 0.05$ ). Dietary TM significantly increased discrimination of  $\Delta^{15}\text{N}$  in all tissues of European perch ( $P < 0.05$ ). Muscle of perch exhibited highest  $\Delta^{15}\text{N}$  among other tissues ( $P < 0.05$ ) (Fig. 2).

The correlation test indicated that there was a significant linear relationship between diet-muscle  $\Delta^{15}\text{N}$  and TGC of perch ( $\text{TGC} = -0.03 \Delta^{15}\text{N} + 0.57$ ,  $P < 0.0001$ , adjusted R-squared = 0.83, *F*-statistic: 74.05), while there was no significant correlation between TGC and  $\Delta^{13}\text{C}$  in muscle ( $P = 0.16$ , adjusted R-squared = 0.07, *F*-statistic: 2.15).

After correcting for ingredient-tissue discrimination factor, the isotopic values of three tissues fell within mixing polygons mapped out by four main ingredients (Fig. 3), providing a sound basis for mixing models, and all important feedstuff were well incorporated in the model. Muscle values tended to fall closely to soy ingredients, while those of liver did to the center of the mixing polygons.

There was considerable variability in diet contribution estimates of the *simmr* model outputs among tissues and across diet treatments (Tables S1, Fig. 4). Regarding muscle development, soy ingredients were predicted to contribute most (63.8–80.3%, mean values) over other feed ingredients in all tissues ( $P_{\text{BIC}} > 0.95$ ) (Fig. 4, Table S1). The contribution of soy was also notably higher than its proportion (43.5%) in experimental diets (Table 1). As expected, increasing replacement of fishmeal with TM significantly reduced the contribution of fishmeal

**Table 2**  
Stable isotope ratios, carbon (C) and nitrogen (N) concentration of main ingredients used in the experimental diets.

	Soy protein	Fishmeal	TM	Soy meal	Corn meal	SEM
$\delta^{13}\text{C}$ (‰)	-25.10 <sup>d</sup>	-20.07 <sup>c</sup>	-16.75 <sup>b</sup>	-25.51 <sup>d</sup>	-12.44 <sup>a</sup>	1.33
$\delta^{15}\text{N}$ (‰)	1.48 <sup>d</sup>	10.57 <sup>a</sup>	3.53 <sup>b</sup>	1.96 <sup>d</sup>	2.85 <sup>c</sup>	0.89
C (%)	45.28 <sup>a</sup>	47.17 <sup>a</sup>	47.54 <sup>a</sup>	41.60 <sup>b</sup>	45.21 <sup>a</sup>	0.06
N (%)	10.58 <sup>c</sup>	12.27 <sup>a</sup>	11.49 <sup>b</sup>	6.76 <sup>d</sup>	1.12 <sup>c</sup>	1.10
C:N ratio	4.28 <sup>b</sup>	3.85 <sup>b</sup>	4.14 <sup>b</sup>	6.16 <sup>b</sup>	40.96 <sup>a</sup>	3.94

Different superscripts indicate significant differences for specific rows. SEM, standard error of mean.

**Table 3**  
Time (days) for feed ingredients reach isotopic equilibrium in muscle of aquatic animals in the literature.

Ingredients	Species	Life stage	Isotopic equilibrium (days)	Reference
Fish meal	Shrimp ( <i>Litopenaeus vannamei</i> )	Postlarvae	22	Martínez-Rocha et al., 2013
Pea meal				
Fish meal	Shrimp ( <i>L. vannamei</i> )	Postlarvae	30	Gamba-Delgado et al., 2014
Poultry by-products				
Fish meal	Shrimp ( <i>L. vannamei</i> )	Postlarvae, juvenile	15	Gamba-Delgado and Le Vay, 2009
Soy concentrate				
Fish meal	Catfish ( <i>Ictalurus punctatus</i> )	Juvenile	30	García-Pérez et al., 2016
Poultry by-products				
Fishmeal	Catfish ( <i>I. gariepinus</i> )	Larvae	30	Enyidi et al., 2013
Corn meal				
Fishmeal	Catfish ( <i>I. gariepinus</i> )	Larvae	28	Enyidi, 2012
Soybean meal				
Fish meal	Cobia ( <i>Rachycentron canadum</i> )	Juvenile	24	Zhou et al., 2016
Soybean meal				
Beer yeast meal				
Corn gluten meal				
Fish meal	Rainbow trout ( <i>Oncorhynchus mykiss</i> )	Fingerling	90	Beltrán et al., 2009
Fish meal	Rainbow trout ( <i>O. mykiss</i> )	Fry	36	Nahon et al., 2020
Corn gluten meal				
Rotifers	Red drum ( <i>Sciaenops ocellatus</i> )	Larvae	10	Herzka and Holt, 2000
Krill	Sockeye salmon ( <i>O. nerka</i> )	Age 1* (9.5–15.3 g)	40	Sakano et al., 2005
Fish meal	Tilapia ( <i>Oreochromis niloticus</i> )	Fry	56	Zhou and Gu, 2020
Soybean meal				

**Table 4**  
Discrimination factor (‰) of feed ingredients and tissues of perch used in the Bayesian mixing model.

Ingredient	Muscle		Liver		Blood	
	$\Delta^{15}\text{N}$	$\Delta^{13}\text{C}$	$\Delta^{15}\text{N}$	$\Delta^{13}\text{C}$	$\Delta^{15}\text{N}$	$\Delta^{13}\text{C}$
Fishmeal	2.91 ± 0.02	1.50 ± 0.01	1.61 ± 0.34	0.77 ± 0.30	1.3 ± 0.2	1.0 ± 0.1
Soy	5.40 ± 0.02	2.80 ± 0.03				
Corn	5.08 ± 0.06	-0.39 ± 0.02				
<i>T. molitor</i>	4.89 ± 0.03	0.68 ± 0.02				

( $P_{BIC} > 0.95$ ) but did not elevate that of the latter ( $P_{BIC} < 0.95$ ) (Table S1). The estimated contribution of these animal-derived ingredients in muscle was considerably lower than their dietary inclusion levels. Except for TM0, corn meal appeared to be a less important ingredient in perch muscle as its contribution remained low (5.7–6.8%) regardless of dietary TM ( $P_{BIC} < 0.95$ ) (Fig. 4, Table S1).

The development of liver of perch fed TM0 received the most significant contribution from corn meal (66.4 ± 3.5%) and significantly higher than fishmeal (30.5 ± 3.6%) and soy (3.1 ± 1.4%) ( $P_{BIC} > 0.95$ ) (Fig. 4, Table S1). TM made up the second-largest proportion to perch liver (25.4–31.1%), following soy (32.5–38.5%), and there is no significant difference in the contribution of these ingredients across TM-containing diets ( $P_{BIC} < 0.95$ ). In similarity to muscle tissue, increasing replacement fishmeal by TM accompanied statistical reduction in proportional contribution of the former to perch liver ( $P_{BIC} > 0.95$ ), yet nonstatistical evidence for the latter ( $P_{BIC} < 0.95$ ). The share of corn meal to the perch liver's growth (16.6–18.7%) was comparable with fishmeal across TM-containing diets.

Fishmeal, in the absence of dietary insect meal, was assimilated in the blood of perch (45.5 ± 4.2%) significantly more than that of soy (30.7 ± 1.8%) and corn meal (23.9 ± 5.2%) ( $P_{BIC} > 0.95$ ). In the TM-containing diets treatments, soy had an immense contribution ( $P_{BIC} > 0.95$ ), followed by fishmeal. TM, together with corn meal, remained a minor contributor to blood composition of perch fed with TM0, TM25 and TM50, but while fed with TM75, TM and fishmeal displayed an equal contribution ( $P_{BIC} < 0.95$ ).

#### 4. Discussion

This was the first study investigating isotopic signatures and proportional contribution of feed components to tissues of fish fed experimental formulations where fishmeal was partially substituted by insect meal. The study provided insightful findings on the importance of particular ingredients to the construction of perch tissues, a result underlies the effects of diet treatments on the production performance and nutrient assimilation in farmed perch. The outputs could offer an additional protein source choice for the future growing percid aquaculture sector (Polcar et al., 2019; Stejskal et al., 2020; Tran et al., 2021). Our findings highlighted that there was a negative correlation between diet-muscle  $\Delta^{15}\text{N}$  and fish growth performance. Insect meal (*T. molitor*) seemed not favorable for perch muscle and blood as its contribution to growth of these tissues was disproportional with increasing inclusion levels. In contrast, the share of TM in the liver remained significant in perch fed TM-containing diets. The present study also proposed helpful information for the ecological study of perch. Accordingly, a non-lethal sample of blood could be useful tissue for investigating food sources and, to a lesser extent, trophic position.

For isotope modeling purposes, dietary sources should obtain distinct isotope values. TM exhibited distinguishable isotope ratios compared to fishmeal and plant ingredients. In farming practice, *T. molitor* is the primary consumer of various plant substrates (Cortes Ortiz et al., 2016) and therefore more enriched in  $^{15}\text{N}$  and  $^{13}\text{C}$  from those diets (DeNiro and Epstein, 1978, 1981). This could explain a significantly higher nitrogenous isotopic signature of TM than soy and corn meal, which are classified as primary producers. On the other hand, fishmeal derived from marine catch enriches a substantial  $^{15}\text{N}$  from marine food sources (Kusche et al., 2018).

Isotopic values of tested tissues strongly reflected those of respective diets, especially for  $\delta^{15}\text{N}$ , while  $\delta^{13}\text{C}$  was slightly modified across tissues from respective diets. Diet-tissue  $\Delta^{15}\text{N}$  for liver (ranged, 1.8–2.84‰) and muscle (2.97–4.63‰) in the present study was similar to an empirical study on *Totoaba macdonaldi* fed compound feeds under controlled conditions (2.8–4.9‰ and 1.2–4.4‰, respectively) (Zapata et al., 2016). Vollaire et al. (2007) reported that  $\Delta^{15}\text{N}$  of 2.88‰ was observed in muscle of perch fed commercial feed, which is in our reported range, but that in liver (0.65‰) was slightly lower than the 1.80–2.84‰ observed in the present study. Regarding diet-tissue  $\Delta^{13}\text{C}$  in muscle and liver, our results, ranged 0.47–0.74‰ and 2.65–3.35‰,

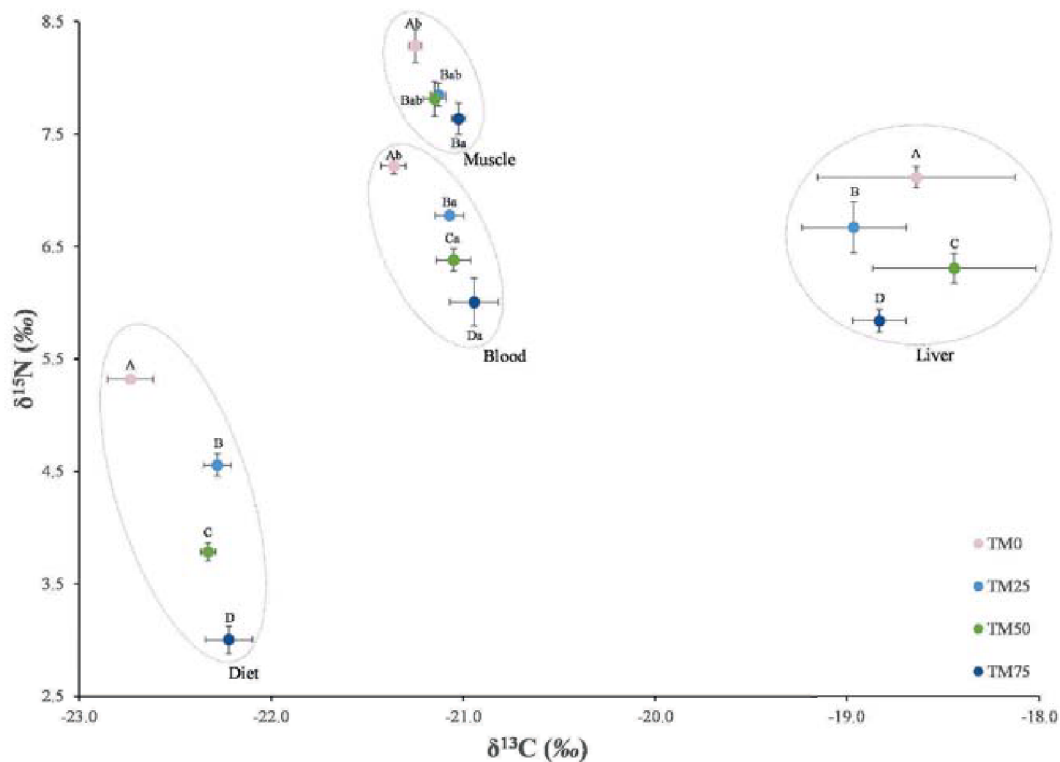


Fig. 1. Isotopic signatures ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) of fillet, liver, and blood of European perch fed four experimental diets. Data were present as mean  $\pm$  SD. Different lowercases and uppercases within the tissue group indicate significant differences in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, respectively.

respectively, indicated relatively lower than published data (4.02‰ and 3.44‰, respectively) (Vollaire et al., 2007). The discrepancies could be due to several factors, including food quality (e.g., protein quality, dietary isotopic values, C/N), physiological status of tested animals, and diet kinetic effects (Vollaire et al., 2007; Kadye et al., 2020; Zhou and Gu, 2020).

The present study also investigated diet-blood discrimination values for both isotopic signatures and found in the range of 1.90–3.00‰ for  $\Delta^{15}\text{N}$  and 0.54–0.65‰ for  $\Delta^{13}\text{C}$ , which is in consent with Chérel et al. (2005) for captive penguins fed fish, and with the published review (Caut et al., 2009) for mammals. Caut et al. (2009) indicated negative relationships between diet-tissue discrimination and respective dietary isotopic values. This was particularly found in our study where the highest  $\delta^{15}\text{N}$  value in narrow discrimination of  $\Delta^{15}\text{N}$  in somatic tissues, a similar phenomenon was observed for  $\delta^{13}\text{C}$  isotopic values in tissues of perch fed TM25. While fish fed low dietary isotopes led to enlarge discrimination values. Moreover, the protein quality of diets strongly influenced nitrogen stable isotope incorporation, thereby  $\Delta^{15}\text{N}$  discrimination (Mohan et al., 2016; Kadye et al., 2020). In the present study, the high nitrogen diet-tissue isotopic differences in all somatic tissues associated with increasing dietary TM could be due to the presence of chitin, which was reported to impair protein digestibility in insect-containing diets (Gasco et al., 2016). This evidence could also explain the negative relationship between growth performance as indicated by TGC of perch and nitrogenous diet-muscle discrimination, which is in agreement with earlier publications (Trueman et al., 2005; Beltrán et al., 2009; Lefebvre and Dubois, 2016).

In comparison with blood and liver in perch fed the same diet, muscle was found to be the most fractionated in diet-tissue nitrogen, which concurs with previous studies (Suzuki et al., 2005; Malpica-CruzLuis et al., 2012; Xia et al., 2013; Mohan et al., 2016; Zapata et al., 2016). The discrepancy in  $\Delta^{15}\text{N}$  among tissues could be ascribed to a

higher accumulation of heavier isotope in muscle than other tissues (Gamboa-Delgado et al., 2020), more essential amino acids contained in the latter than in the former tissues (Mohan et al., 2016), and typical traits of muscle relative to other tissues in fish (Pinnegar and Polunin, 1999).

The higher diet-tissue  $\Delta^{13}\text{C}$  in liver than other tissues regardless of dietary TM observed in our study was in agreement with previously published work (Pinnegar and Polunin, 1999). DeNiro and Epstein (1978) suggested that the magnitude of carbon discrimination primarily relied on tissue's biochemical fractions (lipid, protein and carbohydrate). Liver of perch contained a greater proportion of lipid and glycogen than other tissues (Vollaire et al., 2007), which could explain the high departure of  $\delta^{13}\text{C}$  in perch liver from respective diets in the present study. Our mixing model indicated that liver of perch received a high proportional contribution from TM and corn meal, which are distinguished from FM and soy ingredients, the main contributors to blood and muscle. In addition, we applied carbon isotopic correction (Kiljunen et al., 2006; Skinner et al., 2016) for liver tissue, as lipid synthesis in this tissue depleted the heavy carbon isotope at the expense of the lighter one (DeNiro and Epstein, 1978), this consequently leads to the convergence of  $\delta^{13}\text{C}$  in liver, and thereby of  $\Delta^{13}\text{C}$ . Our study, therefore, implicated that blood and muscle, but not liver, are suitable tissues for evaluating the diet source since it was weekly  $^{13}\text{C}$  enriched. Whereas muscle was particularly enriched in  $^{15}\text{N}$ , rather than liver and blood, thereby this tissue will be useful for determining the trophic levels of perch (Caut et al., 2009). The present study also found that feeding insect-containing diets resulted in significantly lower diet-liver  $\Delta^{13}\text{C}$  than insect-free diet did, which could be linked to diet quality, thereby fish catabolized fatty acid storage in the  $^{13}\text{C}$ -depleted form (Nahon et al., 2020).

The present study provided insights into the incorporation of different ingredients into fish tissues with the presence or absence of

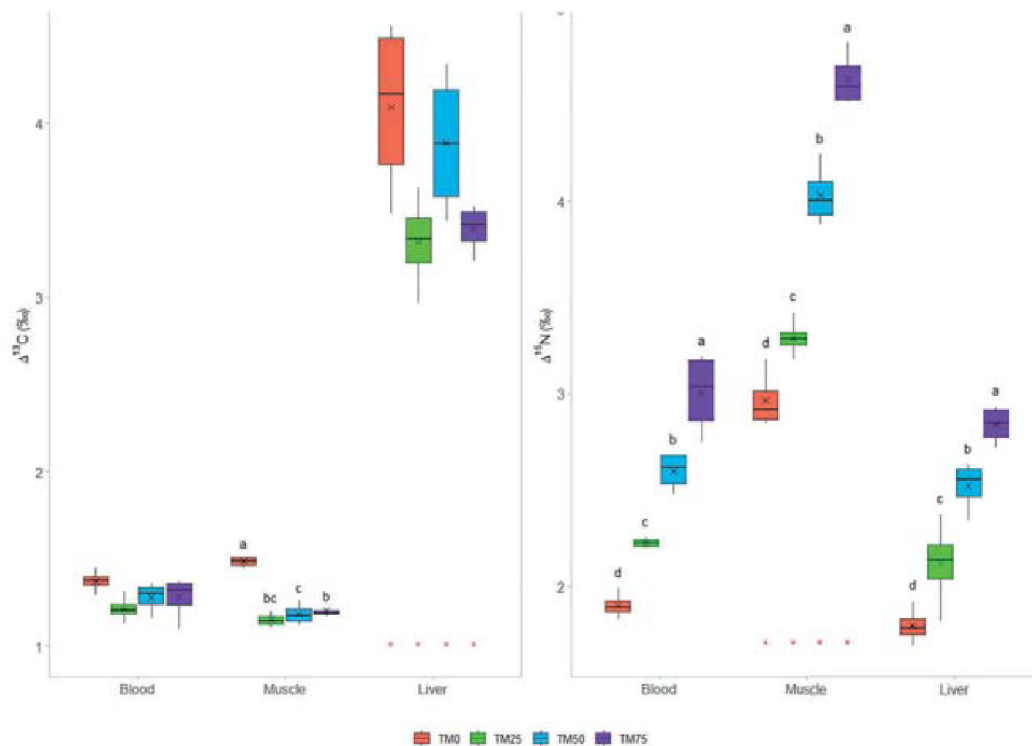


Fig. 2. Discrimination factors,  $\Delta^{13}\text{C}$  (left) and  $\Delta^{15}\text{N}$  (right) of European perch's tissues and experimental diets. The black "x" represents mean value. The horizontal line inside each boxplot represents the median separating the interquartile range. Different lower cases within tissue group indicate significant difference across diet treatments ( $P < 0.05$ ). The red asterisks within diet group indicate significant difference ( $P < 0.05$ ) compared to the other tissues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

insect meal (*T. molitor*). The *simmr* package, a stable isotope mixing model within a Bayesian framework, has been used in the ecological study and aquaculture nutrition to estimate the proportional contribution of ingredients to fish tissue (Nahon et al., 2020). Outputs of the mixing model are highly sensitive and require precise values of ingredient-tissue discrimination factor (Phillips and Gregg, 2001). In the present study, ingredient-muscle discrimination factors were corrected according to the decision diagram described by Caut et al. (2009). A similar approach was reported earlier while estimate ingredient contribution to tissue of cobia (*R. canadum*) (Zhou et al., 2016). A previous study (Nahon et al., 2020) recommended using different carbon and nitrogen discrimination factors in muscle for animal-/plant-derived ingredients. The distinction of our discrimination factors in muscle for animal and plant feedstuff, therefore, fits that assumption. All stable isotope values of perch tissues laid within the mixing polygons, indicating the sound reliability of *simmr* Bayesian mixing model. Overall, the model output showed that soy components, including soy protein and soybean meal, significantly contributed to all tissues, regardless of dietary TM. Indeed, these ingredients were included at a significant proportion in all experimental diets (43.5%). The previous study evidenced that ingredients with a high dietary proportion commonly accompanied a high contribution to sea cucumber due to opportunistic ingestion (Yu et al., 2015). On the other hand, high protein intake was documented to increase protein synthesis in vertebrates (Tsahar et al., 2008).

Perch fed 6.8% TM inclusion in diet received a matching contribution to muscle ( $7.7 \pm 3.8\%$ ), but the utilization of TM in this tissue did not proportionally increase with its higher inclusion in TM50 and TM75 diets. A similar pattern regarding disproportion between dietary inclusion and predicted contribution of TM in blood and liver was observed. This phenomenon could be attributed to the presence of polysaccharides

fraction, namely chitin (Table 1) and the limited ability of perch to degrade this non-protein nitrogen component (Langeland et al., 2016). The earlier study (Yu et al., 2015) has indicated that higher cellulose, one of polysaccharides fraction, and low cellulase activities in the digestive tract impaired seaweed (*Sargassum thunbergia*) utilization by sea cucumber. The unchanged contribution of TM to muscle, despite increased dietary inclusion, could also be linked to amino acid deficiency (Gamboa-Delgado and Le Vay, 2009). The amino acid profile of insect meal of *T. molitor* was found to be imbalanced, as indicated by the essential to non-essential amino acid ratio, being less than 1 (Nogales-Mérida et al., 2019).

Muscle has been confirmed to reflect the majority of isotopic compositions in whole-body fish (Zhou et al., 2016). Therefore, the growth of perch fed dietary groups could be closely linked to the proportional contribution of individual ingredients to muscle. Perch fed TM25, consisting of 45.3% soy ingredients and 6.8% TM, equivalent to proportional contribution to muscle of  $79.9 \pm 1.5\%$  and  $7.7 \pm 3.8\%$ , respectively, supported growth of perch relative to TM0. It appears to be apparent that the nutritional complement among ingredients in TM25 yielded comparable nutritional compositions, e.g., fatty acid profile (Tran et al., 2021), with TM0, thereby supporting the performance of perch. The higher administration of TM accompanying lower fishmeal contribution to muscle, resulted in growth delay of perch compared with TM-free diet. Soy protein has been known to be methionine and lysine deficient (Li et al., 2015), which can affect protein synthesis in fish muscle, thereby depressing fish growth (Abimorad et al., 2014). This suggests that soy, TM and corn meal could not cover the nutritional requirements of perch at the lower fishmeal availability.

Liver of perch tended to assimilate a large amount of corn meal ( $66.4 \pm 3.5\%$ ) in the absence of TM, despite low dietary inclusion level (9.7%) and was significantly higher than that of fishmeal and soy. Lipid and

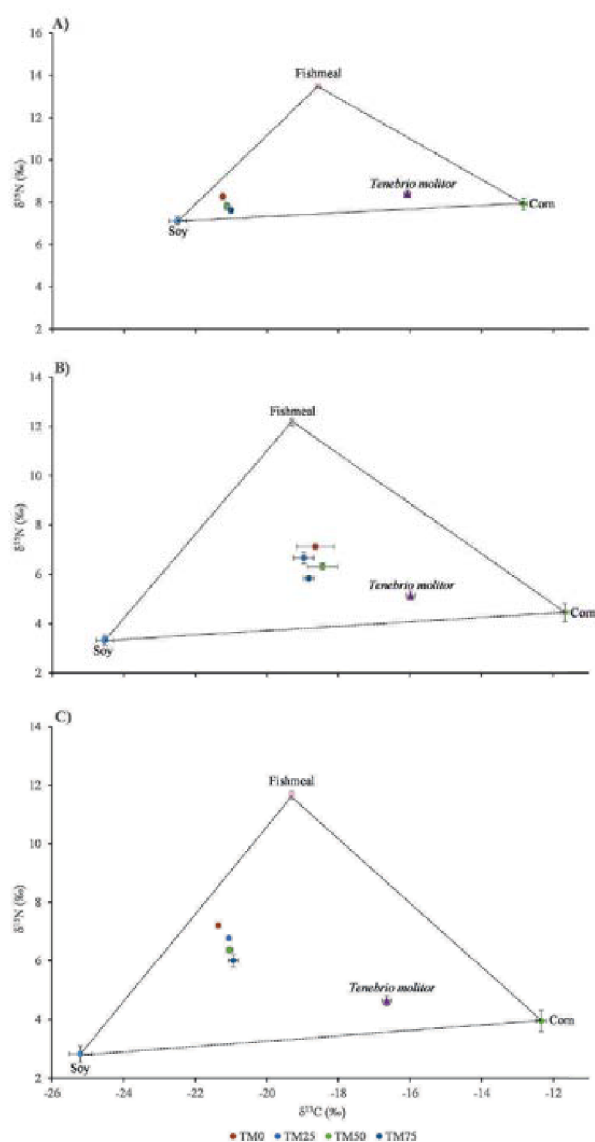


Fig. 3. Isospace plots of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  signatures of four feed ingredients and tissues (muscle (A), liver (B) and blood (C)) of European perch fed for experimental diets.

glycogen were found to be more abundant in liver than other tissues in perch (Vollaire et al., 2007), which are resulted from glucose production (gluconeogenesis) and lipid synthesis (lipogenesis), with amino acids are the primary source of carbon (Ballantyne, 2001). Earlier studies have observed the important role of serine, glycine and alanine, leucine, and valine in these synthesis processes (French et al., 1981; Henderson and Sargent, 1981; Li et al., 2009). Although corn meal provided a comparable carbon quantity with fishmeal, the former contained a relatively higher content of the abovementioned essential amino acids than the latter ingredient (Al-Gaby, 1998; Allan et al., 2000; Herath et al., 2016; Moreno-Arias et al., 2018). Our result may elucidate the higher incorporation of corn meal than fishmeal in perch liver. TM represented a significant contribution to the composition of perch liver and higher compared to its dietary inclusion. This suggests the important role of TM in liver function, which may attribute to its carbon source and some amino acids necessary for the synthesis of biochemical fractions. In

addition, fatty acids, especially oleic and linoleic, which are presented at approximately 60% total fatty acids in defatted TM observed in the present study (Tran et al., 2021), have been reported to be easily incorporated or act as an essential precursor for desaturation and elongation of fatty acid products (Xu et al., 2001; Xu and Kestemont, 2002). The disproportion between higher inclusion levels of TM and its contribution to liver could be explained by the deficiency of highly polyunsaturated fatty acids such as DHA and EPA, found in defatted TM (Nogales-Mérida et al., 2019; Tran et al., 2021). The high dietary insect meal was evidenced to induce lipid peroxidation and hepatic damage (Li et al., 2017).

Unlikely muscle and liver extracted from the lethal specimens. Blood has recently gained attention as a non-lethal sampling approach for bulk stable isotope analyses. The transport of amino acids derived from dietary protein plays an essential function in blood of fish (Barst et al., 2021) and protein was found at a great proportion in the blood of perch (Velíšek et al., 2009; Tran et al., 2021). Therefore, protein content, particularly amino acids, of individual ingredients underlie the relative contribution to blood composition. The present study indicated that fishmeal, at the absence of insect meal, made up  $45.5 \pm 4.2\%$  share, followed by soy and cornmeal to the blood composition of perch. The superior amino acid profile of fishmeal over soy could demonstrate this phenomenon (Nogales-Mérida et al., 2019). However, soy ingredients surpassed fishmeal in terms of relative contribution to blood of perch, at the presence of insect meal. It is speculated that the combination of insect meal and soy ingredient at an appropriate ratio could stimulate the utilization of the latter in blood circulation. The encouragement of soy products in the blood of perch could be linked to the availability of specific amino acids. Leucine and phenylalanine amino acids are known to boost the assimilation and synthesis of protein in animal tissues (Gamboa-Delgado et al., 2020), which was found to be abundant in soy-derived ingredients (Deng et al., 2006) and insect meal (*T. molitor*) (Nogales-Mérida et al., 2019). The limited essential amino acids of TM, especially lysine and methionine, could be the reason that impairs the higher incorporation of TM into blood of perch.

## 5. Conclusion

The present study indicated that yellow mealworm (*Tenebrio molitor*) larvae meal had distinct isotopic signatures from fish meal,  $\text{C}_3$  (soy-derived ingredients) and  $\text{C}_4$  plant ingredients (corn meal), which can be employed in further studies using isotopic mixing models. For aquaculture and ecological studies, diet-tissue discrimination of nitrogen could be a valuable proxy to evaluate the protein quality of aquafeeds, trophic level investigation and the performance of fed organisms whereby muscle is a preferable tissue. In contrast, blood and muscle should be considered important tissues for exploring diet sources for European perch.

An inclusion level of 6.8% or 25% fishmeal replacement with insect meal (*T. molitor*) is recommended in diets for perch to ensure production performance and liver health. The further inclusion did not encourage its relative contribution to the development of three tissues (muscle, liver and blood), thereby impairing growth production. TM appeared to be less critical to the construction of muscle and blood as its dietary inclusion was disproportionately correlated with the relative contribution to these tissues. The role of TM was significant in liver by providing carbon source and important fatty acid, such as oleic and linoleic acids, but substantial inclusion of TM may induce liver damage for perch. The present study's findings revealed that the presence of non-nitrogen protein, chitin were critical factors affecting nutritional assimilation of insect meal in the growth of perch tissue and that the nutritionally complement among feed ingredients could be an important consideration for future feed formulation for perch farming. Our study also hinted at further studies to combine potential insect ingredients, or to supplement insect-containing diets with chitinase-produced bacteria to enhance the benefits of insect meals in aquafeeds.

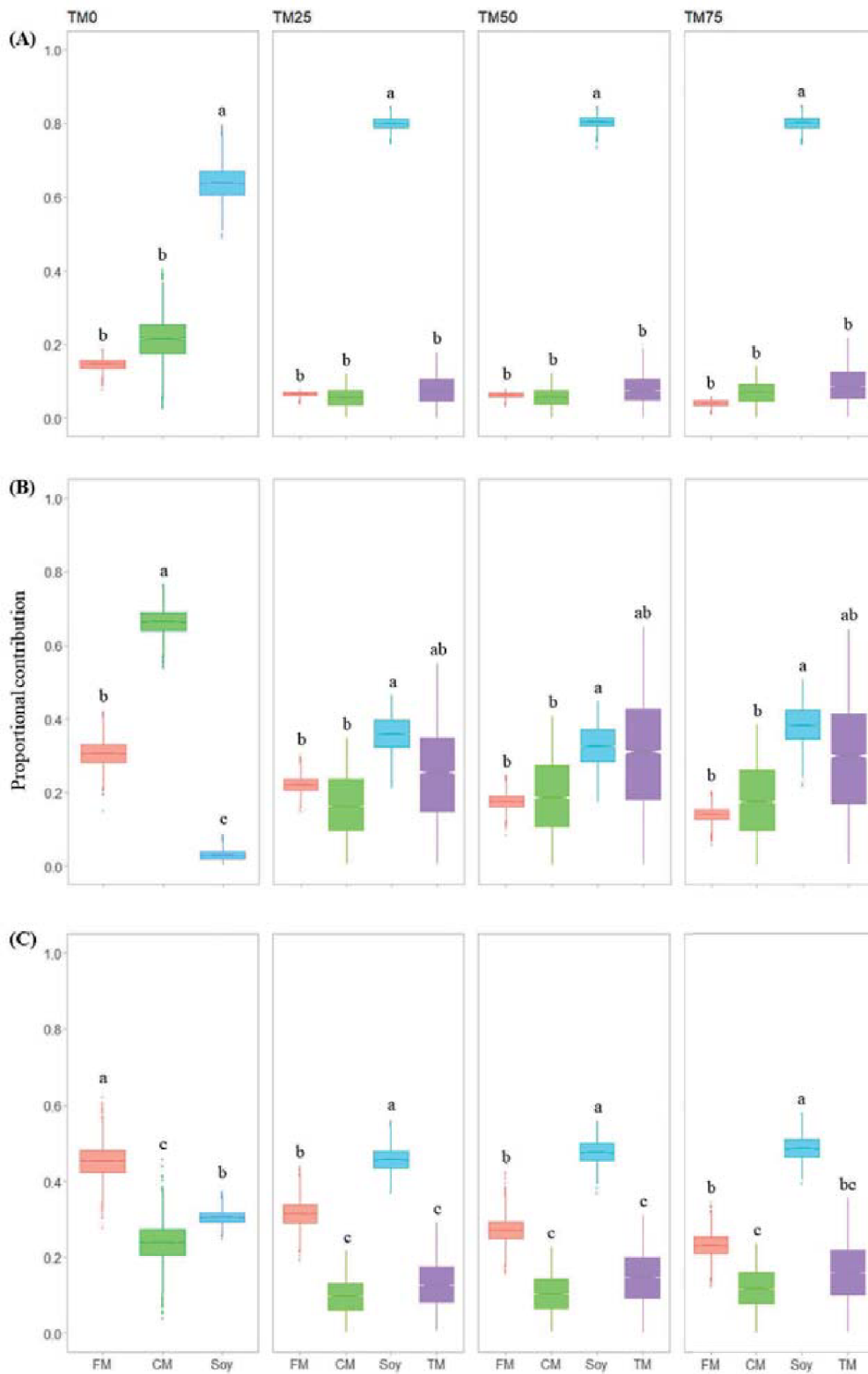


Fig. 4. Boxplots from Bayesian isotopic mixing models representing proportional contribution (mean, interquartile range) of individual feed ingredient to muscle (A), liver (B) and blood (C) tissues of European perch fed experimental diets.



### Authorship statement

The manuscript, entitled “European perch (*Perca fluviatilis*) fed dietary insect meal (*Tenebrio molitor*): from a stable isotope perspective”. All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Journal of Aquaculture.

### Authorship contributions

Vlastimil Stejskal: Conception and the revision of the manuscript.  
Hung Quang Tran: Contribution to design, conduct experiment, analyse of the data, and manuscript preparation.  
Hien Van Doan: Contribution to manuscript preparation.  
Mikko Kiljunen: Contribution to analysis and manuscript preparation.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2021.737265>.

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European perch (*Perca fluviatilis*) fed dietary insect meal (*Tenebrio molitor*):  
From a stable isotope perspective

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## CHAPTER 6

### **HOW DOES PIKEPERCH *Sander lucioperca* RESPOND TO DIETARY INSECT MEAL *Hermetia illucens*? INVESTIGATION ON GUT MICROBIOTA, HISTOMORPHOLOGY, AND ANTIOXIDANT BIOMARKERS**

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# How Does Pikeperch *Sander lucioperca* Respond to Dietary Insect Meal *Hermetia illucens*? Investigation on Gut Microbiota, Histomorphology, and Antioxidant Biomarkers

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Effects of feeding dietary defatted black soldier fly (*Hermetia illucens*) larvae meal (HI) on intestine microbiota, and on histomorphology, oxidative enzyme activities in liver and intestine of pikeperch (*Sander lucioperca*) were investigated. Four isoproteic (45% crude protein) and isolipidic (18% ether extract) diets were formulated to include 0% (CO), 9% (HI9), 18% (HI18) and 36% (HI36) of HI as replacement for fishmeal at 0, 25, 50, and 100%, respectively, and were fed to triplicate groups of juvenile pikeperch (initial body weight, 68.7 ± 7.1 g) for 84 days. No adverse effects were detected on the intestine of pikeperch fed diet groups, in terms of histomorphology ( $P > 0.05$ ), while fish fed free or low levels of HI ( $\leq 9\%$  in diet) showed significant liver degeneration ( $P < 0.05$ ). Dietary HI significantly affected the oxidative enzyme activities of catalase and glutathione peroxidase in the liver, and glutathione S-transferase in the intestine ( $P < 0.05$ ), while activity of superoxide dismutase in both liver and intestine was HI-dose independent ( $P > 0.05$ ). Feeding HI-containing diets positively modulated the richness and diversity of intestinal microbiota, especially for HI18 group ( $P < 0.05$ ). Inclusion HI up to 18% (50% fishmeal replacement) in pikeperch diets increased abundance of *Clostridium*, *Oceanobacillus*, *Bacteroides*, and *Faecalibacterium* genera, whereas the predominant bacterium, *Cetobacterium* was found in control and HI36 groups. This study reveals the potential of HI as an immune and health booster for juvenile pikeperch.

**Keywords:** pikeperch, alternative ingredient, *Hermetia illucens*, microbiota, histomorphology, antioxidant

## INTRODUCTION

Aquaculture is the largest global consumer of fishmeal production, accounting for 68–73% (Shepherd and Jackson, 2013; Tacon and Metian, 2015). Fishmeal is mainly derived from marine capture fisheries (70% in 2018) (FAO, 2020a), which has reached a plateau since the 2000s (Shepherd and Jackson, 2013) and has been projected that the ecological limits of stock will be

reached by 2037 (Froehlich et al., 2018a). Therefore, the current fastest growth of aquaculture in food-producing sectors (FAO, 2020a) and the continuous increasing trend, requires the development of novel aquafeed ingredients. Terrestrial crops have been used in aquafeeds more than other alternatives until recent (Tacon et al., 2011; Tacon and Metian, 2015) and, by 2050, the use of these feedstuffs in aquaculture will rise to twice the current level in a business-as-usual scenario, reaching 91 million tonnes (Froehlich et al., 2018b). However, crop-based feeds for aquatic animals introduce concerns regarding their nutritional properties and environmental consequences. An unbalanced essential amino acid profile, low palatability, and the presence of anti-nutritional substances could impair their inclusion in aquafeeds (Gatlin et al., 2007). Moreover, the expansion and intensification of the production of terrestrial crops will lead to tremendous environmental burdens pertaining to climate change, biodiversity loss, and increasing demand for arable land and water. Among such burdens, land use is considered the one that entails the greatest pressures on the planet (Foley et al., 2005, 2011; Boissy et al., 2011). Beyond terrestrial plant ingredients, fishery by-products and insect meals have shown the greatest potential to be protein-supplied to aquafeeds in the coming years (Hua et al., 2019; Gasco et al., 2020a). Although approximately 34% of the world's fishmeal production will be derived from fish by-products by 2030 (FAO, 2020a), this potential protein source will still not be able to meet the projected aquafeed demand by 2050 (Froehlich et al., 2018a). The efficiency of insect meal as a future aquafeed ingredient has already been identified, especially concerning the feasibility of costs, scalability, and processing technology (Hua et al., 2019). Globally, insect production is on the rise, and will reach approximately 1.2 million tonnes by 2025 and become price-competitive with fishmeal by 2023 (Hua et al., 2019; Gasco et al., 2020a). In addition, the development of production facilities and processing techniques would help to improve the environmental performance of insect meal as a sustainable aquafeed ingredient (van Huis and Oonincx, 2017). The use of seven insect species (two flies, two mealworms, and three cricket species) in fish diets has been authorized by the European Commission (Regulation No. 2017/893). Among these species, black soldier fly (*Hemertia illucens*), which belongs to the Diptera order, has received the most research interest (Hua, 2021). *Hemertia illucens* larvae meal possesses important nutritional profiles, especially amino acid profile which is close to that of fishmeal (Nogales-Mérida et al., 2019). As far as environmental impact is concerned, *H. illucens* production, if obtained using non-valorised substrates, entails significantly less arable land and water use than soybean meal (Šmetana et al., 2019; Gasco et al., 2020b). Moreover, *H. illucens* meal-containing diets have shown lower environmental impacts associated with abiotic depletion, acidification potential, eutrophication potential, climate change, human toxicity potential, and marine aquatic ecotoxicity potential for Arctic char (*Salvelinus alpinus*) (Smárason et al., 2017) and lower water use for European perch (*Perca fluviatilis*) (Stejskal et al., 2020) than insect-free diets.

The substitution of fishmeal with *H. illucens* meal in aquafeeds for the largest fishmeal consumers has already been investigated,

and substitution levels have been achieved that do not delay growth production of the tested species, including, white leg shrimp (*Litopenaeus vannamei*) (60% plausible substitution) (Cummins et al., 2017), Atlantic salmon (*Salmon salar*) (85–100%) (Lock et al., 2016; Belghit et al., 2018, 2019), European seabass (*Dicentrarchus labrax*) (45%) (Magalhães et al., 2017), barramundi (*Lates calcarifer*) (50%) (Katya et al., 2017), and rainbow trout (*Oncorhynchus mykiss*) (45%) (Sealey et al., 2011; Renna et al., 2017; Dumas et al., 2018). In addition, dietary *H. illucens* meal has been proved to modulate bacterial diversity and richness, which play essential roles in nutrition, immunology, and health status of fish, such as rainbow trout (*O. mykiss*) (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019; Rimoldi et al., 2021), and zebrafish (*Danio rerio*) (Zarantoniello et al., 2020b). The gut health benefit of insect-fed fish has been confirmed to be suitable for species that naturally feed on insect (Antonopoulou et al., 2019; Gasco et al., 2020c).

Pikeperch (*Sander lucioperca*) is one of the main percid species that has drawn a great deal of attention in aquaculture (Schulz et al., 2006). Aquaculture production of pikeperch reached 1557 tonnes in 2018, which was doubled that of 2009 (750 tonnes) (FAO, 2020b), and has mainly been established in intensive recirculation systems (Dalsgaard et al., 2013). However, pikeperch and other percid fish have so far received very little attention from feed manufacturers (Bochert, 2020). Although some commercial aquafeeds for percids have become available, salmonids-targeted feeds are more widely used in practice (Stejskal et al., 2016). Since European pikeperch aquaculture is moving toward an established freshwater aquaculture sector (Polcar et al., 2019), it will be necessary to develop suitable and sustainable feeds for aforementioned sector. Dietary protein requirements of at least 43% have been reported for appropriate growth performance and feed utilization of pikeperch fingerling (Nyina-Wamwiza et al., 2005). In the nature, aquatic insects, i.e., larvae of lake flies (*Chironomidae*) (Diptera order), play an important role as food sources for the early ontogenetic stages of pikeperch (Vinni et al., 2009; Ginter et al., 2011; Kashinskaya et al., 2018; Huuskonen et al., 2019). Therefore, the use of *H. illucens* larvae meal has been hypothesised to be suitable for pikeperch aquaculture. The aim of present study is to investigate the effects of dietary defatted black soldier fly (*H. illucens*) (HI) on the diets of juvenile pikeperch (*S. lucioperca*) on intestinal microbiota, histomorphology, and oxidative enzyme activities. The outputs could provide information in the choice of an alternative aquafeed ingredient for the emerging percid farming industry in Europe.

## MATERIALS AND METHODS

### Ethics Statement

The experimental procedures were performed under European Communities Directive (No. 2010/63/EU) on the protection of animals used for scientific purposes and have been approved by the Czech Ministry of Health (MSMT-6744/2018-2).



## Experimental Diets, Rearing Facilities, and Feeding Procedures

The feeding trial was conducted at the wet laboratory of the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Czech Republic. Defatted HI was obtained from a commercial source (Hermetia Geschäftsführungs GmbH, Baruth/Mark, Germany). Four isoproteic (approximately 45% crude protein) and isolipidic (approximately 18% ether extract) diets were formulated, comprising one fishmeal-based diet (CO) and three other diets, where HI was included at 9% (HI9), 18% (HI18), and 36% (HI36) to replace fishmeal at 25, 50, and 100%, respectively (Table 1). Experimental diets were prepared by a commercial feed producer (Exot Hobby s.r.o., Černá v Pošumaví, Czech Republic) using a dual-screw extruder (Saibainuo, China). Chemical composition of HI and experimental diets as well fatty acid (FA) composition of experimental diets are reported in Tables 1, 2, respectively.

TABLE 1 | Ingredients and proximate composition of experimental diets.

Ingredients (g/kg, as it)	HI <sup>a</sup>	CO	HI9	HI18	HI36
Fishmeal <sup>b</sup>		300	225	150	0
HI	-		90	180	360
Soybean protein concentrate	75	75	75	75	75
Corn gluten meal	170	170	170	170	170
Soybean meal	150	150	150	150	150
Wheat meal	80	65	50	20	
Merigel	60	60	60	60	60
Fish oil	60	60	60	60	60
Soybean oil	60	60	60	60	60
Vitamin mixture <sup>c</sup>	10	10	10	10	10
Mineral mixture <sup>d</sup>	10	10	10	10	10
DL-Methionine	7	7	7	7	7
L-Lysine	8	8	8	8	8
Cellite <sup>e</sup>	10	10	10	10	10
Proximate composition					
Dry matter (g/100g)	91.0	94.3	94.9	94.5	94.8
Crude protein (g/100g)	54.5	44.8	45.2	44.7	45.1
Ether extract (g/100g)	8.5	18.9	18.2	18.9	17.4
Ash (g/100g)	7.6	8.7	8.6	8.1	7.4
Chitin (g/100g) <sup>e</sup>	5.34	-	0.47	0.97	1.93
Nitrogen-free extract (g/100g) <sup>f</sup>	24.06	27.60	27.53	27.33	28.17
Gross energy (MJ/kg)	20.20	21.05	20.36	20.32	21.06

<sup>a</sup>Defatted *Hermetia illucens* larvae meal; <sup>b</sup>Purchased from Corpesca S.A. (Santiago, Chile). Proximate composition (g/100g, as fed basis): 91.3 dry matter; 65.8 crude protein; 9.4 ether extract; and 15.5 ash; <sup>c</sup>Vitamin mixture (IU or mg kg<sup>-1</sup> diet): DL- $\alpha$  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<sub>12</sub>, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium pantothenate, 50 mg (purchased from Granda Zootecnici S.r.l., Cuneo, Italy); <sup>d</sup>Mineral mixture (g or mg kg<sup>-1</sup> 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); <sup>e</sup>Estimated as described by Finke (2007); <sup>f</sup>Calculated as 100 - (CP + EE + Ash + Chitin).

The feeding experiment was conducted in a recirculation aquaculture system (total volume 11400 L), consisting of fifteen 250-L round conical plastic tanks (black walls, white bottom) connected to a mechanical drum filter (AEM 15, AEM-Products V.O.F., Lienden, Netherlands), sedimentation tanks (total volume 2600 l), a series of filtration sections (Bioakvacit PPI10), and a moving bed bio-filter (volume 4700 l, media BT10 Ratz Aqua & Polymer Technik, Remscheid, Germany), under controlled rearing conditions, with water temperature of 23.1  $\pm$  1.0°C, photoperiod of 12h light – 12h dark, light intensity of 20–35 Lux, oxygen saturation of 98.4  $\pm$  15.2%, and pH of 6.98  $\pm$  0.28. Moreover, the concentration of nitrite-N, nitrate-N, and ammonia-N concentration were maintained at 0.42  $\pm$  0.24, 48.8  $\pm$  21.3, and 1.89  $\pm$  0.58 mg/l, respectively.

The prepared diets were fed to triplicate groups of juvenile pikeperch (initial body weight 68.7  $\pm$  7.1 g, with 50 individuals per tank) for 84 days. A combined feeding protocol of four meals per day, provided at 07.00, 09.00, 11.00, 13.00, by automatic feeders (EHEIM Twins, Deizisau, Germany), and one hand feeding, at 15.00 was adopted during the trial. Any unconsumed feeds were collected by siphoning and dried in an oven to calculate the exact feed intake.

## Sampling Procedures

### Fish Biometry

At the start and the end of the feeding trial, fish were individually weighed to calculate weight gain (WG) and feed conversion ratio (FCR):

$$\text{WG (g)} = \text{final body weight} - \text{initial body weight}$$

$$\text{FCR} = \text{total feed supplied (g, Dry Matter)} / \text{WG}$$

### Antioxidative Enzyme and Histo-Morphological Analysis

After 84 days of the experiment, a total of 45 fish (3 individuals/tank) were randomly sampled, after 24 h of feed deprivation, and were euthanised by means of overdose anaesthesia (MS222, 125 mg/l).

Dissected livers and intestines from 15 fish/group were stored at –80°C for further antioxidative enzyme analysis. A similar number of samples, taken from another 15 fish/group, were fixed by immersion in a 10% buffered formalin solution for histo-morphological analysis.

### Intestinal Microbiota

At the end of the experiment, three fish were randomly taken from each tank and euthanised by means of overdose anaesthesia (MS222, 125 mg/l). In order to ensure that all sampled fish had digesta throughout the intestinal tract, fish were deprived of feeds 12 h prior to sampling time. Fish exterior was wiped with 70% ethanol before abdomen was opened, whole intestine from each fish was removed from the abdominal cavity and digesta from proximal to distal intestine was squeezed gently into a 1.5 ml aseptic Eppendorf and immediately stored at –80°C for further analysis.

## Analytical Methods

### Diet Chemical Composition

Analysis of HI defatted meal and experimental diets for dry matter, crude protein, crude lipid, ash, and fatty acids (FAs) were performed as described elsewhere (Tran et al., 2021). Gross energy was determined by mean of a calorimetric bomb (IKA C7000, Stufen, Germany).

### Oxidative Stress in Livers and Intestines

Oxidative stress biomarkers were evaluated in liver and intestine of each fish sample by means of spectrophotometer analysis (Varian Cary spectrophotometer, Santa Clara, CA, United States) as previously described by Elia et al. (2018). Briefly, superoxide dismutase (SOD) activity was measured in 50 mM Na<sub>2</sub>CO<sub>3</sub>, pH 10, 0.1 mM EDTA, 500 mM cytochrome C, and 1 mM hypoxanthine and xanthine oxidase. Reduction of cytochrome C by the xanthine/hypoxanthine system was measured versus a standard curve of SOD units at 550 nm. Catalase (CAT) activity was measured as the decrease in absorbance at 240 nm due to the consumption of H<sub>2</sub>O<sub>2</sub>. The assay was performed in an NaH<sub>2</sub>PO<sub>4</sub> + Na<sub>2</sub>HPO<sub>4</sub> buffer (100 mM, pH 7) and 12 mM H<sub>2</sub>O<sub>2</sub>. Glutathione peroxidase (SeGPx's) activities were measured by following the oxidation of NADPH at 340 nm and using 0.6 mM H<sub>2</sub>O<sub>2</sub> or 0.8 mM cumene hydroperoxides (tot GPx) as substrates. Glutathione S-transferase (GST) was measured at 340 nm using as a substrate 1-chloro-2,4-dinitrobenzene (CDNB).

### Histo-Morphological Analysis of Intestine and Liver

Samples of the anterior intestine were excised and flushed with a 0.9% saline solution to remove all the content. The collected samples were fixed in a 10% buffered formalin solution, routinely embedded in paraffin wax blocks, sectioned at a 5 μm thickness,

mounted onto glass slides and stained with Haematoxylin & Eosin (HE). One slide per intestinal segment was examined by means of light microscopy and captured with a Nikon DS-Fi1 digital camera, coupled to a Zeiss Axiophot microscope, using a 2.5× objective lens. NIS-Elements F software was used to capture images.

Morphometric analysis was performed using Image®-Pro Plus software on ten well-oriented and intact villi. The evaluated morphometric indices were villi height (from the villus tip to submucosa) and villi width (across the base of the villus, but not including the brush border).

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). Histopathological findings in intestine were assessed separately for each segment for mucosa (inflammatory infiltrates) and submucosa [inflammatory infiltrates and Gut-Associated Lymphoid Tissue (GALT) activation]. The total score of each gut segment was obtained by adding to the mucosa and submucosa scores. All the slides were blind assessed by two independent observers, and any discordant cases were re-examined, using a multi-head microscope, until unanimous consensus was reached.

## Microbiome Analysis

### DNA Extraction and 16S rRNA Amplicon Target Sequencing

Nucleic acid was extracted from the intestine content (500 mg as starting materials). Total DNA from the samples was extracted using a RNeasy Power Microbiome KIT (Qiagen, Milan, Italy), according to the manufacturer's instructions. One microlitre of RNase (Illumina Inc, San Diego, CA, United States) was added to

**TABLE 2 |** Fatty acid (FA) composition (as mg/g total FAs) of experimental diets.

*FAs	Experimental diets			
	CO	HI9	HI18	HI36
C12:0	0.4 ± 0 <sup>a</sup>	16.1 ± 0.3 <sup>b</sup>	25.7 ± 0.7 <sup>c</sup>	61.8 ± 3.4 <sup>d</sup>
C14:0	17.2 ± 0.1 <sup>a</sup>	20.1 ± 0.1 <sup>b</sup>	21.2 ± 0.1 <sup>c</sup>	27.5 ± 0.7 <sup>d</sup>
C16:0	102.7 ± 0.5 <sup>a</sup>	106.8 ± 0.3 <sup>b</sup>	105.2 ± 1.6 <sup>b</sup>	106.2 ± 0.9 <sup>b</sup>
C16:1	23.7 ± 0 <sup>a</sup>	23.9 ± 0 <sup>ab</sup>	24.0 ± 0.1 <sup>b</sup>	24.1 ± 0.1 <sup>b</sup>
C18:0	29.9 ± 0.2	30.2 ± 0.3	30.3 ± 1.7	28.1 ± 0.2
C18:1n9	201.3 ± 0.8 <sup>c</sup>	196 ± 0.2 <sup>b</sup>	195.6 ± 0.3 <sup>b</sup>	188.5 ± 0.9 <sup>a</sup>
C18:1n7	206.2 ± 3.5 <sup>b</sup>	196 ± 0.2 <sup>a</sup>	197.9 ± 3.8 <sup>a</sup>	194.5 ± 0.9 <sup>a</sup>
C18:2n6	257.6 ± 0.9 <sup>d</sup>	254.1 ± 0.4 <sup>c</sup>	251 ± 1.8 <sup>b</sup>	241.8 ± 1.0 <sup>a</sup>
C18:3n3	38.9 ± 0.2 <sup>c</sup>	37.3 ± 0 <sup>b</sup>	37 ± 0.2 <sup>b</sup>	34.3 ± 0.2 <sup>a</sup>
C20:1n9	33.0 ± 0.3 <sup>c</sup>	31.2 ± 0.1 <sup>b</sup>	31.0 ± 0.2 <sup>b</sup>	27.5 ± 0.1 <sup>a</sup>
C20:5n3 (EPA)	3.20 ± 0.01 <sup>d</sup>	3.10 ± 0.01 <sup>c</sup>	3.00 ± 0.01 <sup>b</sup>	2.60 ± 0.01 <sup>a</sup>
C22:6n3 (DHA)	48.2 ± 0.5 <sup>d</sup>	45.5 ± 0.2 <sup>c</sup>	39.1 ± 0.2 <sup>b</sup>	26.7 ± 0.5 <sup>a</sup>
∑n-3	91.4 ± 0.7 <sup>d</sup>	86.9 ± 0.3 <sup>c</sup>	80.1 ± 0.4 <sup>b</sup>	64.4 ± 0.6 <sup>a</sup>
∑n-6	268.1 ± 1.0 <sup>d</sup>	264 ± 0.5 <sup>c</sup>	259.8 ± 1.8 <sup>b</sup>	248.3 ± 1.0 <sup>a</sup>
∑SFA	164.6 ± 0.9 <sup>a</sup>	190.6 ± 0.7 <sup>b</sup>	200 ± 4.4 <sup>c</sup>	239.5 ± 4.4 <sup>d</sup>
∑MUFA	470.9 ± 2.5 <sup>c</sup>	453.6 ± 0.3 <sup>b</sup>	454.8 ± 3.6 <sup>b</sup>	440.2 ± 1.9 <sup>a</sup>
∑PUFA	360 ± 1.7 <sup>d</sup>	351.4 ± 0.7 <sup>c</sup>	340.4 ± 2.2 <sup>b</sup>	316.0 ± 4.9 <sup>a</sup>

\*Only FAs > 10 mg/g total FAs (except for EPA) are presented; Different letters denote significant differences among the experimental groups ( $P < 0.05$ ).

# How does pikeperch *Sander lucioperca* respond to dietary insect meal *Hermetia illucens*? Investigation on gut microbiota, histomorphology, and antioxidant biomarkers

Tran et al.

Pikeperch Fed Defatted Insect Meal

digest the RNA in the DNA samples for an incubation period of 1 h at 37°C. DNA was quantified using Qubit ds and standardised at 5 ng/ $\mu$ l.

DNA extracted directly from digesta samples was used to assess the microbiota, through amplification of the V3–V4 region of the 16S rRNA gene (Klindworth et al., 2012). The PCR products were purified according to the Illumina metagenomic standard procedure (Illumina Inc, San Diego, CA, United States). Sequencing was performed with an MiSeq Illumina instrument, with V3 chemistry, and 250 bp paired-end reads were generated according to the manufacturer's instructions.

## Statistical Analysis

All data for antioxidative enzyme activities were tested for homogeneity of variance using Cochran, Hartley, Bartlett test. The effects of diet on oxidative stress in different organs were analysed separately, by means of one-way ANOVA, followed by Tukey test. Statistical analyses were performed using STATISTICA 12.0, with  $P$ -value < 0.05 as the significant difference.

Raw reads of microbiota were first joined, after sequencing, using FLASH software (Magoč and Salzberg, 2011), with default parameters, and were filtered, using QIIME 1.9.0 software and the pipeline as recently described (Biasato et al., 2018). Briefly, shorter reads (<300 bp) were discarded, using Prinseq. USEARCH software (version 8.1) was used for chimera filtering, and the Operational Taxonomic Units (OTUs) were picked, at a threshold of 97% similarity, using UCLUST algorithms. Taxonomy was assigned against 16S rRNA from Greengenes. The OTU table was rarefied at 10,144 sequences/sample. The OTU table displays the highest taxonomy resolution that was reached. When the taxonomy assignment was not able to reach the genus level, the family or phyla were displayed. R software was used to calculate the alpha diversity, while Weighted and Unweighted UniFrac distance matrix and OTUs table were used to find differences between samples, using permutational multivariate analysis of variance (Anosim) and analysis of similarity (Adonis) statistical test, considering the same function in R environment. Pairwise Wilcoxon test were used to determine any significant

differences in alpha diversity or OTU abundance as a function of dietary insect meal. Principal component analysis (PCA) were plotted, using the *dudi.pca* function, through the *made4* package of R environment. Non-normally distributed variables were presented as median values (interquartile range, IR), and box plots represented the interquartile range between the first and the third quartile, with the error bars showing the lowest and the highest value. Pairwise Kruskal-Wallis tests were used to find any significant differences in microbial taxa abundance according to the dietary treatment.  $P$ -values were adjusted for multiple testing, and a false discovery rate (FDR) < 0.05 was considered as significant. The data generated from sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under the BioProject Accession Number PRJNA704237.

GraphPad Prism® software (version 8.0) was used to perform statistical analysis, for histo-morphometrical investigations. The Shapiro–Wilk test was used to test the normality of the data distribution before statistical analyses. Data were described by mean and standard deviation (SD), or median and IR depending on data distribution. Bivariate analysis was performed, by means of one way-ANOVA or Kruskal Wallis tests, to compare the intestine morphology and organs histopathology among different diet groups.  $P$ -values < 0.05 were considered statistically significant.

## RESULTS

### Diet Composition and Growth Production of Pikeperch

Formulated diets had a similar proximate composition, except for chitin which increased with the increase of HI inclusion (Table 1). The inclusion of dietary HI significantly altered the FA profile of experimental diets. As regards saturated FAs (SFA), lauric (C12:0), myristic (C14:0), and palmitic acid (C16:0) significantly increased with the increase of HI inclusion ( $P$  < 0.05). Monounsaturated FAs (MUFA), dominated by palmitoleic acid (C16:1), C18:1n9 and C18:1n7, were found to be significantly higher in CO than H36 ( $P$  < 0.05), while MUFAs

TABLE 3 | Growth performances and histopathological traits divided by diet groups.

	Experimental diets				P-value
	CO	HI9	HI18	HI36	
<b>Growth performances</b>					
Weight gain (g), mean (SD)	85.3 <sup>a</sup> (24.1)	84.8 <sup>a</sup> (23.7)	83.2 <sup>a</sup> (26.4)	62.8 <sup>b</sup> (18.3)	<0.001
FCR, mean (SD)	1.27 <sup>b</sup> (0.06)	1.28 <sup>b</sup> (0.07)	1.29 <sup>b</sup> (0.03)	1.81 <sup>a</sup> (0.15)	<0.001
<b>Anterior gut</b>					
Villi height (mm), mean (SD)	0.31 (0.07)	0.32 (0.07)	0.29 (0.05)	0.28 (0.07)	0.979
Villi width (mm), mean (SD)	0.03 (0.005)	0.03 (0.006)	0.03 (0.008)	0.11 (0.34)	0.065
Inflammation, median (IR)	0.00 (0.0–0.5)	0.00 (0.0–0.3)	0.00 (0.0–0.5)	0.00 (0.0–0.5)	0.967
<b>Liver</b>					
Degeneration, median (IR)	3.00 <sup>a</sup> (3.0–3.0)	3.00 <sup>a</sup> (2.0–3.0)	2.50 <sup>b</sup> (1.0–3.0)	2.50 <sup>b</sup> (1.0–3.0)	0.015
Inflammation	Absence of alterations				

SD, standard deviation; FCR, feed conversion ratio; IR, interquartile range. Values in the same row not sharing common superscript letter are significantly different.

in H9 and H18 remained comparable ( $P > 0.05$ ). Increasing inclusion level of HI significantly reduced polyunsaturated FAs (PUFA) ( $P < 0.05$ ). A similar trend was observed for EPA, DHA, linoleic acid, alpha-linolenic acid ( $P < 0.05$ ) (Table 2).

At the end of the feeding trial, WG in fish fed HI36 (62.8, mean value) was significantly lower than the control group (85.3 g) ( $P < 0.05$ ), whereas pikeperch fed HI9 (84.8 g) and HI18 (83.2 g) did not show significant difference with CO ( $P > 0.05$ ). FCR of the CO group (1.27) was comparable with that of HI9 (1.28) and HI18 (1.29) ( $P > 0.05$ ), but significantly lower than HI36 (1.81) ( $P < 0.05$ ) (Table 3).

### Oxidative Stress in Liver and Intestine

The results of oxidative biomarkers, SOD, CAT, SeGPx, and GST, in liver and intestine of pikeperch fed experimental diets are depicted in Figure 1. Dietary HI did not alter the SOD activities in either liver or intestine, CAT activities in liver, SeGPx activities in intestine, or GST activities in liver of pikeperch ( $P > 0.05$ ). No significant difference was observed across experimental groups ( $P > 0.05$ ) for liver, as regards CAT activities, whereas this biomarker was significantly lower in HI18 and HI36 than in HI9 ( $P < 0.05$ ), but remained similar to CO ( $P > 0.05$ ) in intestine. Even if did not differ from the CO group, among fish fed HI-containing diets, HI9 produced highest SeGPx activity in liver ( $P < 0.05$ ), while the lowest activity was found in HI36 group ( $P < 0.05$ ). A significant increase in the GST concentration was observed in intestine of pikeperch fed HI-containing diets, compared to CO ( $P < 0.05$ ). Of the different insect-fed groups, HI9 showed a higher GST than HI18 ( $P < 0.05$ ), while HI36 was remained intermediate position.

### Histo-Morphology

Data regarding histopathological evaluation are reported in Table 3. Only few differences were observed for morphometry and histopathology of intestine among diet groups. Although there was no significant difference, a trend could be observed ( $P = 0.065$ ) with HI36 group recording wider villi than the other groups. Thus, dietary HI inclusion did not induce any significant morphological changes in the pikeperch intestine, thereby suggesting no negative influence of such dietary HI on the physiological development of intestine.

Mild to severe multifocal to diffuse liver vacuolar degeneration was recorded in all treatments, and it was found to be greater in CO and HI9 group than in the HI18 and HI36 ones. Dietary HI did not show any evidence of inflammation of the liver of pikeperch (Table 3 and Figure 2).

### Microbiota

The total number of high-quality paired-end sequences obtained from 16S rRNA sequencing reached 1.916.822 raw reads. After the filtering, 1.295.693 reads passed the filters applied by QIIME, with a median value of  $37.559 \pm 15.565$  reads/sample, and a mean sequence length of 443 bp. The rarefaction analysis and Good's coverage, expressed as a median percentage (97%), also indicated satisfactory coverage of all samples.

The result of the OTUs analysis showed that there was no significant difference in Shannon index ( $P > 0.05$ ) among diet

groups, while alpha-diversity of intestinal bacteria, associated with Chao1 and observed OTUs, in fish fed HI18 significantly increased relative to CO diet ( $P < 0.05$ ) (Figure 3).

Adonis and Anosim statistical tests, based on weighted and on unweighted UniFrac distance matrix using the OTUs table, showed significant differences between diet groups as a administration of HI ( $P < 0.002$ ). These differences were also observed when the PCA plot was produced at a genus level (Figure 4). It was also possible to observe a certain degree of separation, following diet groups. Microbiota of CO diet was near to the insect meal inclusion of 9%, while the microbiota of fish fed with 18 and 36% of HI was well separated (Figure 4).

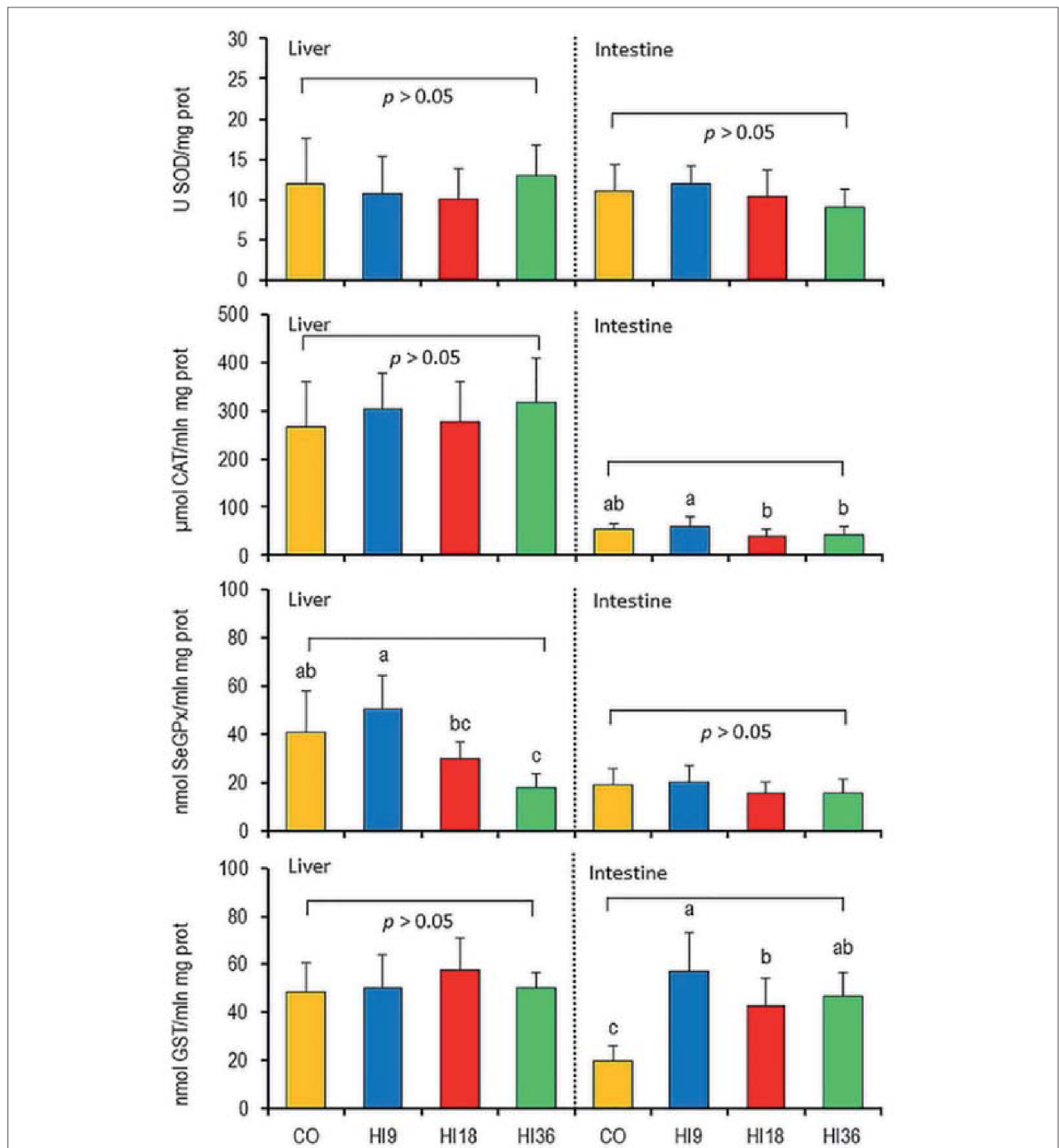
The dominant OTUs, at the phyla level, were *Firmicutes* (mean values, 45–75%), regardless to dietary HI. Perch fed CO diet was enriched with *Proteobacteria* (26%), while *Bacteroidetes* (7–13%) was the prevalent phyla in fish fed HI-containing diets. As a result, *Clostridiaceae*, *Enterococcaceae*, and *Bacillaceae* were found to be the predominant families across fish fed diet groups. *Clostridium*, *Acetobacter*, *Cetobacterium*, *Plesiomonas*, *Acetobacter*, *Peptostreptococcaceae*, *Bacteroides*, and *Oceanobacillus* were, at the genus level, the most abundant genera found in intestine of perch considered in our study (Figure 5).

Dietary HI positively affected relative abundance of almost OTUs, compared with CO (FDR  $< 0.05$ ), excepted for *Bacillus*, *Burkholderia*, and *Sporosarcina*, which were dominant in the CO group (Figure 6).

## DISCUSSION

### Oxidative Enzymes

Reactive oxygen species (ROS) is the production of aerobic metabolism processes, including superoxide, hydrogen peroxide, and lipid peroxides (Buetler et al., 2004). Excessive ROS compounds cause cellular and tissue damages (Rosa et al., 2008). The balance of ROS production ensures the normal physical function of any organism and is regulated by antioxidant systems (Rosa et al., 2008) involving two mechanisms, (i) enzymes that remove ROS, including SOD, CAT, and SeGPx; and (ii) antioxidative compounds, i.e., ascorbate, glutathione, scavenge free radicals (Passi et al., 2002). Antioxidative enzyme activities were documented to be tissue-specific in pikeperch, and liver was the most sensitive organ to the diet manipulation under recirculating aquaculture system (Polcar et al., 2016). In the case of detoxification in the intestine, however, certain enzymes such as SOD were known to play a vital role (Tang et al., 2013). This study indicates that in liver of pikeperch dietary HI did not alter the SOD, CAT, or GST oxidative enzymes, while significantly reduced SeGPx activity, a result that is in agreement with those of previous study (Elia et al., 2018), who performed a trial on rainbow trout fed dietary HI. The significant reduction in the catalytic SeGPx efficiency in liver of pikeperch fed dietary HI could be explained by the presence of chitin (Elia et al., 2018). Indeed, increasing inclusion levels of HI increased chitin levels in diets (Table 1). In addition, declining in SeGPx activities, as a result of increasing dietary HI, could be attributed to different dietary PUFA levels

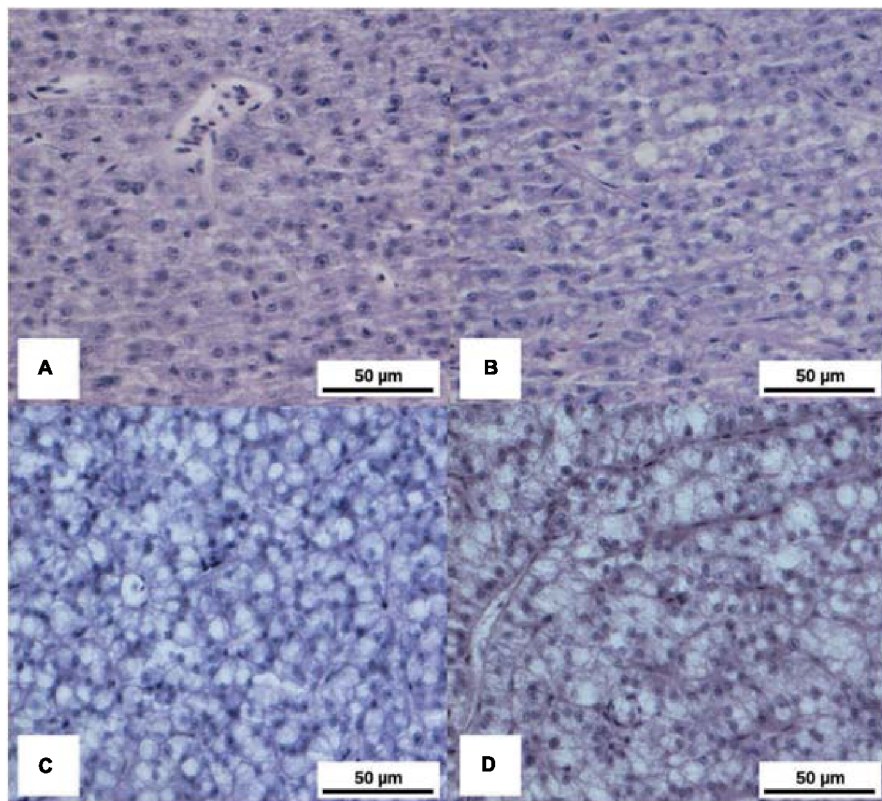


**FIGURE 1** | Oxidative biomarkers in liver and intestine of pikeperch (*Sander lucioperca*) fed experimental diets. Values are presented as means ± standard deviation. Means with different letters are significantly different ( $P < 0.05$ ) from each other.

(Table 2), which are highly susceptible to oxidation. In fact, Tocher et al. (2002) reported that a high dietary PUFA content increased lipid peroxidation in fish tissues, and consequently the SeGPx enzyme activity involved in reducing peroxides, including

FA hydroperoxides and hydrogen peroxide, will be also high (Passi et al., 2002).

The present study indicates that the CAT activity in intestine of pikeperch was significantly higher for HI9 than



**FIGURE 2 |** Histopathological alteration of liver of pikeperch considered in the present study. **(A)** Normal liver, Haematoxylin & Eosin (H-e) stain, 40× magnification, for HI36 diet. **(B)** Mild and multifocal vacuolar degeneration (grade 1), H-e, 40× magnification, for HI18 diet. **(C)** Moderate and multifocal vacuolar degeneration (grade 2), H-e, 40× magnification, for the HI9 diet. **(D)** Severe and diffuse vacuolar degeneration (grade 3), H-e, 40× magnification, for CO diet.

for HI18 and HI36 groups. A similar phenomenon was reported for CAT activity in the intestine of rainbow trout fed insect meal (*T. molitor*), where a substitution level of 25% fishmeal displayed higher activity than the 50% level (Henry et al., 2018a). The CAT and SeGPx activities in the present study were similar for CO and HI9, and lower than for HI18, HI36 groups. This result indicates that substantial substitution of fishmeal with HI reduced antioxidant enzyme activities in pikeperch. This is in line with a previous finding pertaining to rainbow trout (*O. mykiss*) (Elia et al., 2018). The decline of these biomarkers in HI18 and HI36 groups could be related to an imbalance between ROS production and antioxidant capacity. A suitable concentration of antioxidants, such as chitin and other bioactive compounds (Ngo and Kim, 2014), may support antioxidant enzyme activities in HI9 compared to the other HI-contained diets (Henry et al., 2018a).

Glutathione S-transferase plays an essential role in scavenging free radicals and xenobiotics detoxification (Aksnes and Njaa, 1981; Li et al., 2010). Increased glutathione S-transferase activity in intestine, but not liver, was observed across diet groups in the present study (Figure 1), thus implicating that some of the compounds in HI may have stimulated the biotransformation

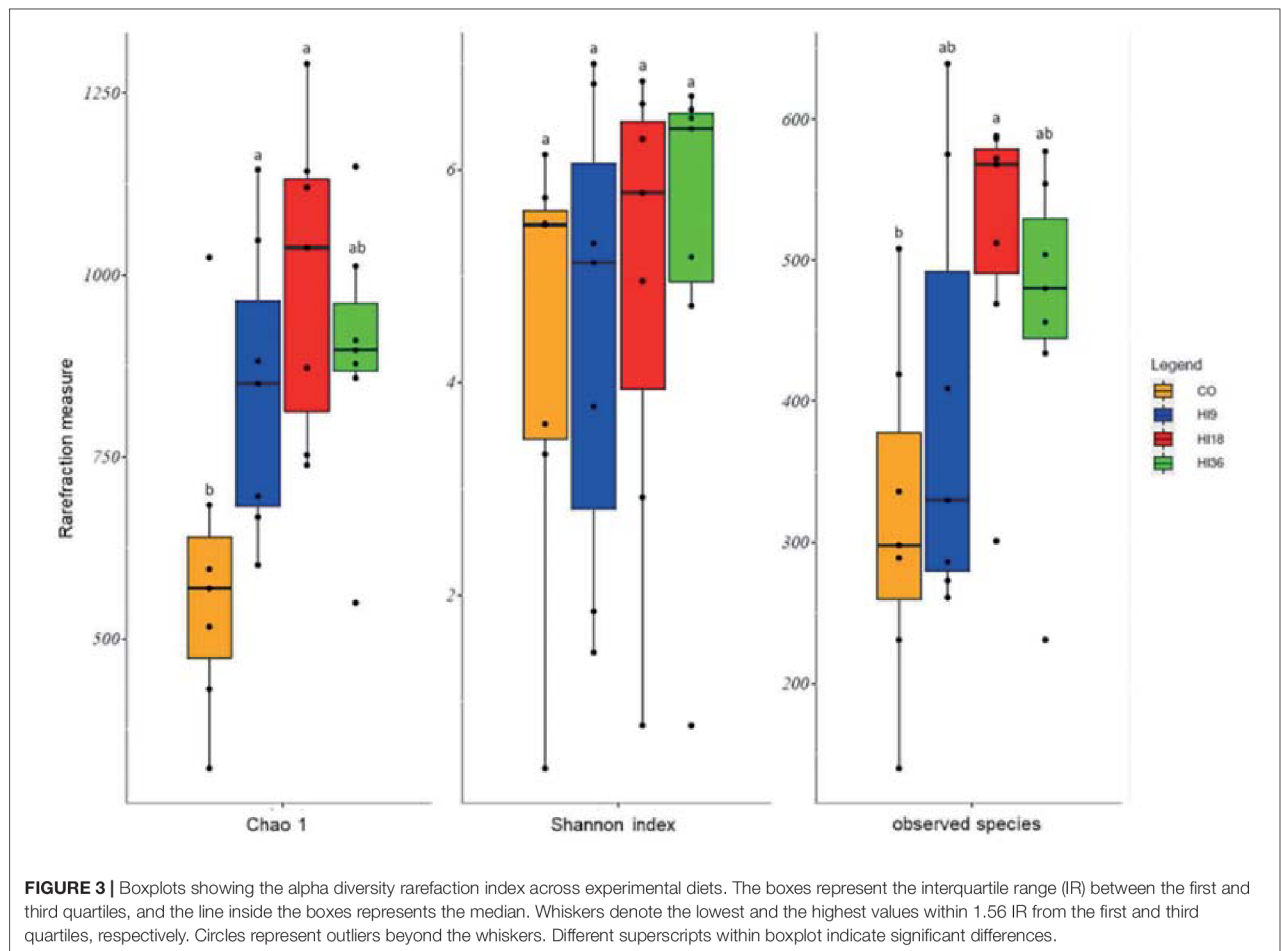
pathway in intestine of pikeperch, which was also found in liver of tilapia (*Oreochromis niloticus*) fed cricket-based feeds (Ogunji et al., 2007). In fact, insect meals may contain harmful substances, i.e., heavy metals and pesticides (van der Spiegel et al., 2013). The absence of an alteration of the hepatic GST activities after administration of HI could be the result of factors other than xenobiotics (Collier and Varanasi, 1991) or tissue-specific response (Martínez-Álvarez et al., 2005).

We also observed numerically higher oxidative biomarkers in liver of pikeperch than in intestine (Figure 1), which was in agreement with recent findings (Polcar et al., 2016), reporting that liver was one of the most susceptible tissue in response to artificial nutrition and controlled conditions.

### Histo-Morphology

Dietary HI in our study did not induce any morphological or inflammatory changes in the intestine of pikeperch, a result that is in agreement with previous studies conducted on different fish species fed dietary insect meals (Elia et al., 2018; Zarantoniello et al., 2019; Zarantoniello et al., 2020a). The absence of intestinal and hepatic inflammation could be linked to anti-inflammatory properties regulated by dietary saturated fatty acids content, especially lauric acid (C12:0) and chitin

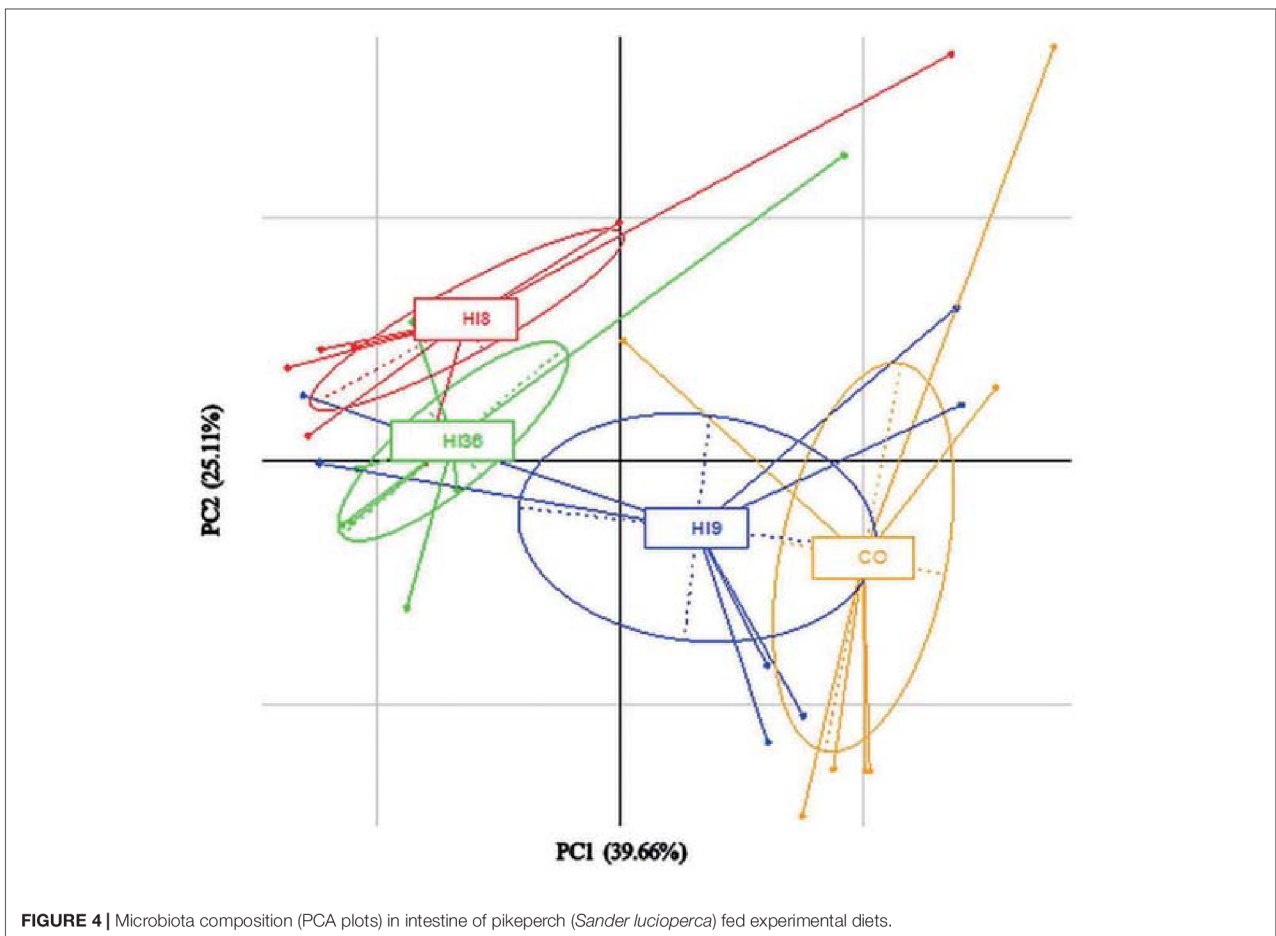
# How does pikeperch *Sander lucioperca* respond to dietary insect meal *Hermetia illucens*? Investigation on gut microbiota, histomorphology, and antioxidant biomarkers



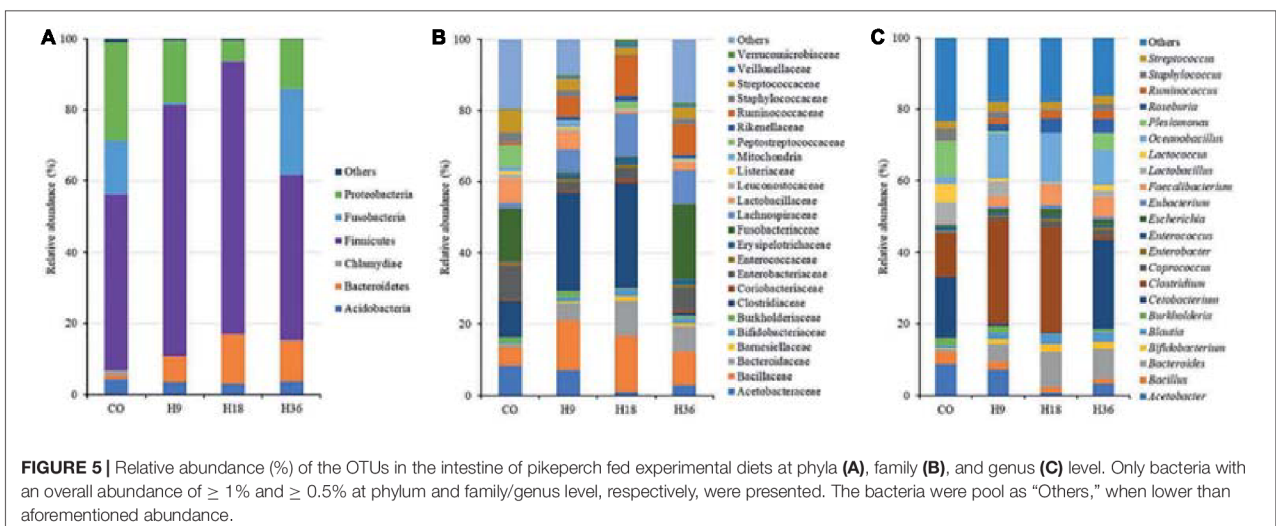
component (Henry et al., 2018b; Vargas-Abundez et al., 2019; Zarantoniello et al., 2019; Gasco et al., 2020b,c) which were found to be particularly high in HI and HI-containing diets in the present study. Although there were no significant differences (at  $P$ -value < 0.05), the villi were more expanded in the HI36 group than in the other groups (Table 3), and this was attributed to the presence of chitin. Chitin could stimulate the growth of villi thickness in tilapia (*O. niloticus*), probably due to its viscosity and water holding capacity (Kihara and Sakata, 1997). Chitin also induced the production of short-chain fatty acids, such as acetate, propionate and n-butyrate, and n-butyrate in particular was observed in intestine of tilapia (Kihara and Sakata, 1997), thereby increasing intestinal histo-morphology of fish, e.g., villi length and weight (Dawood, 2021). The large quantity of *Paenibacillus* genus in intestinal digesta of fish fed HI36 (Figure 6) could act as a probiotic for aquatic animal species (Midhun et al., 2017; Chen et al., 2019; Amoah et al., 2020), consequently enhancing intestinal health indices, including histomorphology (Dawood, 2021).

In contrast to recent findings, which reported that an increasing inclusion of insect meals induced a higher degree of hepatic vacuolization degeneration in fish (Li et al., 2017;

Zarantoniello et al., 2019), the present study indicates that feeding pikeperch with  $\leq 9\%$  HI caused more severe hepatic degeneration than 18 or 36% did (Table 3), which could be related to a fatty liver status. Schulz et al. (2005) reported that a low level of palmitic acid (C16:0) yielded a higher hepatic lipid content. In the present study, the significantly lower palmitic acid in the control group than in the HI-containing groups could partly explain the hepatocellular vacuolization phenomenon. The mechanism to which palmitic acid affecting hepatic tissues remained to be elucidated. However, this FA promotes hepatocyte proliferation (Wang et al., 2011) and possess anti-inflammatory and antiviral effects (Librán-Pérez et al., 2019). On the other hand, the high content of dietary lauric acid (C12:0), high oxidation and low tissue deposition, was found to decrease liver lipid storage in freshwater Atlantic salmon (Belghit et al., 2019). This could explain the reduction in the adipose liver in pikeperch fed HI18 and HI36, compared to the control and HI9 diets (Table 3). Two FAs, linoleic and oleic acids, were confirmed to induce the occurrence of hepatic steatosis in sea bream (*Sparus aurata*) (Caballero et al., 2004). Moreover, owing to large molecular weight, oleic acid could produce a large lipid droplet while inrush hepatocyte (Bradbury, 2006). These FAs were found to



**FIGURE 4 |** Microbiota composition (PCA plots) in intestine of pikeperch (*Sander lucioperca*) fed experimental diets.



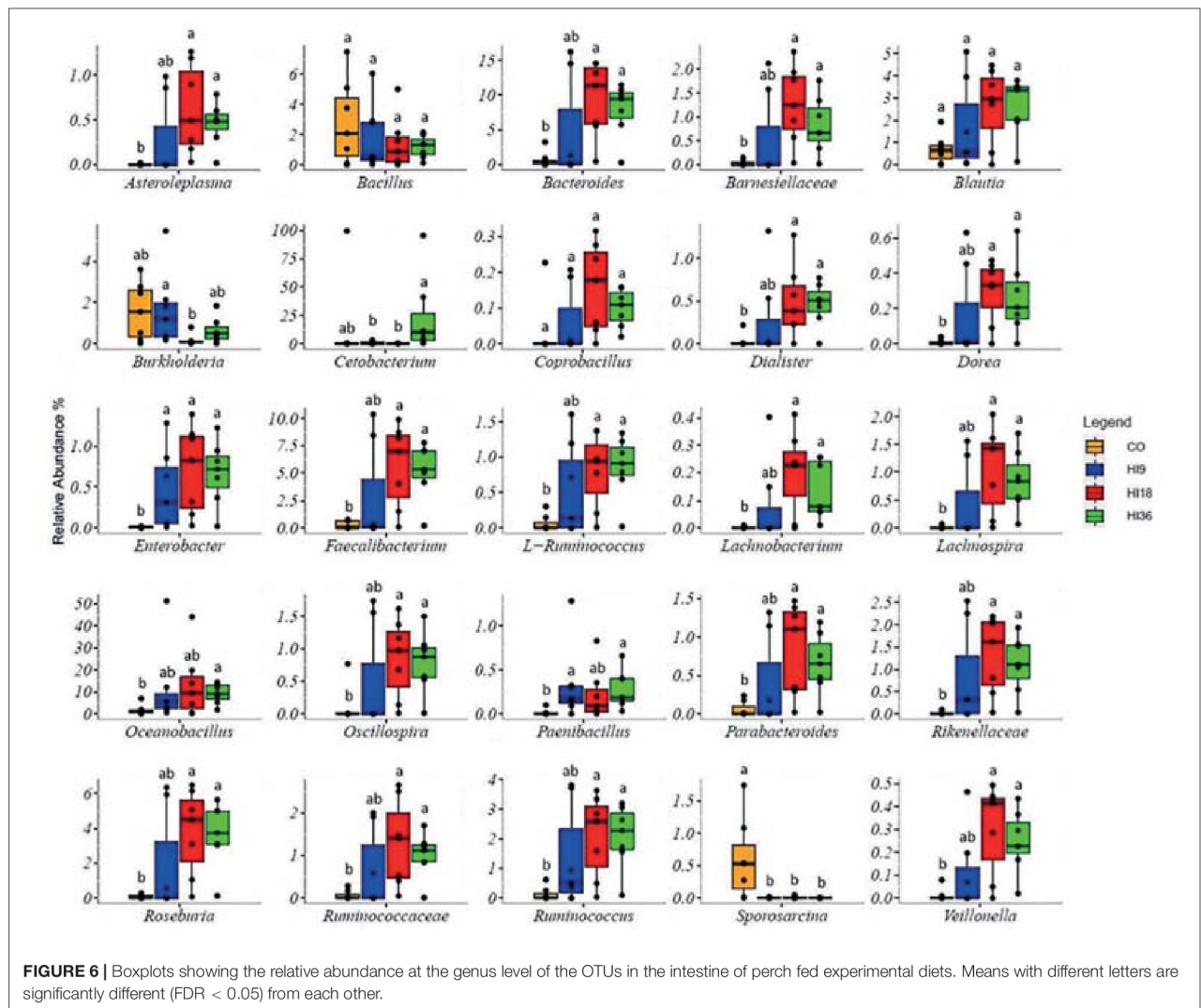
**FIGURE 5 |** Relative abundance (%) of the OTUs in the intestine of pikeperch fed experimental diets at phyla (A), family (B), and genus (C) level. Only bacteria with an overall abundance of  $\geq 1\%$  and  $\geq 0.5\%$  at phylum and family/genus level, respectively, were presented. The bacteria were pool as “Others,” when lower than aforementioned abundance.

be significantly higher in CO than in HI18, HI36 (Table 2), which could indicate severe steatosis in livers of the former group (Table 3). High intakes of eicosapentaenoic acid (EPA) and

docosahexaenoic acid (DHA) are known to an inhibitor of lipid accumulation in livers of sea bream (*S. aurata*) (Caballero et al., 2004). Therefore, the change in the percentage of the different



# How does pikeperch *Sander lucioperca* respond to dietary insect meal *Hermetia illucens*? Investigation on gut microbiota, histomorphology, and antioxidant biomarkers



FAs in the experimental diets, due to the inclusion of HI, could further explain the severity of hepatic vacuolization degeneration observed in perch fed CO and H9 diets.

## Microbiota

The present study reveals that dietary HI enhanced microbial biodiversity indices in intestine of pikeperch, compared with insect-free diet, a result that is in line with recent findings on rainbow trout (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019), thereby contributing to gut health and health status of the host.

In agreement with previous studies on intestinal microbiota of percid fish and freshwater species, the present study reveals that *Firmicutes*, *Proteobacteria*, *Bacteroidetes* were the most dominant phyla in the intestine of pikeperch, regardless of the HI inclusion level (Li et al., 2014; Kashinskaya et al., 2018; Terova et al., 2019).

Our results show an abundance of *Clostridium* genus in fish fed HI9 and HI18, which was even greater than those fed

CO and HI36. Members of the *Clostridium* genus are common effective microorganism used as probiotics in aquaculture (Nayak, 2010a,b). *Clostridium butyricum* has been shown to possess a pathogenic inhibition capacity in farmed fishes (Pan et al., 2008a,b; Gao et al., 2013), improve feed efficiency in shrimp (Duan et al., 2017; Li et al., 2019), and to be suitable for use as probiotics in farmed fish (Hai, 2015; Zorriehzaha et al., 2016). The greater prevalence of *Clostridium* and other probiotic-used bacteria in HI9, such as *Lactobaccillus* and *Bacillus* genera, than in HI36, could explain the difference in feed conversion ratio between these diets in present study. The *Bacteroides* and *Clostridium* genera are known to be the main taxa involved in production of fatty acids and vitamins (Balcázar et al., 2006). The abundant presence of these taxa could partially compensate for nutritional insufficiencies in HI-containing diets, and consequently resulted in a comparable growth rate among control, HI9 and HI18 diets, yet the offset may be not efficient for HI36 group.

It is worth noting that *Cetobacterium*, the most predominant bacterium in intestine of natural pikeperch (Kashinskaya et al., 2018) and other freshwater fish (Larsen et al., 2014), was detected in our captive pikeperch fed dietary HI. Similar findings were also observed in rainbow trout (Etyemez and Balcázar, 2015), common carp (van Kessel et al., 2011), and giant arapaima (Ramírez et al., 2018) fed commercial aquafeeds. It seems relevant that *Cetobacterium* is among the core bacteria in pikeperch.

Insect meal, in general, is a chitin-rich ingredient. The degradation and digestion of this substance require binary enzymes, including chitinase and  $\beta$ -N-acetylglucosaminidase, and involve various microbacteria derived from digestive tract of fish with a chitinase-produced capacity (Ray et al., 2012; Ringø et al., 2012). Among these chitin-degraded bacteria, the *Plesiomonas* and *Bacillus* genus were detected across treatment groups at a particularly low abundance (Figure 4). This finding implicates that pikeperch may not be able to degrade chitin. A limited presence of chitinase-producing bacteria was also observed in rainbow trout (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019) and this may help to explain the low or absent chitin digestibility in this species (St-Hilaire et al., 2007; Henry et al., 2015; Renna et al., 2017; Caimi et al., 2020).

In conclusion, HI, fed as a partial or total replacement of fishmeal did not induce any inflammation of liver or intestine, or any intestine degeneration, but did show signs of severe hepatic steatosis of pikeperch fed CO and HI9 groups. Dietary HI promotes antioxidative enzyme activities of CAT, GPx and GST, but not of SOD, in liver and, to a lesser extent, in intestine of pikeperch. The inclusion of HI up to 18% or 50% fishmeal replacement in pikeperch diets increased abundance of *Clostridium*, *Oceanobacillus*, *Bacteroides*, and *Faecalibacterium*, whereas the predominant bacterium, *Cetobacterium* was found in the control and HI36 groups. Because of the absence of inflammation in tissues, the evolution of antioxidative enzyme, and modification of the favourable microbiota observed in the

present study, it is possible to assume that defatted HI could have an immunological effect on juvenile pikeperch. Further study on immune response and disease resistance of pikeperch fed insect meal could help to explore this efficiency.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Czech Ministry of Health (MSMT-6744/2018-2).

## AUTHOR CONTRIBUTIONS

LG and VS: planning the experiment and editing the manuscript. HT: data analysis, writing, and editing of the manuscript. MP, MZ, and TG: wrote the manuscript. AE, EC, IF, CC, and FG: analysis and the first draft. All authors contributed to the article and approved the submitted version.

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# How does pikeperch *Sander lucioperca* respond to dietary insect meal *Hermetia illucens*? Investigation on gut microbiota, histomorphology, and antioxidant biomarkers

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## CHAPTER 7

### **DOES DIETARY *Tenebrio molitor* AFFECT SWIMMING CAPACITY, ENERGY USE, AND PHYSIOLOGICAL RESPONSES OF EUROPEAN PERCH *Perca fluviatilis*?**

Tran, H.Q., Van Doan, H., Stejskal, V., 2021. Does dietary *Tenebrio molitor* affect swimming capacity, energy use, and physiological responses of European perch *Perca fluviatilis*? *Aquaculture* 539, 736610.

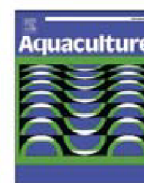
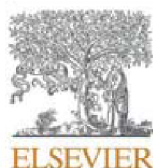
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## Does dietary *Tenebrio molitor* affect swimming capacity, energy use, and physiological responses of European perch *Perca fluviatilis*?

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### ABSTRACT

We assessed swimming capacity, energy expenditure, and physiological responses of European perch (*Perca fluviatilis*) fed four isonitrogenous and isoenergetic diets containing yellow mealworm (*Tenebrio molitor*) larvae meal at 0, 25, 50, and 75% substitution for fishmeal (abbreviated diets, TM0, TM25, TM50, and TM75). Each diet was fed to quadruplicate group of perch (initial biometrics, body weight  $20.81 \pm 3.36$  g, total length  $11.77 \pm 0.72$  cm) for 119 days. At the terminal of feeding trial following 24 h starvation, eighty fish (20 fish/diet group) were individually selected for swimming performance tests, which were conducted in a 10 L enclosed swimming tunnel with velocity increased from 5 cm/s in 2 cm/s increments every 60 s. Exercised fish, fish experienced swimming tests, and non-exercised fish, fish not involved in swimming tests were, at the same time, sampled for serum biochemistry, muscle traits. Whole-body of non-exercised fish were also analyzed for proximate composition and fatty acid profile.

Critical swimming speed ( $U_{crit}$ , cm/s and body length/s), oxygen consumption ( $MO_2$ , mg/kg/h), and energy cost of transport (COT, J/kg/m) of perch did not differ among diet treatments. Exercised perch significantly increased serum glucose and cortisol compared to non-exercised fish. Substitution of fishmeal by *T. molitor* larvae meal induced significant changes in aspartate aminotransferase across treatment groups, lactate dehydrogenase in TM0 and TM75,  $K^+$  concentration in fish fed TM75, and muscle water content in TM50 of exercised compared to non-exercised perch. Oleic acid of whole-body fish had a significant linear correlation with the critical swimming speed of European perch. Since fish swimming behavior is an indicator of animal welfare, our findings suggest that dietary insect meals could ensure the welfare of farmed fish.

### 1. Introduction

The continuous growth of aquaculture industry as a consequence of increasing global seafood demand has pressured aquafeed ingredient inputs, which traditionally rely on finite marine fish for dietary protein sources (Froehlich et al., 2018; FAO, 2020). Insect meal possesses essential properties, namely good nutritional content, environmental suitability, consumer acceptance, scalability, and price competition, and thereby has been identified as the greatest potential as protein sources for aquafeeds over the next decades (Hua et al., 2019; Gasco et al., 2020b; Gasco et al., 2020a). Among seven insect species (two flies, two worms, and three crickets) approved for use in aquafeeds by the European Commission (Regulation 2017/893, 24 May 2017), black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), and yellow

mealworm (*Tenebrio molitor*) have received the most interests of research (Mastoraki et al., 2020; Hua, 2021).

The use of *T. molitor* larvae meal (TM) in fish feed has been investigated extensively. Substantial replacement of fishmeal by TM without detrimental impact on growth performance or feed conversion ratio has been reported, for example, red sea bream (*Pagrus major*) (100% substitution) (Ido et al., 2019), whiteleg shrimp (*Litopenaeus vannamei*) (100%) (Panini et al., 2017), African catfish (*Clarias gariepinus*) (80%) (Ng et al., 2001), yellow catfish (*Pelteobagrus fulvidraco*) (75%) (Su et al., 2017), and rainbow trout (*Oncorhynchus mykiss*) (67%) (Belforti et al., 2015). Dietary TM has been shown to alter fish immune responses (Su et al., 2017; Henry et al., 2018b; Henry et al., 2018a; Sankian et al., 2018; Song et al., 2018), gut microbiota diversity (Antonopoulou et al., 2019; Józefiak et al., 2019) and meat traits, including fatty acid profile,

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in mandarin fish (*Siniperca scherzeri*) (Sankian et al., 2018), rainbow trout (Belforti et al., 2015; Iaconisi et al., 2018), *P. bogaraveo* (Iaconisi et al., 2017), Nile tilapia (*Oreochromis niloticus*) (Sánchez-Muros et al., 2016), and European sea bass (*Dicentrarchus labrax*) (Gasco et al., 2016). Consequently, these alterations may influence the physiology of insect meal-fed aquatic animals. Swimming performance and metabolic activity assessment are considered important proxy for the physiological traits of fishes (Allen et al., 2021). The former variable is commonly assessed via critical swimming speed ( $U_{crit}$ ) (Brett, 1964), while oxygen consumption ( $MO_2$ ) is the measurement of the latter, and of other metabolic endpoints, such as cost of transport (COT), the energy cost to transport unit of body mass over one unit of distance (McPhee and Janz, 2014). Those variables are sensitive indicators of physiological stress (Brett, 1972), dietary nutrition (Martos-Sitcha et al., 2018), and meat trait alteration of fish (Hammer, 1995; McKenzie et al., 1998; Wagner et al., 2004; Chatelier et al., 2006). In particular, muscle fatty acid profile was reported to affect swimming performance of Atlantic salmon (*Salmo salar*) (McKenzie et al., 1998; Wagner et al., 2004), European seabass (Chatelier et al., 2006), and Arctic charr (*Salvelinus alpinus*) (Pettersson et al., 2010). In addition, the fast-growing of aquaculture has placed the importance of introducing new farmed candidates and animal welfare, physiological indicators of swimming performance and metabolic rate are, therefore, beneficial for aquaculture guidelines, such as system design, towards better growth, health, and welfare (Martins et al., 2012; Allen et al., 2021).

European perch (*Perca fluviatilis*) is a novel candidate for aquaculture diversification in Europe and, along with other percids fish species, will promptly become an established aquaculture sector in Europe (Polcar et al., 2019). Fillets of intensively cultured perch have excellent nutritional value, particularly beneficial fatty acids (Stejskal et al., 2011). Dietary insect meal (*Hermetia illucens*) was reported to significantly modify meat quality, especially the fatty acid profile of European perch relative to insect-free diet (Stejskal et al., 2020), which could alter the above-mentioned physiological indicators. These indicators become more critical for grow-out production of percids mainly held in recirculated aquaculture system (RAS), which is moving towards optimal operation system (Steenfeldt et al., 2015; Polcar et al., 2019).

Thus far, the measure of swimming performance, metabolic activities of insect meal-fed European perch could provide useful information for the future farming practice of percid fish, especially RAS designed system and for other insect-fed fish species. The goal of the present study was to determine critical swimming speed, oxygen consumption, cost of transport, and physiological response of European perch fed dietary defatted mealworm *T. molitor* larvae meal. We also explored predictable factors which may influence the swimming performance of European perch.

## 2. Materials and methods

### 2.1. Ethics statement

The experimental procedures were performed under guidelines of the European Communities Directive (No. 2010/63/EU) on the protection of animals used for scientific purposes and have been approved by the Czech Ministry of Health (MSMT-6744/2018-2).

### 2.2. Experimental diets

Defatted TM was obtained from a commercial source (NovoProtein, FISHAG EDELHOF GmbH, Wien, Austria). Nutritional composition and fatty acid profile of TM are presented in Tables 1, 3.

Four isonitrogenous and isoenergetic diets were formulated, with the control diet (TM0) containing fishmeal as the main protein source and three diets with TM larvae substituted for fishmeal at 25, 50, and 75% (TM25, TM50, TM75) (Table 1). The experimental diets were produced at the EXOT HOBBY s.r.o., Czech Republic, using a commercial twin-

**Table 1**

Ingredients and proximate composition of *Tenebrio molitor* larvae meal and experimental diets.

	Fishmeal	TM	TM0	TM25	TM50	TM75
<i>Ingredients (%)</i>						
Soybean concentrate			29.0	29.0	29.0	29.0
Fishmeal	27.1	20.3	13.5	6.8		
<i>Tenebrio molitor</i>	0.0	6.8	13.5	20.3		
Soybean meal	14.5	14.5	14.5	14.5		
Corn flour	9.7	9.7	9.7	9.7		
Fish oil	7.7	7.7	7.7	7.7		
Rapeseed oil	5.8	5.8	5.8	5.8		
Methionine <sup>a</sup>	0.8	0.8	0.8	0.8		
Lysine <sup>b</sup>	0.5	0.5	0.5	0.5		
Valine <sup>c</sup>	0.2	0.2	0.2	0.2		
L-Threonine <sup>d</sup>	0.05	0.05	0.05	0.05		
Vitamins & minerals <sup>e</sup>	0.8	0.8	0.8	0.8		
Additives <sup>f</sup>	4.0	4.0	4.0	4.0		
<i>Proximate composition (dry basis)</i>						
Dry matter (%)	96.5	95.0	94.8	95.7	95.6	95.6
Crude protein (%)	71.2	71.1	47.5	48.7	47.4	47.2
Crude lipid (%)	7.9	8.5	16.3	13.9	15.6	17.0
Ash (%)	14.0	7.1	8.9	9.0	8.3	7.6
Fibre (%)	1.24	2.8	2.0	2.0	2.2	2.3
Nitrogen-free extract (%) <sup>g</sup>	1.3	5.5	19.5	21.8	22.3	21.6
Gross energy (MJ/kg) <sup>h</sup>	20.1	21.1	21.0	20.8	21.2	21.5

<sup>a</sup> Adisseo, China.

<sup>b</sup> Inner Mongolia Eppen Biotech Co., Ltd.

<sup>c</sup> Ajinomoto Animal Nutrition Europe.

<sup>d</sup> Ningxia Eppen Biotech, China.

<sup>e</sup> Aminovitan Sak, Trouw Nutrition Biofaktory s.r.o, Czech Republic.

<sup>f</sup> Feed limestone (0.5%); Pentasodium triphosphate (Fosfa a.s, Czech Republic) (0.5%) and binder (NutriBind, Adisseo, China) (3.0%).

<sup>g</sup> Nitrogen-free extracts (NFE) = dry matter - (crude protein + crude lipid + ash + fibre).

<sup>h</sup> Gross energy (MJ/kg) as gross energy content of protein (23.6 MJ/kg), lipid (39.5 MJ/kg) and NFE (17.2 MJ/kg).

screw extruder (Saibainuo, China). All finely grounded ingredients were mixed in a feed mixer HLJ-700/C (Saibainuo, China), followed by adding oil and water to form a mixture, subsequently extruded with 2-mm diameter pellets. Temperature during the extrusion process ranged 96–106 °C.

### 2.3. Fish and rearing facilities

European perch juveniles were obtained by artificial propagation (Anapartner, Prague, Czech Republic) and transported in oxygenated 1 m<sup>3</sup> tanks to the Research Institute of Fish Culture and Hydrobiology. Fish underwent two weeks of adaptation to the experimental facility and were fed a commercial diet.

Eighty-two fish (body weight 20.81 ± 3.36 g, total length 11.77 ± 0.72 cm) were randomly assigned to each of sixteen black circular 180 L tanks (four per diet) connected in RAS. Water inflow of 6.5 L/min in combination with stone aeration created a constant clockwise flow of 4.6 cm/s. Other parameters included photoperiod of 12 h:12 h (light:dark), light intensity 58.6 lx, water temperature 22.44 ± 0.66 °C, pH 7.00 ± 0.29, oxygen saturation 80.41 ± 8.02%, ammonia-N 0.28 ± 0.16 mg/L, nitrite nitrogen <0.45 mg/L.

Fish were fed daily at 7.00, 9.00, 11.00, 13.00, and 15.00 using automatic feeders (EHEIM Twins, Deizisau, Germany) for 119 days. Unconsumed feed was removed after each feeding and dried to calculate daily feed intake.

### 2.4. Swimming experiment

At the conclusion of rearing, evaluation of swimming performance was conducted in a 10 L 40 × 10 × 10 cm swimming tunnel respirometer

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(Loligo systems, Tjele, Denmark) submerged in a buffer tank that was connected to an aerated temperature-controlled 100 L reservoir tank allowing continuous water exchange. To ensure adequate dissolved oxygen concentration during swimming performance testing, the swimming chamber was connected to a buffer tank via a flush pump (20 L/min, Eheim GmbH, Deizisau, Germany). Throughout the swimming trial, dissolved oxygen remained above 70%, ensuring sufficient oxygen during swimming test (Hammer, 1995; Tudorache et al., 2008; Thomas and Janz, 2011). Dissolved oxygen and temperature in the swimming chamber were continuously recorded, using a fiberoptic oxygen probe and a temperature probe connected to a Witrox 1 (Loligo Systems, Tjele, Denmark). Water temperature was maintained at  $23 \pm 0.15$  °C and light intensity at  $\sim 60$  lx at the surface of the swimming system. Because of the sensitivity of perch to external stressors (Jentoft et al., 2005; Langeland et al., 2016), the system was covered by black plastic sheeting to prevent disturbance from surroundings. The system was connected to AutoResp® software (Loligo systems, Tjele, Denmark) to control and record water velocity and dissolved oxygen in the swimming chamber.

Eighty fish (20 fish/diet group) were used in swimming tests following 24 h without feeding. Fish were selected rotationally among diet treatments as described previously (Wagner et al., 2004) to minimize time differences and any potential additional growth among groups.

Fish were lightly anesthetized with MS 222 (50 mg/L), and body weight was measured to the nearest 0.01 g and total length, width, and depth to the nearest 0.01 cm (Table 2). Individual fish were immediately transferred to the swimming tunnel and acclimated to test conditions for 30 mins with water flow velocity of 5 cm/s, which closely approximated that of the rearing tank.

At the start of the swimming test, the tunnel was completely closed (no water exchange with the surrounding bath). The initial velocity was set at 5 cm/s and increased in 2 cm/s increments every 60 s until fatigue. The small increments of velocity and time in our protocol were set to minimize stress of tested fish. A similar protocol has been conducted by Peterson (1974). The swimming test was terminated when the fish remained at the rear grid for more than 10 s.

Critical swimming speed ( $U_{crit}$ , cm/s) was calculated according to Brett (1964):

$U_{crit} = U_{max} + (T_{max}/T_{interval} * U_{interval})$ , where  $U_{max}$  is highest velocity recorded at fatigue (cm/s);  $U_{interval}$  is velocity interval (2 cm/s);  $T_{max}$  is spent time at fatigued velocity; and  $T_{interval}$  is the time interval (60 s).

Oxygen consumption ( $MO_2$ , mg/kg/h) was calculated as:

$MO_2 = ([O_2]_{t0} - [O_2]_{t1}) * (V/t) * (1/BW)$ , where  $[O_2]_{t0}$  is oxygen concentration at the start of the swimming test (mg  $O_2$ /L),  $[O_2]_{t1}$  is oxygen concentration at the terminal of the swimming test (fish fatigue) (mg  $O_2$ /L);  $V$  is volume of swimming chamber minus volume of fish (L). In fish, 1 kg is equivalent to 1 L (Boldsen et al., 2013);  $t = t1 - t0$  (hours); BW, body weight.

Cost of transport (COT, J/kg/m) was calculated as described by McPhee and Janz (2014).

$COT = (MO_2 * 14.2)/U_{swim}$ , where oxy-caloric value of 14.1 J/mg  $O_2$ ;  $U_{swim}$  is corresponding swimming speed (m/s).

**Table 2**  
Morphometrics (Mean  $\pm$  SD,  $n = 20$ /group) of European perch fed experimental diets for 119 days used in the swimming performance test.

Diets	Body weight g	Total length cm	Condition factor <sup>a</sup>
TM0	84.22 $\pm$ 9.85 <sup>a</sup>	18.40 $\pm$ 0.62 <sup>a</sup>	1.35 $\pm$ 0.08
TM25	84.82 $\pm$ 10.33 <sup>a</sup>	18.19 $\pm$ 0.72 <sup>a</sup>	1.41 $\pm$ 0.11
TM50	82.86 $\pm$ 10.25 <sup>a</sup>	18.03 $\pm$ 0.60 <sup>a</sup>	1.41 $\pm$ 0.10
TM75	70.70 $\pm$ 8.01 <sup>b</sup>	17.16 $\pm$ 0.56 <sup>b</sup>	1.40 $\pm$ 0.11

Different superscripts within a column indicate significant difference at  $P < 0.05$ .

<sup>a</sup>Condition factor = (Body weight/total length<sup>3</sup>)  $\times$  100.

## 2.5. Sampling procedures

After a 119-day feeding trial fish not used for swimming tests, hereafter non-exercised fish, and fish experienced swimming tests till fatigue, hereafter exercised fish, were sampled as following procedures:

Twelve non-exercised fish (12 fish/diet group) and exercised fish (20 fish/diet group) were euthanized using an overdose of 125 mg/L (Dezfuli et al., 2013) MS222. Approximate 1 mL of blood was drawn from the caudal vein and centrifuged at 3000 RPM for 10 min with obtained serum was held at  $-80$  °C for further analysis. Subsequently, those fish were dissected for assay of post-mortem pH and water content. Fish muscle was assessed for pH using Testo 206-pH 2 (Testo Inc., Lenzkirch, Germany) inserting into muscle, then scale-off muscle was ground in a mincer (IKA Mill A11 Basic, Staufen, Germany), subsequently oven-dried (105 °C) to constant weight for water content.

Forty-eight non-exercised fish (12 fish/group) were taken and stored at  $-20$  °C for subsequent analysis of whole-body proximate composition.

## 2.6. Chemical and biochemical analyses

Feed samples were finely ground (IKA Mill A11 Basic, Staufen, Germany) and analyzed for dry matter (AOAC, n. 934.01), crude protein (AOAC, n. 984.13), crude fibre by the Henneberg-Stohmann method (AOAC, n. 920.86), and ash content (AOAC, n. 942.05) according to (AOAC, 2000). Lipid and fatty acid profiles in both diets and whole-body fish were determined according to the methodology described by Mráz and Pickova (2009).

Serum biochemical parameters and blood ions ( $Na^+$ ,  $K^+$ ,  $Cl^-$ ) were analyzed using an auto-analyzer Architect c4000 (Abbott Laboratories, Illinois, USA) with commercial reagent kits (Abbott Diagnostics, Illinois, USA). Cortisol concentration was analyzed using a reagent kit and automated immune-analyzer Immulite 2000 XPI (Siemens Healthineers, Siemens Healthcare GmbH, Erlangen, Germany).

## 2.7. Statistical analyses

All statistical analyses were performed using the R Statistic Package; R Development Core Team 2009–2019, available at [www.r-project.org/](http://www.r-project.org/). All data were assessed for normality using Shapiro-Wilk test and homogeneity of variance using Levene's test. Effect of fish weight, length, width, depth on critical swimming speed, oxygen consumption, and cost of transport was tested with Covariance Analysis (ANCOVA), and no significant influence [ $U_{crit}$ , (weight:  $F$ -statistic = 0.85,  $P$ -value = 0.36; length: 0.75, 0.39; width: 0.36, 0.55; depth: 1.49, 0.23);  $MO_2$  (weight: 2.97, 0.09; length: 0.34, 0.94, width: 2.0, 0.16; depth: 1.56, 0.22), COT (weight: 0.77, 0.08; length: 0.75, 0.39; width: 0.54, 0.37; depth: 0.36, 0.87)] was found. Significant differences in swimming speed, oxygen consumption, cost of transport, blood biochemistry, meat traits, diet composition among diet treatments were verified using one-way ANOVA followed Tukey's honestly significant difference (HSD) as a post hoc test, when appropriate. Student's  $t$ -tests were used to test significant differences in blood biochemistry, muscle traits between exercised and non-exercised groups. Correlation matrix analysis with respect to critical swimming speed was performed on 'ggcorrplot' package (Kassambara, 2019). Correlation between nutritional composition (of diets, fish muscle, and whole-body fish) vs.  $U_{crit}$  as well as between  $MO_2$ , COT vs.  $U_{crit}$  were assessed with linear models using 'lm' function. Significant differences were considered at  $P < 0.05$ .

## 3. Results

### 3.1. Fish growth and proximate composition

After a 119-day feeding trial, fish fed experimental diets had a similar survival rate, ranged 98.48–99.09%. Morphometrics of fish used

in swimming tests is shown in Table 2. European perch fed TM75 showed significantly lower growth performance than other treatment groups ( $P < 0.05$ ). Although fish growth was impaired when *T. molitor* larvae meal was substituted for 75% of fishmeal, condition factor did not differ among dietary treatments ( $P > 0.05$ ). Feed conversion ratio significantly higher in fish fed TM75 diet (1.77) than does TM0 (1.15), TM25 (1.19), and TM50 (1.33) (standard error of the mean, 0.07).

TM contained a high amount of monounsaturated constitute of oleic acid, accounting for 35.10% total fatty acids, while possessed low levels of essential fatty acids, docosahexaenoic acid (DHA) (0.01%) and eicosapentaenoic acid (EPA) (0.04%) (Table 3). Replacement fishmeal by TM significantly increased oleic acid (OA), linoleic acid (LA), and decreased EPA, DHA ( $P < 0.05$ ) (Table 3).

There was no significant difference ( $P > 0.05$ ) on proximate composition of whole-body perch fed experimental diets, except for moisture, palmitic acid, and total saturated fatty acids (Table 4). Feeding perch with 50% fishmeal replaced by TM resulted in significantly higher moisture content compared to the fishmeal group ( $P < 0.05$ ) and significantly reduced C16:0 and total SFA ( $P < 0.05$ ). (See Table 5.)

Water content and pH were not statistically different in both specimen groups across diet treatments ( $P > 0.05$ ) (Table 5).

### 3.2. Swimming performance

Critical swimming speed, both in cm/s and BL/s, did not differ across treatment groups ( $P > 0.05$ ) (Fig. 1). The values of  $U_{crit}$  in the present study were 106.4 cm/s (interquartile range, 97.42–117.23) and 5.94 BL/s (5.43–6.49).

### 3.3. Oxygen consumption and cost of transport

No significant difference on  $MO_2$  and COT across diet treatments was found ( $P > 0.05$ ). There was a positive quadratic relationship ( $P < 0.001$ ,  $F = 36.01$ , adjusted  $R$ -square = 0.47) for oxygen consumption and respected critical swimming speed. In contrast, negative quadratic model ( $P < 0.001$ ,  $F = 169.5$ , adjusted  $R$ -square = 0.81) was observed for cost of transport (Fig. 2).

### 3.4. Physiological responses

Serum biochemistry indices of exercised and non-exercised fish fed

**Table 4**

Whole-body composition (% as wet weight) and fatty acid profile (as a percentage of total fatty acid) of European perch *Perca fluviatilis* fed experimental diets for 119 days.

	TM0	TM25	TM50	TM75	SEM
Moisture	66.66 <sup>b</sup>	67.27 <sup>ab</sup>	68.81 <sup>a</sup>	67.92 <sup>ab</sup>	0.27
Crude protein	19.66	19.71	19.82	19.07	0.24
Crude lipid	11.85	11.57	10.85	11.80	0.24
Ash	3.58	3.27	3.42	3.25	0.10
<i>Fatty acid profile</i>					
C16:0	14.43 <sup>ab</sup>	15.86 <sup>a</sup>	14.92 <sup>ab</sup>	13.25 <sup>b</sup>	0.33
C16:1	6.30	7.51	7.26	6.23	0.27
C18:0	1.22	1.20	1.24	0.99	0.04
C18:1n9	44.07	42.10	41.52	39.08	0.92
C18:2n6	14.74	14.56	15.07	15.14	0.11
C18:3n3	3.03	3.26	3.10	3.16	0.22
C20:5n3 (EPA)	1.79	1.74	1.96	1.69	0.07
C22:6n3 (DHA)	6.82	6.27	6.98	5.88	0.28
SFA	19.08 <sup>ab</sup>	20.44 <sup>a</sup>	19.61 <sup>ab</sup>	17.14 <sup>b</sup>	0.46
MUFA	52.24	51.50	50.78	47.18	1.19
PUFA	27.87	27.31	28.61	35.23	1.62
n-3	11.87	11.48	12.23	10.90	0.40
n-6	16.00	15.82	16.39	24.33	1.94
PUFA/SFA	1.46	1.34	1.46	2.22	0.17
n-3/n-6	0.74	0.73	0.75	0.59	0.04

Fatty acids with less than 1% total fatty acids in experimental diets (C10:0, C12:0, C13:0, C14:1, C15:0, C15:1, C17:0, C17:1, C16:3, C18:1n9 trans, C18:1n7, C18:2n6 trans, C18:3n6, C20:0, C21:0, C20:3n6, C20:3n3, C20:4n6, C22:0, C24:0, C24:1n9, C22:5n6) were not presented in the table but included in fatty acids group calculation. Data are means and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly ( $P < 0.05$ ).

experimental diets were found insect meal-dose independent ( $P > 0.05$ ), except serum  $Cl^-$  where exercised fish fed TM75 showed significantly higher concentration than does control group ( $P < 0.05$ ) (Fig. 3).

The fatigued swimmers significantly increased serum AST, glucose, and cortisol regardless of dietary treatment compared to non-swimmers ( $P < 0.05$ ). This was also observed for serum  $K^+$  concentration in TM50 ( $P < 0.05$ ). Exercised fish fed TM0 and TM75 also significantly elevated LDH serum activities relative to non-exercised specimens ( $P < 0.05$ ), while this pattern was not significant in TM25 and TM50 groups ( $P > 0.05$ ).

Exercised fish significantly increased muscle water content compared with non-exercised fish fed TM50 ( $P < 0.05$ ).

**Table 3**

Fatty acid composition (as a percentage of total fatty acid) of fishmeal, defatted *Tenebrio molitor* larvae meal and experimental diets.

	Fishmeal <sup>a</sup>	TM	TM0	TM25	TM50	TM75	SEM
C14:0	7.9	1.30	1.57	1.57	1.49	1.47	0.03
C16:0	23.0	20.68	9.30	9.18	8.95	9.14	0.07
C16:1		2.05	2.04	2.00	1.94	1.98	0.02
C18:0	5.3	9.08	2.68	2.57	2.59	2.69	0.03
C18:1n9 (OA)	8.4	35.10	46.65 <sup>b</sup>	46.07 <sup>ab</sup>	47.27 <sup>ab</sup>	49.77 <sup>a</sup>	0.52
C18:2n6 (LA)	1.1	25.02	17.14 <sup>b</sup>	17.43 <sup>b</sup>	17.80 <sup>b</sup>	18.89 <sup>b</sup>	0.22
C18:3n3 (LNA)	0.2	0.96	6.19	6.07	6.23	4.31	0.51
C20:1n9	0.3	0.26	3.02 <sup>a</sup>	2.92 <sup>a</sup>	2.03 <sup>ab</sup>	0.41 <sup>b</sup>	0.36
C20:5n3 (EPA)	14.1	0.04	2.54 <sup>a</sup>	2.38 <sup>b</sup>	2.15 <sup>c</sup>	2.06 <sup>c</sup>	0.06
C22:6n3 (DHA)	16.1	0.01	4.26 <sup>a</sup>	3.96 <sup>b</sup>	3.43 <sup>c</sup>	3.23 <sup>c</sup>	0.13
SFA	36.1	33.32	15.25	14.96	14.62	14.95	0.12
MUFA	20.6	38.95	52.70	53.06	53.63	54.42	0.35
PUFA	37.3	26.55	31.89	31.51	31.01	29.90	0.45
n-3	34.7	1.05	13.26	12.67	12.08	9.88	0.57
n-6	2.7	25.50	18.63 <sup>b</sup>	18.84 <sup>b</sup>	18.93 <sup>b</sup>	20.01 <sup>a</sup>	0.17
PUFA/SFA	1.03	0.77	1.69	1.70	1.69	1.71	0.04
n-3/n-6	12.9	0.04	0.71	0.67	0.64	0.49	0.03

<sup>a</sup> Barroso et al. (2014) (Proximate composition (% dry matter), crude protein: 73%; ether extract: 8.2%; ash: 18%; nitrogen-free extract: 0.8%). Fatty acids with less than 1% total fatty acids in experimental diets (C10:0, C12:0, C13:0, C14:1, C15:0, C15:1, C17:0, C17:1, C16:3, C18:1n9 trans, C18:1n7, C18:2n6 trans, C18:3n6, C20:0, C21:0, C20:3n6, C20:3n3, C20:4n6, C22:0, C24:0, C24:1n9, C22:5n6) were not presented in the table but included in fatty acids group calculation. LNA: linolenic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. Data are means and pooled standard error of the mean (SEM). Means in the same row with different superscripts letters differ significantly ( $P < 0.05$ ).

# Does dietary *Tenebrio molitor* affect swimming capacity, energy use, and physiological responses of European perch *Perca fluviatilis*?

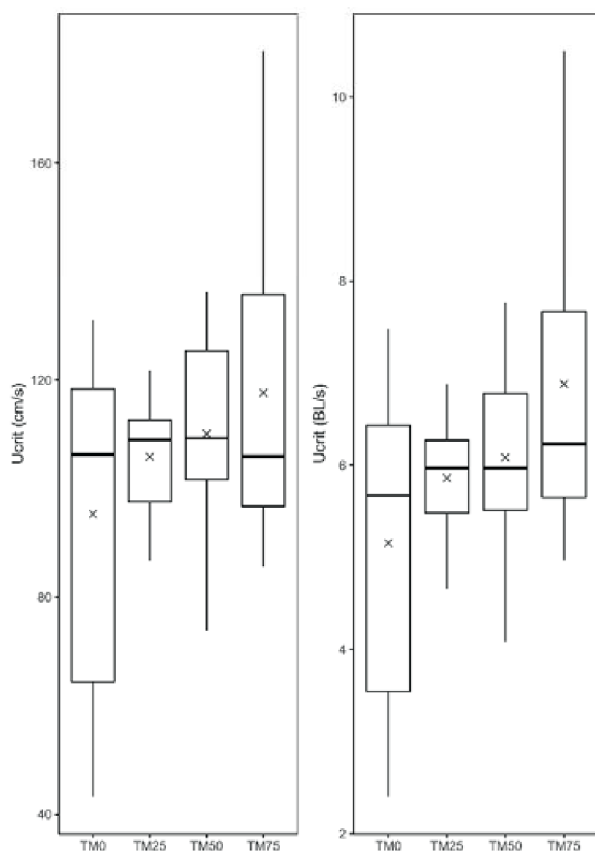
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**Table 5**  
Muscle properties of exercised ( $n = 48$ ) and non-exercised ( $n = 48$ ) European perch fed *T. molitor* larvae meal diets. Data are mean  $\pm$  SD.

Variable	Experimental diets			
	TM0	TM25	TM50	TM75
<b>pH</b>				
Non-exercised	6.74 $\pm$ 0.04	6.74 $\pm$ 0.06	6.77 $\pm$ 0.04	6.80 $\pm$ 0.01
Exercised	6.77 $\pm$ 0.03	6.71 $\pm$ 0.10	6.78 $\pm$ 0.01	6.73 $\pm$ 0.10
<b>Water content (%)</b>				
Non-exercised	74.44 $\pm$ 0.16	74.27 $\pm$ 0.36	74.72 $\pm$ 0.54 <sup>b</sup>	73.96 $\pm$ 1.98
Exercised	74.76 $\pm$ 0.64	75.53 $\pm$ 1.06	75.61 $\pm$ 0.59 <sup>a</sup>	75.28 $\pm$ 0.81

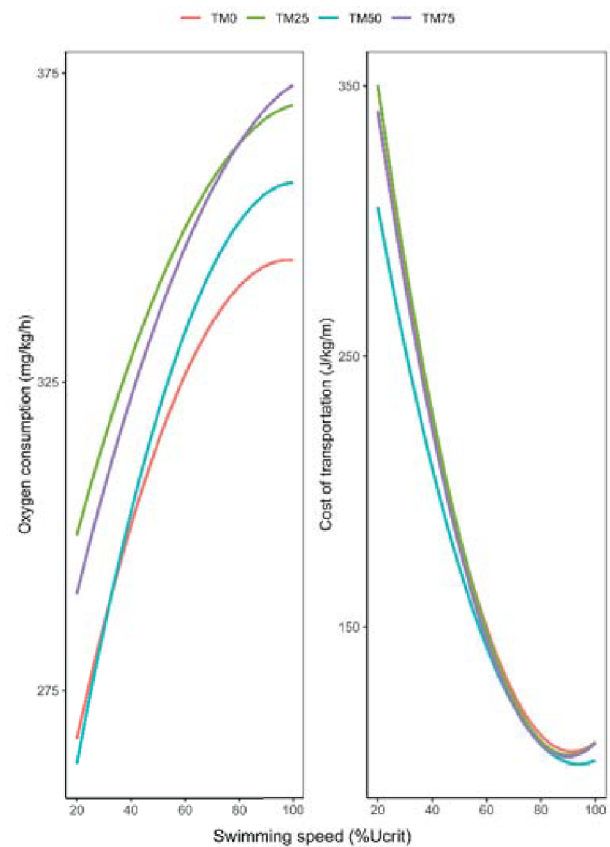
Superscripts indicate significant differences between exercised and non-exercised perch within a diet group ( $P < 0.05$ ).



**Fig. 1.** Critical swimming speed ( $U_{crit}$ , cm/s and BL/s) of European perch (*Perca fluviatilis*) fed experimental diets. The black 'x' in boxes represents the mean value, the horizontal line within boxes represents median separating interquartile range (upper quartile and lower quartile).

### 3.5. Correlation matrix between nutritional factors and critical swimming speed

A significant correlation in both diet and whole-body composition relative to swimming performance was observed (Fig. 4). Three factors that influenced the perch's swimming performance in the present study included DHA, EPA, and MUFA matrixes. There was a negative linear correlation between DHA and  $U_{crit}$  (estimated correlation =  $-0.96$ ,  $P = 0.04$ ), similar model was found EPA (estimated correlation =  $-0.96$ ,  $P = 0.04$ ). While  $U_{crit}$  increased linearly with increasing dietary MUFA (estimated correlation =  $0.96$ ,  $P = 0.04$ ). No significant relationship



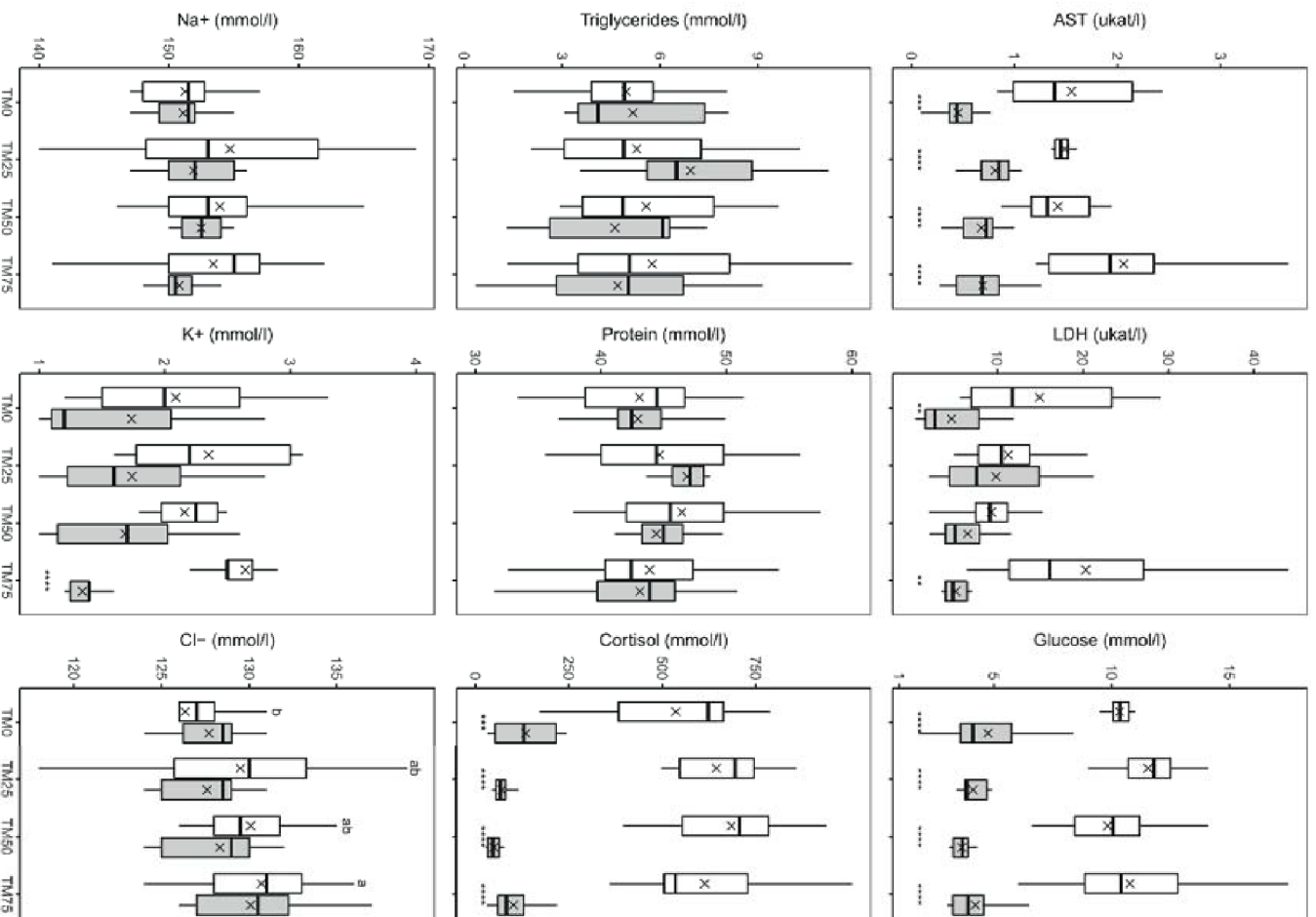
**Fig. 2.** Oxygen consumption ( $MO_2$ , mg/kg/h) and cost of transport (COT, J/kg/h) with respect to critical swimming speed of European perch (*Perca fluviatilis*) fed experimental diets.

among other dietary composition (protein, lipid, gross energy) on critical swimming speed ( $P > 0.05$ ). We found a significant and strong linear correlation between oleic acid in whole-body fish and swimming capacity (estimated correlation =  $-0.98$ ,  $P = 0.02$ ). Other whole-body proximate composition did not significantly influence fish swimming ( $P > 0.05$ ) (Fig. 4).

### 4. Discussion

As a part of many published works investigating the effects of insect meals as the alternative protein source in diets for aquatic animals, our work contributed findings on swimming performance, metabolism rate, cost of transport of European perch, which was insect meal levels independent. This could be an important implication for future percid aquaculture gearing towards established aquaculture sector in Europe (Polcar et al., 2019), since swimming performance of fish represents a useful indicator of farmed fish welfare (Martins et al., 2012), the use of insect meals in aquafeeds could benefit future aquaculture industry in term of animal welfare. We also explored insights on dietary and fish composition factors which could influence swimming capacity of fish and found that fatty acids rather than other macronutrients in diets and fish body significantly was the significant influencers.

In accordance with our results, previous studies have confirmed that dietary protein sources did not affect swimming capacity (Wilson et al., 2007; Chai et al., 2013), cost of transport (Wilson et al., 2007), and oxygen consumption (Gerle and Pirhonen, 2017) of fish. Known as a long-distance migrant species, *P. fluviatilis* exhibited high swimming



**Fig. 3.** Serum biochemistry of exercised (white boxplots) and non-exercised (grey boxplots) European perch (*Percu flavescens*) fed experimental diets. The black ‘\*’ shows the significant difference between exercised and non-exercised fish (Student’s *t*-test, \* $P < 0.001$ , \*\* $P < 0.0001$ , \*\*\* $P < 0.00001$ , \*\*\*\* $P < 0.000001$ ). Different lowercase letters denote significant differences in serum biochemistry indices of exercised fish fed experimental diets ( $P < 0.01$ ). Significant differences in serum biochemistry in non-exercised perch were absent. AST = aspartate aminotransferase; LDH = lactate dehydrogenase.

speeds. Tudorache et al. (2008) evaluated the critical swimming speed of 17.8 cm perch and reported a  $U_{crit}$  of 113.04 cm/s or 6.35 BL/s. Our findings were also consistent with recent findings (Cano-Barbacid et al., 2020), reporting  $U_{crit}$  of 97.7 cm/s or 5.97 BL/s for 16.37 cm length perch, similar to our data (interquartile range, 97.42–117.23 cm/s or 5.43–6.49 BL/s). Štejskal et al. (2009) reported oxygen consumption of

European perch (48.3–333.6 g, body weight) at 23 °C was 261.9–279.7 mg O<sub>2</sub>/kg/h. Similarly, perch (18.5–56.5 g) kept at the same temperature consumed 150.1–278.5 mg O<sub>2</sub>/kg/h (Zakcs et al., 2003). Those were aligned with our oxygen consumption results at 20%  $U_{crit}$  (interquartile range, 265.2–302.0 mg/kg/h).

The contrasting trends, positive for MO<sub>2</sub> and negative for COT

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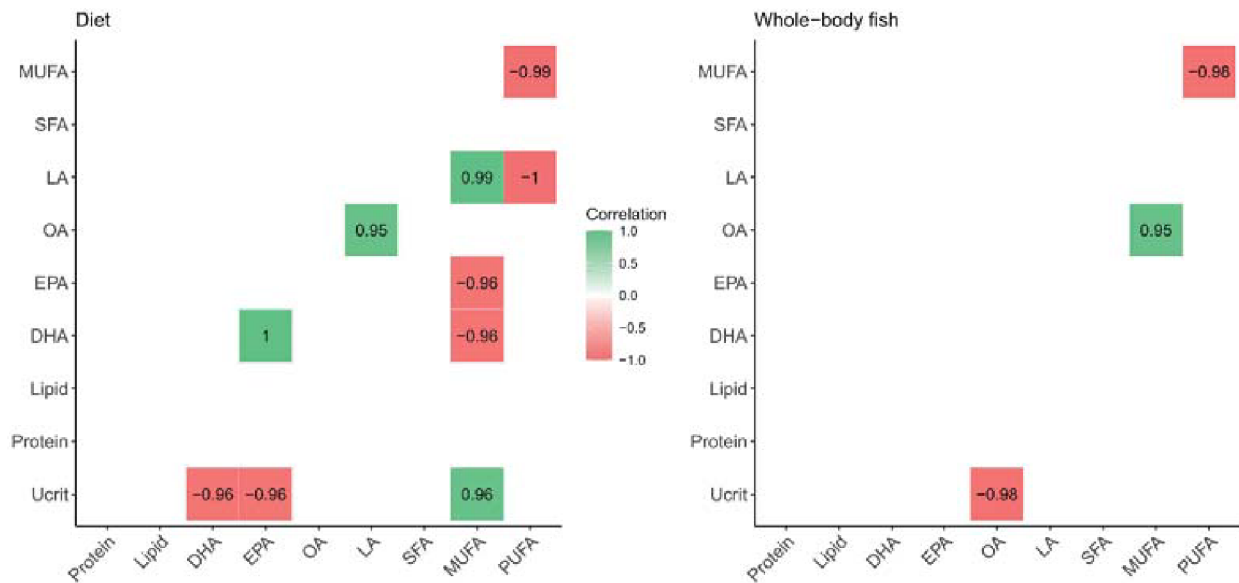


Fig. 4. Correlation matrix between critical swimming speed and nutritional composition of diet, whole-body fish. The red and blue boxes indicate significantly negative and positive correlations, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

relative to critical swimming speed, in our study has also been reported earlier in fish species (Brett, 1964; Thomas and Janz, 2011; Yan et al., 2013; McPhee and Janz, 2014; Martos-Sitcha et al., 2018; Rubio-Gracia et al., 2020). In any case, oxygen consumption increased with the velocity acceleration of the swimmer in response to current flow drag (Martos-Sitcha et al., 2018). This demonstrates that perch tended to take up more oxygen when swimming against the higher water flow while using less energy to transport one unit of body mass over one unit of distance. The relationship between  $U_{crit}$  and  $MO_2$  in our study was well described by a positive quadratic model, to which oxygen consumption of perch sharply rocketed until reaching approximately 70–80%  $U_{crit}$  and then slightly increased till fatigue. This is a common trend for many fishes to experience swimming exercise as there was a shift from aerobic to anaerobic energy use at this critical swimming speed point (Webb, 1971; Moves and West, 1995; Burgetz et al., 1998).

During aerobic swimming, fish generate energy from triglyceride catabolism up to about 80% critical swimming speed, while anaerobic activities initiated, fuelling glycogen from glycogenolysis through glucose, subsequently transited through circulation and distributed to targeted tissues (Hammer, 1995; Moves and West, 1995). Therefore, the measure of triglycerides and glucose in serum could provide a useful explanation of how perch mediates energy during their swimming exercise. We found the concentration of triglycerides in serum was consistent in non-exercised fish as well as in exercised groups, a similar pattern was also observed for glucose. This could explain similar swimming capacity of perch fed dietary insect meal. In addition, the high swimming capacity of fish across diet groups could also be attributed to continuous exposure to mild water velocity of 4.6 cm/s for 119 days of experimental fish, as this form of training had a positive effect on  $U_{crit}$  (Hammer, 1995).

At the dietary level, our matrix analysis indicated that critical swimming speed is dietary protein, lipid independent (Fig. 4). The studies on the effect of protein and lipid sources on swimming capacity strongly supported our findings (Wilson et al., 2007; Regan et al., 2010; Gerile and Pirhonen, 2017). We also highlighted that dietary fatty acids, particularly DHA, EPA, and monounsaturated fatty acids, rather than other macro-nutrients, significantly affected European perch swimming

performance. This is consistent with a previous study on Atlantic salmon (McKenzie et al., 1998). The negative relationship of dietary n-3 PUFA relative to swimming speed in our study is unexpected and contradicts with the result from Wagner et al. (2004). In fact, diet-mediated alterations in the fatty acid composition of fish were documented to significantly affect physiological performance, including critical swimming capacity in seabass (Chatelier et al., 2006). Fatty acid composition of fish generally mirrors that of diet (Turchini et al., 2009), but the extent to which this occurs depends on the number of factors. The present study, however, found slightly modified DHA, EPA, and some MUFA constituents of whole-body fish from the respective diet (Tables 3, 4). This was because European perch exhibited a high capacity of biosynthesis of DHA from EPA and C18 precursors (Xu et al., 2001; Xu and Kestemont, 2002). Consequently, a considerable increase in DHA, and a remarked decrease in EPA and linolenic acid in whole-body perch compared to respective diets (Tables 3, 4) were observed. McKenzie et al. (1998) suggested that 18 carbon unsaturated fatty acids were responsible for altering the swimming capacity of salmon. Our correlation analysis indicated that oleic acid strongly influenced exercise activities of European perch, following a negative correlation, which is in agreement with the finding of Wagner et al. (2004). Accordingly, oleic acid may, in combination with other fatty acids, impair carnitine palmitoyl transferase activities - the enzyme enhancing fatty acid metabolism in the red muscle (Wagner et al., 2004).

Fish after exposure to exhaustive exercise or stressors, regulated assortment of physiological changes involving primary, secondary, and tertiary response (Barton, 2002). The primary response was the elevation of cortisol level from the head kidney into blood, while the second response resulted in alteration in blood chemistry, tissue composition, ion concentrations, and increased glucose, and the whole-body organism performance was referred to as the third response (Eissa and Wang, 2016). As expected, exercised perch in our study showed nearly double glucose and ten-fold the cortisol concentration of non-exercised perch (Fig. 3). The previous studies on perch *P. fluviatilis* following acute stress also reported significant elevation of these products (Acerete et al., 2004; Jentoft et al., 2005). Cortisol levels of non-exercised perch in our study (interquartile, 46.9–116.0) were comparable with the 45 ng/mL

(124 mmol/L) reported by Acerete et al. (2004) and relatively higher than the basal level in fish (Barton, 2002). This suggests the high susceptibility of European perch with stressors and low capacity of acclimatization. Increase glucose concentration in blood excreted from the liver is a typical stress response of fish to source fuel energy during exercise (Maves and West, 1995).

Electrolyte Na<sup>+</sup> was consistent across diet treatments and within fish treatment group, while K<sup>+</sup> in fish fed TM75 diet was significantly increased in exercised compared to non-exercised fish. Increased potassium concentration has been recorded in exercised rainbow trout, possibly resulting from loss of K<sup>+</sup> from swimming-involved muscles during depolarization, which replaces K<sup>+</sup> with NH<sub>4</sub><sup>+</sup>, and subsequent K<sup>+</sup> take-up by erythrocytes (Nielsen and Lykkeboe, 1992; Wicks et al., 2002). This elevation could also be related to decreased blood pH and oxygen pressure (Cnaani et al., 2014) and osmoregulatory dysfunction (Imanpoor et al., 2017). We also observed significant elevation on Cl<sup>-</sup> concentration in exercised groups fed TM75 compared to TM0. This may be due to the absorption of Cl<sup>-</sup> during lactate and/or anion in the white muscle (Wood, 1991).

In the present study, the independence of hematological indices on dietary mealworm meal supports previous studies of common carp (*Cyprinus carpio* var. Jian) (total protein, AST, glucose, and triglyceride) (Li et al., 2017), *D. labrax* (glucose, protein, and triglyceride) (Magalhães et al., 2017), *S. scherzeri* (total protein, AST, and triglyceride) (Sankian et al., 2018), rockfish (*Sebastes schlegelii*) (triglyceride, protein, and AST) (Khosravi et al., 2018), and *O. niloticus* (total protein, glucose) (Tubin et al., 2020). Aspartate aminotransferase is a non-plasma-specific enzyme used as a proxy for liver damage (Gharaei et al., 2011), involved in protein synthesis (Masola et al., 2008) and glucose production via gluconeogenesis (Tejpal et al., 2009). We evaluated aminotransferase activity in serum of exercised and non-exercised perch and found fatigued perch fed experimental diets significantly elevated AST activities relative to non-exercised specimens (Fig. 3). The present work also showed no significant effects on hematological glucose or protein concentration of either exercised or non-exercised perch fed diet treatments. Therefore, the source of AST's elevation could be hepatic cell damage, with subsequent release into the blood circulation. Acute exercise induced hepatic injury, thereby increased AST enzyme activity in mammals (Zhao et al., 2010; Ruhee et al., 2020). This, in conjunction with significantly higher lactate dehydrogenase, a biomarker of cell injury (Gharaei et al., 2011), in TM0 and TM75 (Fig. 4), could further confirm the susceptibility of cells of perch fed fishmeal and insect meal diets.

Dietary *T. molitor* meal had no significant effect on muscle water content of non-exercised perch (Table 5), confirming results observed in blackspot sea bream (Iaconisi et al., 2017), mandarin fish (Sankian et al., 2018), rainbow trout (Iaconisi et al., 2018), and rockfish (Khosravi et al., 2018). Similarly, muscle water content of fatigued perch did not differ among experimental diets (Table 5). This is in agreement with Regan et al. (2010), who reported no significant effect of diet on muscle water content of exercised chinook salmon held in freshwater. The significant difference in muscle water content of two groups of perch fed TM50 could be linked to lipid depletion, as they show an inverse relationship (Kause et al., 2002). The muscle pH of non-exercised perch in the present study was similar to that reported by (Komolka et al., 2020) for farmed European perch. Our diets did not affect muscle pH, which is similar to observations of rainbow trout (Iaconisi et al., 2018), but Iaconisi et al. (2017) reported significantly lower pH in the muscle of sea bream fed a diet containing 50% *T. molitor* replacement of fishmeal compared to a fishmeal diet. Basal pH value is species-specific as well as associated with dietary stressors, anaerobic glycolysis, and buffer substances or free amino acid retention in exercised fish (Bugeon et al., 2003; Iaconisi et al., 2017; Komolka et al., 2020).

Our study suggests the potential use of insect meal as an aquafeed ingredient for farmed fish welfare. Future aquaculture of percid fish could consider the high swimming behavior and susceptibility of

European perch to stressors for an adequately designed and operated RAS system. Attention should also be paid to diet formulation, especially for the fatty acid profile, which was the critical factor that affects critical swimming speed of European perch.

#### Authorship statement

Manuscript title: Does dietary *Tenebrio molitor* affect swimming capacity, energy use, and physiological responses of European perch *Perca fluviatilis*? All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Aquaculture Journal.

#### Authorship contributions

Tran Quang Hung: Contribution to design, conduct experiment, analyze of the data, and manuscript preparation.

Vlastimil Stejskal: Conception and the revision of the manuscript.

Hien Van Doan: Contribution to manuscript preparation.

#### Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2021.736610>.

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## CHAPTER 8

### **PARTIALLY DEFATTED *Hermetia illucens* LARVA MEAL IN DIET OF EURASIAN PERCH (*Perca fluviatilis*) JUVENILES**

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My share on this work was 40%.





Article

# Partially Defatted *Hermetia illucens* Larva Meal in Diet of Eurasian Perch (*Perca fluviatilis*) Juveniles

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**Simple Summary:** The replacement of fishmeal by insect meal is a promising strategy to obtain more sustainable fish feeds, a major goal in aquaculture. Black soldier fly *Hermetia illucens* larva meal has a high crude protein and fat content, essential for omnivorous and carnivorous fish. We used partially defatted *H. illucens* larva meal as a substitute for 20%, 40% and 60% of the fishmeal in standard diets for Eurasian perch and measured its effect on growth performance, feed utilization, body indices, fish body composition and blood indices. We found no significant differences in survival, size heterogeneity, hematology indices; or in whole-body dry matter, crude protein and ether extract content. The 60% inclusion reduced final body weight, specific growth rate, feeding rate, protein efficiency ratio, condition factor and hepatosomatic index. The fish-in-fish-out index decreased proportionally with increased *H. illucens* meal inclusion. Partially defatted *H. illucens* larva meal seems to be a promising alternative to fishmeal for Eurasian perch nutrition at moderate inclusion level.

**Abstract:** Insect meal is gaining increased attention in aquafeed formulations due to high protein content and an essential amino acid profile similar to that of fishmeal. To investigate insect meal in feed for European perch *Perca fluviatilis*, a promising candidate for European intensive culture, we replaced standard fishmeal with partially defatted black soldier fly *Hermetia illucens* larva meal at rates of 0%, 20%, 40% and 60% (groups CON, H20, H40 and H60, respectively) and compared growth performance, somatic indices, hematological parameters, whole-body proximate composition and occurrence of spleen lipidosis. In addition, we assessed the economic and environmental sustainability of the tested feeds by calculating economic conversion ratio (ECR) and economic profit index (EPI). The tested groups did not differ in survival rate. Significant differences were documented in final body weight and specific growth rate, with the highest values in CON, H20 and H40. The proximate composition of fish whole-body at the end of the experiment did not differ in dry matter, crude protein or ether extract, while organic matter, ash and gross energy composition showed significant differences. The fatty acid content and n-3/n-6 ratio showed a decreasing trend with increasing *H. illucens* larva meal inclusion. No differences were found in hematological parameters among tested groups. The *H. illucens* larva meal inclusion significantly affected ECR and EPI, even at 20% inclusion level the cost of diets did not differ from the control fish meal based diet. Results suggested that 40% inclusion of *H. illucens* larva meal can be used successfully in standard diets for perch.

**Keywords:** alternative feed; insect meal; splenic lipidosis; economic and environmental sustainability

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## 1. Introduction

Intensive culture of the carnivorous freshwater Eurasian perch (*Perca fluviatilis* L.) is increasing in recirculating aquaculture systems (RAS) and represents an expanding branch of commercial fish farming in Europe. Nevertheless, as a relatively new aquaculture species, production is low [1]. It is commonly reared on feed formulated primarily for salmonids or marine fish species [2]. Diets for carnivorous species contain high levels of protein, which have been obtained from marine fishmeal (FM), considered optimal because of its balanced nutritional composition [3,4]. Currently, with FM increased cost and unsustainability [5], plant protein sources, especially soybean meal, are being used in aquaculture to decrease the dependency on FM and reduce feed costs [4]. High levels of plant protein in feeds can reduce growth performance or induce fish health issues, chiefly due to imbalance in essential amino acid (EAA) content, low feed acceptance and the presence of anti-nutritional factors [3,6,7]. Processed animal proteins (PAP) such as poultry by-product meal, meat meal and meat and bone meal are valid proteins for aquaculture feeds but their use is limited by legislation. In the EU, PAP from poultry and swine have only recently been reintroduced into aquafeed (EC No. 56/2013) after more than 10 years of ban due to Bovine Spongiform Encephalopathy (EC No 999/2001), while in other parts of the world, its use is common practice [8].

Recently, interest has turned to PAPs from insects as a component of aquafeeds [9,10]. Insect larva meals are rich in proteins and their EAA profile is close to that of FM and considered superior to that of plant proteins [9]. The use of insect PAP has recently been sanctioned by the European Commission (Brussels, Belgium) (Regulation 2017/893/EC, 2017).

The black soldier fly *Hermetia illucens* belongs to the family *Stratiomyidae* and is among the most promising insect species for mass-rearing for animal feed [11]. Commercial *H. illucens* meal has an average protein content of 55% dry matter (DM) with lipid content ranging from 5% to 35% DM, depending on the defatting process applied during meal production. Research into its efficacy has thus far been contradictory: Similar or better growth performance to that of fish fed conventional protein sources (mainly FM or soybean meal) using commercial *H. illucens* meal at inclusion levels from 2.5% to 40% were obtained for Atlantic salmon (*Salmo salar*) [12–14], rainbow trout (*Oncorhynchus mykiss*) [15,16], European sea bass (*Dicentrarchus labrax*) [17], yellow catfish (*Pelteobagrus fulvidraco*) [18] and rice field eel (*Monopterus albus*) [19]. Conversely, other authors reported reduced acceptance and growth [20,21], with high levels of inclusion. Divergence in results is likely due to the differences among *H. illucens* meals and the level of inclusion in the diet and also suggest species differences in adaptation to insect meals. The use of *H. illucens* meal in perch diets has not been investigated.

The goal of this research was to determine the effects of partially defatted *H. illucens* meal as partial substitute for FM on growth performance, somatic indices, occurrence of splenic lipidosis, hematological parameters and proximate whole-body composition of juvenile *P. fluviatilis*. The research also aimed to provide new data on the economic and environmental sustainability of this novel protein source.

## 2. Materials and Methods

An 84-day growth trial was carried out at the Faculty of Fisheries and Water Protection of the University of South Bohemia (České Budějovice, Czech Republic). The trial was designed and carried out in accordance with the Czech and European Communities Directive (2010/63/EU) on the protection of animals used for scientific purposes, protocol number MSMT-6744/2018-2.

### 2.1. Experimental Diets

Four experimental diets were formulated to be isonitrogenous (crude protein, CP: ~54 g 100 g DM); isolipidic (ether extract, EE: ~13 g 100 g DM); and isoenergetic (gross energy, GE: ~23 MJ kg DM). An FM-based diet was used as control (CON) and three additional diets included FM replacement with 20% (H20), 40% (H40) and 60% (H60) partially defatted *H. illucens* larva meal obtained with a mechanical process performed using high pressure and without solvents was provided by Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, Germany). In order to ensure that diets were isonitrogenous, isolipidic and isoenergetic, the proportion of wheat meal and fish oil was reduced with increase in *H. illucens*.

The experimental feeds were prepared at the Department of Agricultural, Forest and Food Sciences experimental facility. Finely ground ingredients and fish oil were thoroughly mixed with water and pelleted using a 2 mm meat grinder and dried at 50 °C for 48 h. Feeds were stored in dark bags at –20 °C until use. The ingredients of the experimental diets are reported in Table 1. An additional control group (BIO) was fed a commercial extruded diet (BioMar Inicio 2 mm, BioMar A/S, Brande, Denmark) containing fish meal, wheat gluten, wheat, pea protein, soybean concentrate, rapeseed oil, fish oil and yeast extract as main ingredients. Proximate composition (on a wet basis) according to manufacturer's label was CP 52%, crude lipid 23%, carbohydrates 12%, ash 8.7%, fiber 0.9%, total phosphorus (P) 1.2% and GE 23.5 MJ/kg.

**Table 1.** Ingredients and proximate composition of *Hermetia illucens* larva meal and experimental diets.

Ingredients (g/kg)	<i>H. illucens</i> Larva Meal	CON	H20	H40	H60
FM (Chile, super prime) <sup>a</sup>	-	720	570	420	270
<i>H. illucens</i> larva meal <sup>b</sup>	-	0	200	400	600
Wheat meal	-	120	90	60	30
Fish oil	-	60	40	20	0
Starch, D500	-	80	80	80	80
Mineral mixture <sup>c</sup>	-	10	10	10	10
Vitamin mixture <sup>d</sup>	-	10	10	10	10
Proximate composition <sup>e</sup>					
DM (g/100 g)	94.18	88.74	90.76	90.59	90.51
CP (g/100 g DM)	55.34	54.50	54.37	54.10	53.91
EE (g/100 g DM)	17.97	11.92	11.95	11.62	11.64
Ash (g/100 g DM)	7.12	14.77	13.70	12.44	11.41
Chitin (g/100 g DM)	5.00	-	0.98	2.12	3.15
NFE (g/100 g DM) <sup>f</sup>	14.57	18.81	19.02	19.72	19.89
Gross energy (MJ/kg DM) <sup>g</sup>		22.90	22.54	23.02	23.26

FM, fishmeal; DM, dry matter; CP, crude protein; EE, ether extract; NFE, nitrogen free extracts, groups CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of *H. illucens* meal, respectively. <sup>a</sup> Fishmeal was purchased from Corpesca S.A. (Santiago, Chile). Proximate composition (% as-fed basis): 90.4 DM; 66.7 CP; 8.3 EE; 14.9 Ash. <sup>b</sup> *Hermetia illucens* larvae meal purchased from Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, Germany). <sup>c</sup> Mineral mixture (g or mg/kg diet): bicalcium 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 (Granda Zootecnica, Cuneo, Italy). <sup>d</sup> Vitamin mixture (IU or mg/kg diet): DL-tocopherolacetate, 60 IU; sodium menadione bisulphate, 5 mg; retinylacetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamine, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; vitamin B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg (Granda Zootecnica, Cuneo, Italy). <sup>e</sup> Values are reported as mean of triplicate analyses. <sup>f</sup> Calculated as 100 – (CP + EE + Ash + Chitin). <sup>g</sup> Determined by bomb calorimetry.

### 2.2. Fish and Feeding Trial

Eurasian perch juveniles were obtained from pond-reared larvae and intensively reared juveniles in an RAS [22]. The RAS (4360 L total water volume) included fifteen 75 L rearing tanks, a mechanical drum filter AEM 15 (AEM-Products V.O.F., Lienden, The Netherlands), a 1620 L tank with a series of filtration sections, Bioakvacit PP10 (Jezírka Banát s.r.o., Hněvotín, Czech Republic), a moving bed biofilter (1620 L) with media BT10 (Ratz Aqua & Polymer Technik, Remscheid, Germany), UV treatment AquaForte 55 W (AquaForte, Veghel, The Netherlands) and an Eheim Jäger Thermocontrol 300 flow-through

heater (Eheim GmbH & Co KG, Stuttgart, Germany) incorporated directly into the recirculation flow. The flow rate in the tanks was approximately 80 L h<sup>-1</sup> with light aeration. Photoperiod was set at 12:12 h (dark: light) with light intensity of 500–700 Lx at the surface. Oxygen saturation (83.7 ± 6.2%), pH (6.83 ± 0.52) and water temperature (22.5 ± 0.7 °C) (HACH HQ 40, Germany) were measured daily at 08.00 and 16.00. Ammonia, nitrate and nitrite concentrations were analyzed at two-day intervals with kits (HACH, LCK 304, LCK 339, LCK 341), using a HACH DR2800 spectrophotometer. The concentration of nitrite-N, nitrate-N and ammonia-N were 0.62 ± 0.44 mg L<sup>-1</sup>, 88.88 ± 57.31 mg L<sup>-1</sup> and 2.07 ± 1.02 mg L<sup>-1</sup>, respectively.

A total of 750 juvenile European perch were lightly anaesthetized (0.3 mL L<sup>-1</sup> of clove oil), individually weighed (initial body weight (BWi) 21.9 ± 4.2 g) using a digital balance (Pioneer, Ohaus Corporation, Parsippany, NJ, USA, d = 0.01 g) and randomly allocated to one of the fifteen 75 L rectangular plastic tanks at a stocking density of 14.6 kg m<sup>-3</sup>. The four experimental diet groups and the BIO group were randomly allocated to the fifteen tanks, with each diet tested in triplicate. Fish were fed manually to subjectively-judged satiation five times daily (09:00, 11:00, 13:00, 15:00 and 17:00 h). Care was taken to avoid feed waste and to ensure that all supplied feed was consumed. The feeding trial lasted 84 days.

### 2.3. Growth Performance

At the end of the trial, all fish were individually weighed and growth performance was calculated using following equations:

$$\text{Survival (S, \%)} = 100 \times N_f (N_i - N_s)^{-1}$$

$$\text{Initial coefficient of variation (ICV, \%)} = (\text{SD}/\text{BWi}) \times 100$$

$$\text{Final coefficient of variation (FCV, \%)} = (\text{SD}/\text{BWf}) \times 100$$

$$\text{Specific growth rate (SGR, \% day}^{-1}\text{)} = ((\ln\text{BWf} - \ln\text{BWi})/\text{Nd}) \times 100$$

$$\text{Feed conversion ratio (FCR)} = (\text{TFS}/\text{WG})$$

$$\text{Protein efficiency ratio (PER)} = (\text{WG (g)}/\text{TPS total protein fed (g, DM)})$$

$$\text{Feeding rate (FR, \%}/\text{d)} = ((\text{TFS} \times 100/\text{Nd})/(\text{e} (\ln\text{BWf} + \ln\text{BWi}) \times 0.5)),$$

where  $N_i$  and  $N_f$  = initial and final number of fish per tank,  $N_s$  = number of sampled fish per tank,  $\text{BWf}$  = final body weight (g),  $\text{BWi}$  = initial body weight (g),  $\text{Nd}$  = number of feeding days,  $\text{TFS}$  = total feed supplied (g),  $\text{TPS}$  = total protein supplied (g, DM),  $\text{SD}$  = standard deviation of subsample BW,  $\text{BWi}$  = initial mean body weight,  $\text{BWf}$  = final mean body weight,  $\ln\text{BWf}$  = natural logarithm of final body weight,  $\ln\text{BWi}$  = natural logarithm of initial body weight,  $\text{DM}$  = dry matter,  $\text{WG}$  = weight gain.

### 2.4. Condition Factor, Somatic Indexes and Occurrence of Spleen Lipidosis

To calculate condition factor (K), at the end of the growth trial, fifty fish from each tank were anaesthetized (0.3 mL L<sup>-1</sup> of clove oil) and individually weighed and measured for total length (TL, mm) and standard length (SL, mm) within 1 mm using a ruler.

K was calculated as:

$$K = (\text{BWf}/\text{TL}) \times 100,$$

where  $\text{BWf}$  = final body weight (g),  $\text{TLf}$  = final body length (cm).



At the end of the trial, 30 fish/tank were killed by overdosing of anesthesia with clove oil and wet weight of liver, spleen, viscera and perivisceral fat recorded ( $\pm 0.01$  g) for calculation of somatic indices using the equations:

$$\text{Hepatosomatic index (HSI)} = W_l (\text{weight, g}) \times 100/\text{BW (body weight, g)}$$

$$\text{Splenosomatic index (SSI)} = W_s (\text{weight, g}) \times 100/\text{BW (body weight, g)}$$

$$\text{Viscerosomatic index (VSI)} = W_v (\text{weight, g}) \times 100/\text{BW (body weight, g)}$$

$$\text{Perivisceral fat index (PFI)} = W_{\text{Ppf}} (\text{weight, g}) \times 100/\text{BW (body weight, g)},$$

where  $W_l$  = liver weight (g),  $W_s$  = spleen weight (g),  $W_v$  = viscera weight (g),  $W_{\text{Ppf}}$  perivisceral fat weight (g).

Frequency of occurrence splenic lipidosis [2], was calculated according to the equation:

$$\text{SL} = 100/\text{Nt} \times \text{Nsl},$$

where  $N_t$  is total number of investigated fish and  $N_{sl}$  is number of fish with spleen lipidosis.

### 2.5. Chemical Analyses

The *H. illucens* larva meal chemical analysis was obtained from Renna et al. [15]. The proximate composition and energy of diets are reported in Table 1. Feed samples were finely ground (MLI 204; Bühler AG, Uzwil, Switzerland) and analyzed for DM (AOAC, n. 934.01), CP (AOAC, n. 984.13) and ash (AOAC, n. 942.05) content according to AOAC International [23]. The EE content (AOAC, n. 2003.05) was analyzed according to AOAC International [24]. The GE content was determined using an adiabatic bomb calorimeter (C7000; IKA, Staufen, Germany). Chitin content was determined following Finke [25], by correcting for the amino acid (AA) content of the acid fiber detergent (ADF) fraction and assuming the remainder of the ADF fraction to be chitin. The AA composition of *H. illucens* larva meal and FM used in the experimental diets is shown in Table 2. Amino acid quantification was conducted according to De Marco et al. [26]. After 22 h hydrolysis in 6N HCl at 112 °C under a nitrogen atmosphere, the AA content in the hydrolysate was assessed by HPLC after post-column derivatization. Performic acid oxidation occurred prior to acid hydrolysis for methionine and cysteine. Tryptophan was not determined.

At the end of the trial, whole-body homogenate of six fish from each group was analyzed for DM, CP, EE, organic matter (OM) and ash content according to the procedure used for feed analyses [23,24]. The DM content was measured according to AOAC (n. 934.01; [23]).

Fatty acid profiles were determined both in feed and fish whole-body homogenate (3 fish/tank, 9 fish/group), according to the method of Sampels et al. [27]. Initially, lipids were extracted by the hexan-isopropanol method according to Hara and Radin [28]. Fatty acid methyl esters (FAME) were prepared by the  $\text{BF}_3$  method according to Appelqvist [29] and analyzed using FAME C 11:0 as an internal standard by a gas chromatograph (Trace Ultra FID; Thermo Scientific, Milan, Italy) equipped with a flame ionization detector, using a BPX 70 column (length 50 m, i.d. 0.22 mm, film thickness 0.25  $\mu\text{m}$ ) (SGE Inc., Austin, TX, USA). The peaks were identified using Thermo Xcalibur 3.0.63 (Thermo Fisher Scientific Inc., Waltham, MA, USA) software and quantification was achieved by comparing sample retention times and peak areas to retention times and peak area in 7 levels (1000  $\mu\text{g/mL}$ –15  $\mu\text{g/mL}$ ) of the standard mixture Supelco 37 component FAME mix (Sigma-Aldrich, St. Louis, MO, USA). Fatty acid profiles for feed are shown in Table 3, analysis were performed in triplicate.

**Table 2.** Amino acid (AA) profile (% of protein) of *Hermetia illucens* larva meal and experimental diets.

	<i>H. illucens</i>	CON	H20	H40	H60
<b>Essential AA</b>					
Arginine	3.9	6.2	5.7	5.2	4.7
Histidine	2.2	2.4	2.4	2.3	2.3
Isoleucine	3.3	4.2	4.0	3.8	3.6
Leucine	5.2	7.3	6.8	6.4	5.9
Lysine	3.8	7.4	6.7	5.9	5.1
Methionine	2.1	2.7	2.5	2.2	2.0
Cysteine	0.1	0.9	0.7	0.5	0.4
<b>Non-essential AA</b>					
Phenylalanine	3.0	4.0	3.7	3.5	3.3
Tyrosine	4.8	3.1	3.4	3.8	4.1
Threonine	3.1	4.1	3.9	3.7	3.5
Valine	4.9	4.9	4.9	4.9	4.9
Alanine	6.2	6.1	6.1	6.1	4.9
Aspartic acid	6.7	8.8	8.4	7.9	7.5
Glycine	4.2	0.9	1.6	2.2	2.9
Glutamic acid	8.8	7.0	7.3	7.6	7.9
Proline	5.5	12.3	10.9	9.5	8.0
Serine	3.7	4.1	4.0	3.9	3.8

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of *H. illucens* larva meal, respectively.

**Table 3.** Fatty acid profile of experimental diets for Eurasian perch. Data are expressed as percent of total FAs (mean  $\pm$  SD, n = 3).

FA	CON	H20	H40	H60
C12:0	2.54 $\pm$ 0.27	11.89 $\pm$ 0.17	24.47 $\pm$ 1.81	34.37 $\pm$ 1.23
C14:0	4.76 $\pm$ 0.04	6.54 $\pm$ 0.06	8.77 $\pm$ 0.11	10.74 $\pm$ 0.38
C14:1	0.05 $\pm$ 0.01	0.13 $\pm$ 0.01	0.24 $\pm$ 0.01	0.33 $\pm$ 0.01
C15:0	0.40 $\pm$ 0.01	0.35 $\pm$ 0.01	0.28 $\pm$ 0.01	0.23 $\pm$ 0.01
C16:0	15.38 $\pm$ 0.04	16.01 $\pm$ 0.05	16.58 $\pm$ 0.32	17.21 $\pm$ 0.69
C16:1	4.21 $\pm$ 0.01	4.49 $\pm$ 0.01	4.79 $\pm$ 0.10	5.08 $\pm$ 0.11
C18:0	3.77 $\pm$ 0.03	3.63 $\pm$ 0.04	3.22 $\pm$ 0.07	3.02 $\pm$ 0.13
C18:1n9trans	0.08 $\pm$ 0.01	0.07 $\pm$ 0.01	0.07 $\pm$ 0.01	0.06 $\pm$ 0.01
C18:1n9	26.60 $\pm$ 0.20	22.08 $\pm$ 0.02	16.20 $\pm$ 0.36	11.29 $\pm$ 0.27
C18:1n7	3.23 $\pm$ 0.02	2.57 $\pm$ 0.01	1.68 $\pm$ 0.04	0.94 $\pm$ 0.04
C18:2n6	9.18 $\pm$ 0.03	8.61 $\pm$ 0.01	7.79 $\pm$ 0.18	7.20 $\pm$ 0.14
C18:3n6	0.20 $\pm$ 0.01	0.16 $\pm$ 0.01	0.11 $\pm$ 0.01	0.07 $\pm$ 0.01
C18:3n3	3.15 $\pm$ 0.01	2.56 $\pm$ 0.01	1.80 $\pm$ 0.05	1.14 $\pm$ 0.03
C20:0	0.32 $\pm$ 0.01	0.27 $\pm$ 0.01	0.21 $\pm$ 0.01	0.15 $\pm$ 0.01
C20:1n9	2.55 $\pm$ 0.02	1.91 $\pm$ 0.01	1.05 $\pm$ 0.03	0.32 $\pm$ 0.03
C20:3n6	0.76 $\pm$ 0.01	0.56 $\pm$ 0.01	0.31 $\pm$ 0.01	0.09 $\pm$ 0.01
C20:3n3	0.71 $\pm$ 0.01	0.56 $\pm$ 0.01	0.38 $\pm$ 0.01	0.23 $\pm$ 0.01
C20:4n6	0.32 $\pm$ 0.01	0.24 $\pm$ 0.01	0.13 $\pm$ 0.01	0.03 $\pm$ 0.01
C22:0	0.16 $\pm$ 0.01	0.13 $\pm$ 0.01	0.08 $\pm$ 0.01	0.05 $\pm$ 0.01
C22:1n9	0.37 $\pm$ 0.01	0.27 $\pm$ 0.01	0.15 $\pm$ 0.01	0.04 $\pm$ 0.01
C20:5n3	6.80 $\pm$ 0.03	5.44 $\pm$ 0.01	3.72 $\pm$ 0.10	2.21 $\pm$ 0.01
C22:2	0.07 $\pm$ 0.01	0.05 $\pm$ 0.01	0.32 $\pm$ 0.50	0.65 $\pm$ 0.56
C24:0	0.17 $\pm$ 0.01	0.13 $\pm$ 0.01	0.10 $\pm$ 0.01	0.05 $\pm$ 0.03
C24:1n9	0.55 $\pm$ 0.01	0.44 $\pm$ 0.01	0.29 $\pm$ 0.01	0.16 $\pm$ 0.01
C22:5n3	1.44 $\pm$ 0.01	1.06 $\pm$ 0.02	0.69 $\pm$ 0.02	0.31 $\pm$ 0.02
C22:6n3	12.23 $\pm$ 0.18	9.85 $\pm$ 0.11	6.61 $\pm$ 0.15	4.03 $\pm$ 0.14
SFA	27.58 $\pm$ 0.34	39.00 $\pm$ 0.12	54.02 $\pm$ 1.06	66.47 $\pm$ 0.77
MUFA	37.63 $\pm$ 0.23	31.96 $\pm$ 0.03	24.45 $\pm$ 0.54	18.23 $\pm$ 0.40
PUFA	34.79 $\pm$ 0.21	29.04 $\pm$ 0.11	21.53 $\pm$ 0.51	15.30 $\pm$ 0.38
n-3	24.33 $\pm$ 0.22	19.47 $\pm$ 0.12	13.20 $\pm$ 0.32	7.90 $\pm$ 0.28
n-6	10.46 $\pm$ 0.03	9.57 $\pm$ 0.01	8.33 $\pm$ 0.19	7.39 $\pm$ 0.15
n-3/n-6	2.33 $\pm$ 0.02	2.03 $\pm$ 0.01	1.58 $\pm$ 0.01	1.07 $\pm$ 0.03

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of *H. illucens* larva meal, respectively; SD, standard deviation, FA fatty acid, SFA saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids.

## 2.6. Haematological Analyses

At the end of experiment, three fish per tank (nine fish from each group) were over-anaesthetized with clove oil and blood samples were taken for hematological analysis. Red blood cell count (RBCC), hematocrit (HCT), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were measured according to Svobodova et al. [30].

## 2.7. Economic Analysis and Environmental Sustainability of Feeds

To determine the relative efficacy and benefits of tested diets, economic conversion ratio (ECR) and economic profit index (EPI) for each tested group was calculated by the following equations:

$$\text{ECR (€ kg of fish}^{-1}\text{)} = \text{FCR} \times \text{DP}$$

$$\text{EPI (€ fish}^{-1}\text{)} = (\text{WG} \times \text{SP}) - (\text{WG} \times \text{DP}),$$

where FCR is feed conversion ratio (kg feed per kg fish); DP is cost per kg feed; WG is weight gain. The per kilogram cost in euros, excluding labor and taxes, of all components from commercial retailers was as follows: FM = € 1.48; *H. illucens* larva meal = € 3.5; wheat meal = € 0.61; fish oil = € 1.32; gelatinized starch = € 0.75; mineral mixture = € 0.49; vitamin mixture = € 3.85. This resulted in per kg feed cost of CON = € 1.31; H20 = € 1.75; H40 = 2.18; H60 = € 2.61; and BIO = € 2.53. Eurasian perch sale price (SP) was calculated at € 6.50 kg<sup>-1</sup>.

Fish-in fish-out (FIFO) ratio was used as a practical measure of the quantity of live fish from capture fisheries required for each unit of farmed fish produced [31]. This indicator of environmental sustainability of feeds was calculated as follows:

$$\text{FIFO} = (\text{LFM} + \text{LFO})/(\text{YFMw} + \text{YFOw}) \times \text{FCR},$$

where LFM is level of fishmeal in the diet; LFO is level of fish oil in the diet; YFMw is yield of fishmeal from wild fish; YFOw is yield of fish oil from wild fish; FCR is feed conversion ratio.

We estimated the impact of FM substitution with *H. illucens* larva meal reported to Metric Tons (MT) on freshwater demand (WD, m<sup>3</sup>/MT), land demand (LD, ha/MT), energy use (EU, GJ/MT) and greenhouse gas production (GWP, kg CO<sub>2</sub><sup>-eq</sup>). Mean WD, LD and EU for FM, wheat, fish oil, starch and mineral and vitamin mixes were obtained from Chatvijitkul et al. [32]. Data of WD, LD, EU and GWP for *H. illucens* larva meal was retrieved from Roffeis et al. [33]. Finally, GWP for FM was sourced from Thevenot et al. [34] and GWP for wheat meal from Heusala et al. [35].

## 2.8. Statistical Analyses

All data were tested for homogeneity of variance using Cochran, Hartley and Bartlett tests. Normality of data was tested by Shapiro-Wilk test. Perivisceral fat index, splenosomatic index, some minor fatty acids, hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and economic profit index were analyzed using Kruskal-Wallis non-parametric test as these data does not show normality. All other remaining parameter results were analyzed separately by one-way ANOVA. Differences were considered significant at  $p \leq 0.05$  (post-hoc test: Tukey test). The data were expressed as mean  $\pm$  SD and statistical analyses were performed using STATISTICA 12.0 (StatSoft CR, Prague, Czech Republic). As BIO was a completely different diet, not comparable with respect to composition, nutrient and energy contents, it was not included in the statistical analyses.

### 3. Results

#### 3.1. Diet Composition

Diets were comparable in proximate composition, which reflected the calculated one. The amino acid profile of *H. illucens* larva meal and experimental diets is presented in Table 2. Leucine, tyrosine and valine were the most common EAAs in the *H. illucens* larva meal, with the non-essential AAs glutamic and aspartic acid showing the highest content. *Hermetia illucens* larva meal showed similar values for histidine and lower values for arginine and lysine than observed in FM [36]. With increasing dietary *H. illucens* proportions, all EAAs decreased except valine and tyrosine, which remained constant and increased, respectively.

#### 3.2. Growth Trial

Fish survival and growth performance are shown in Table 4. With all diets, fish tripled their initial body weight. Fish readily accepted the feeds and no rejection was recorded. At the end of the 84-day experiment, no significant differences in survival were observed among diets. There were no significant differences among experimental groups in BW<sub>i</sub>, ICV and FCV. On the other hand, BW<sub>f</sub>, SGR, PER and FR differed significantly with diet, with the H60 treatment showing lower values compared to other treatments.

**Table 4.** Survival and growth performance of Eurasian perch fed experimental diets and the commercial control diet (mean ± SD; n = 3).

Items	CON	H20	H40	H60	SEM	p-Value	BIO *
Survival, %	98.7 ± 2.3	98.7 ± 2.3	98.0 ± 1.2	99.3 ± 1.2	0.512	0.878	96.0 ± 4.0
BW <sub>i</sub> , g	21.9 ± 0.1	22.0 ± 0.1	22.1 ± 0.1	22.0 ± 0.1	0.023	0.195	22.0 ± 0.1
BW <sub>f</sub> , g	63.8 ± 1.2 <sup>a</sup>	67.1 ± 2.0 <sup>a</sup>	68.1 ± 1.8 <sup>a</sup>	58.0 ± 3.2 <sup>b</sup>	1.305	0.002	74.1 ± 6.0
WG, g	41.8 ± 1.0 <sup>a</sup>	45.1 ± 2.0 <sup>a</sup>	46.0 ± 1.7 <sup>a</sup>	36.0 ± 3.2 <sup>b</sup>	1.296	0.002	52.1 ± 5.9
ICV, %	19.4 ± 0.6	19.6 ± 0.8	19.5 ± 0.9	19.3 ± 0.9	0.205	0.981	19.0 ± 0.9
FCV, %	37.9 ± 1.6	32.9 ± 7.0	34.3 ± 7.1	38.3 ± 1.8	1.439	0.525	42.4 ± 9.1
SGR, %/d	1.25 ± 0.06 <sup>a,b</sup>	1.30 ± 0.03 <sup>a</sup>	1.30 ± 0.04 <sup>a</sup>	1.14 ± 0.03 <sup>b</sup>	1.331	0.000	1.39 ± 0.11
FCR	1.00 ± 0.07 <sup>a,b</sup>	0.91 ± 0.05 <sup>b</sup>	0.91 ± 0.04 <sup>b</sup>	1.12 ± 0.06 <sup>a</sup>	0.029	0.006	0.96 ± 0.13
PER	1.72 ± 0.12 <sup>a,b</sup>	1.91 ± 0.11 <sup>a</sup>	1.90 ± 0.08 <sup>a</sup>	1.55 ± 0.08 <sup>b</sup>	0.050	0.000	1.88 ± 0.23
FR, %/d	1.36 ± 0.03 <sup>a,b</sup>	1.30 ± 0.04 <sup>a</sup>	1.30 ± 0.01 <sup>a</sup>	1.39 ± 0.04 <sup>b</sup>	0.014	0.023	1.47 ± 0.06

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of *H. illucens* larva meal respectively; BIO is a commercial diet (BioMar Inicio, Brande, Denmark). SD, standard deviation, SEM, standard error of the mean; BW<sub>i</sub>, initial body weight; BW<sub>f</sub>, final body weight; WG, weight gain; ICV, initial coefficient of variation of weight; FCV, final coefficient of variation of weight; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; FR, feeding rate. Different letters within a row indicate significant differences ( $p \leq 0.05$ ). \* Statistical analysis did not include BIO.

#### 3.3. Condition Factor, Somatic Indices and Occurrence of Spleen Lipidosis

Fish fed H60 showed lower K and HSI compared to fish fed the CON diet, while no differences among treatments were recorded in any other parameter (Table 5). No splenic lipidosis was recorded in fish fed insect meal, while a high occurrence was recorded in fish fed the BIO diet.

**Table 5.** Condition factor (n = 45), somatic indices and occurrence of splenic lipidosis (n = 90) of Eurasian perch juveniles fed experimental diets and the commercial control diet (mean ± SD).

Items	CON	H20	H40	H60	SEM	p-Value	BIO *
K	1.20 ± 0.02 <sup>a,b</sup>	1.22 ± 0.02 <sup>a</sup>	1.19 ± 0.01 <sup>a,b</sup>	1.15 ± 0.01 <sup>b</sup>	0.008	0.020	1.28 ± 0.03
HSI	1.76 ± 0.20 <sup>a</sup>	1.41 ± 0.12 <sup>a,b</sup>	1.48 ± 0.10 <sup>a,b</sup>	1.21 ± 0.07 <sup>b</sup>	0.067	0.006	1.37 ± 0.04
SSI	0.12 ± 0.04	0.11 ± 0.04	0.10 ± 0.04	0.11 ± 0.05	0.010	0.964	0.13 ± 0.02
VSI	2.91 ± 0.38	2.79 ± 0.17	2.94 ± 0.19	3.06 ± 0.05	0.063	0.608	2.90 ± 0.19
PFI	6.19 ± 0.68	5.63 ± 0.19	6.06 ± 0.78	5.53 ± 0.25	0.157	0.826	9.43 ± 0.97
SL	3.9 ± 6.71	NF	NF	NF	-	-	19.5 ± 13.6

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of *H. illucens* larva meal, respectively, BIO is a commercial diet (BioMar Inicio, Brande, Denmark). SD, standard deviation, SEM, standard error of the mean; K, condition factor; HSI, hepatosomatic index; SSI, splenosomatic index; VSI, visceromatic index; PFI, perivisceral fat index; SL, splenic lipidosis; NF, not found. Different letters within a row indicate significant difference ( $p \leq 0.05$ ). \* Statistical analysis did not include BIO. No statistical analysis was performed for SL, as some diet groups did not show lipidosis.

### 3.4. Proximate and Fatty Acid Composition of Whole Fish Homogenate

The proximate composition of the whole fish homogenates showed no significant differences in DM, CP and EE content (Table 6). On the other hand, OM and GE content showed a decreasing trend with increased the *H. illucens* meal in the feed, while the opposite was recorded for ash content.

**Table 6.** Proximate composition of whole-body homogenate of Eurasian perch fed experimental diets and the commercial control diet (mean ± SD, n = 6).

Items	CON	H20	H40	H60	SEM	p-Value	BIO *
DM (g/100 g)	33.3 ± 1.0	32.9 ± 0.6	32.5 ± 0.6	32.1 ± 0.5	0.186	0.142	36.6 ± 1.1
CP (g/100 g DM)	24.1 ± 3.1	21.8 ± 0.9	21.6 ± 0.6	20.7 ± 0.3	0.466	0.065	22.4 ± 0.5
EE (g/100 g DM)	10.1 ± 1.3	9.5 ± 0.2	8.7 ± 0.5	8.5 ± 0.8	0.232	0.052	13.5 ± 1.0
OM (g/100 g DM)	28.6 ± 1.0 <sup>a</sup>	27.9 ± 0.6 <sup>a</sup>	27.2 ± 0.6 <sup>a</sup>	26.4 ± 0.8 <sup>b</sup>	0.254	0.001	32.1 ± 0.9
Ash (g/100 g DM)	4.7 ± 0.3 <sup>b</sup>	5.0 ± 0.2 <sup>b</sup>	5.3 ± 0.2 <sup>b</sup>	5.6 ± 0.3 <sup>a</sup>	0.098	0.003	4.5 ± 0.4
GE (MJ/kg DM)	0.81 ± 0.04 <sup>a</sup>	0.78 ± 0.01 <sup>a</sup>	0.75 ± 0.02 <sup>a</sup>	0.74 ± 0.03 <sup>b</sup>	0.009	0.014	0.95 ± 0.04

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of *H. illucens* larva meal, respectively; BIO is a commercial diet (BioMar Inicio, Brande, Denmark). SD, standard deviation, SEM, standard error of the mean; DM, dry matter; CP, crude protein; EE, ether extract; OM, organic matter; GE, gross energy. Different letters within a row indicate significant difference ( $p \leq 0.05$ ). \* Statistical analysis did not include BIO.

The fatty acid composition of Eurasian perch was significantly affected by the feed (Table 7). In general, saturated fatty acids (SFA) content tended to increase with increased *H. illucens* larva meal proportions with exception of C15:0 and C20:0. A trend similar to SFA was observed for monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Significant differences were found both in omega-6 and omega-3 content that decreased among tested *H. illucens* larva meal diets and consequently, the n-3/n-6 ratio decreased with increasing *H. illucens* larva meal inclusion.

**Table 7.** Fatty acid (FA) profile of whole-body homogenate Eurasian perch fed experimental and commercial control diet. Data are expressed as percent of total FAs (mean  $\pm$  SD, n = 9).

FA	CON	H20	H40	H60	SEM	p-Value	BIO *
C12:0	1.00 $\pm$ 1.56 <sup>b</sup>	4.76 $\pm$ 1.81 <sup>b</sup>	8.83 $\pm$ 1.24 <sup>a,b</sup>	12.03 $\pm$ 1.49 <sup>a</sup>	0.79	<0.001	0.20 $\pm$ 0.20
C14:0	4.67 $\pm$ 0.61 <sup>d</sup>	5.96 $\pm$ 0.64 <sup>c</sup>	7.41 $\pm$ 0.35 <sup>b</sup>	9.14 $\pm$ 0.72 <sup>a</sup>	0.315	<0.001	5.52 $\pm$ 0.18
C14:1	0.54 $\pm$ 0.07 <sup>d</sup>	0.63 $\pm$ 0.05 <sup>c</sup>	0.79 $\pm$ 0.03 <sup>b</sup>	0.96 $\pm$ 0.06 <sup>a</sup>	0.03	<0.001	0.66 $\pm$ 0.03
C15:0	0.42 $\pm$ 0.02 <sup>a</sup>	0.36 $\pm$ 0.03 <sup>a,b</sup>	0.34 $\pm$ 0.02 <sup>b</sup>	0.34 $\pm$ 0.05 <sup>b</sup>	0.008	<0.002	0.49 $\pm$ 0.02
C16:0	17.49 $\pm$ 1.04	18.45 $\pm$ 0.79	17.81 $\pm$ 0.67	18.3 $\pm$ 1.79	0.206	0.335	18.98 $\pm$ 1.03
C16:1	9.46 $\pm$ 0.57 <sup>b</sup>	9.9 $\pm$ 0.46 <sup>a,b</sup>	10.35 $\pm$ 0.42 <sup>a</sup>	10.44 $\pm$ 0.51 <sup>a</sup>	0.109	0.001	10.81 $\pm$ 0.41
C18:0	1.6 $\pm$ 0.07	1.20 $\pm$ 0.74	0.96 $\pm$ 0.80	1.70 $\pm$ 0.40	0.112	0.262	1.23 $\pm$ 0.08
C18:1n9trans	1.06 $\pm$ 0.10 <sup>a</sup>	0.84 $\pm$ 0.16 <sup>b</sup>	0.84 $\pm$ 0.12 <sup>b</sup>	0.74 $\pm$ 0.13 <sup>b</sup>	0.03	<0.001	2.57 $\pm$ 0.13
C18:1n9	27.34 $\pm$ 0.96 <sup>a</sup>	26.34 $\pm$ 0.79 <sup>a</sup>	23.99 $\pm$ 1.04 <sup>b</sup>	21.42 $\pm$ 0.68 <sup>c</sup>	0.436	<0.001	21.16 $\pm$ 0.36
C18:1n7	3.07 $\pm$ 0.20 <sup>a</sup>	2.00 $\pm$ 1.24 <sup>a,b</sup>	1.70 $\pm$ 1.05 <sup>b</sup>	2.07 $\pm$ 0.17 <sup>a</sup>	0.167	<0.001	2.76 $\pm$ 0.08
C18:2n6	8.21 $\pm$ 0.40 <sup>a</sup>	7.74 $\pm$ 0.28 <sup>a,b</sup>	7.79 $\pm$ 0.40 <sup>a</sup>	7.28 $\pm$ 0.40 <sup>b</sup>	0.087	<0.001	7.15 $\pm$ 0.24
C18:3n6	0.08 $\pm$ 0.09 <sup>a,b</sup>	0.06 $\pm$ 0.08 <sup>b</sup>	0.04 $\pm$ 0.07 <sup>b</sup>	0.17 $\pm$ 0.01 <sup>a</sup>	0.015	0.005	0.15 $\pm$ 0.01
C18:3n3	2.23 $\pm$ 0.16 <sup>a</sup>	1.99 $\pm$ 0.09 <sup>b</sup>	1.65 $\pm$ 0.08 <sup>c</sup>	1.30 $\pm$ 0.07 <sup>d</sup>	0.065	<0.001	1.77 $\pm$ 0.05
C20:0	1.02 $\pm$ 0.38 <sup>a</sup>	1.07 $\pm$ 0.04 <sup>a</sup>	0.97 $\pm$ 0.09 <sup>a</sup>	0.42 $\pm$ 0.37 <sup>b</sup>	0.065	0.001	0.98 $\pm$ 0.95
C20:1n9	2.21 $\pm$ 0.23 <sup>a</sup>	1.73 $\pm$ 0.23 <sup>a,b</sup>	1.58 $\pm$ 0.22 <sup>b</sup>	1.64 $\pm$ 0.36 <sup>b</sup>	0.063	0.003	4.25 $\pm$ 0.16
C20:3n6	0.11 $\pm$ 0.05 <sup>a</sup>	0.07 $\pm$ 0.06 <sup>a,b</sup>	0.06 $\pm$ 0.04 <sup>b</sup>	0.09 $\pm$ 0.02 <sup>a,b</sup>	0.008	0.035	0.06 $\pm$ 0.03
C20:3n3	0.38 $\pm$ 0.25 <sup>a</sup>	0.32 $\pm$ 0.20 <sup>a</sup>	0.18 $\pm$ 0.19 <sup>b</sup>	0.30 $\pm$ 0.04 <sup>a</sup>	0.034	0.050	0.41 $\pm$ 0.04
C20:4n6	0.19 $\pm$ 0.08 <sup>a</sup>	0.09 $\pm$ 0.10 <sup>a,b</sup>	0.05 $\pm$ 0.07 <sup>b</sup>	0.11 $\pm$ 0.03 <sup>a,b</sup>	0.015	0.009	0.14 $\pm$ 0.02
C22:0	nd	nd	nd	nd			nd
C22:1n9	nd	nd	nd	nd			nd
C20:5n3	0.55 $\pm$ 0.47	0.58 $\pm$ 0.26	0.74 $\pm$ 0.14	0.56 $\pm$ 0.36	0.057	0.629	1.06 $\pm$ 1.47
C22:2	4.23 $\pm$ 0.39 <sup>a</sup>	3.58 $\pm$ 0.33 <sup>b</sup>	3.14 $\pm$ 0.38 <sup>b</sup>	2.62 $\pm$ 0.34 <sup>c</sup>	0.122	<0.001	5.99 $\pm$ 0.18
C24:0	nd	nd	nd	nd			nd
C24:1n9	nd	nd	nd	nd			nd
C22:5n3	1.16 $\pm$ 0.15 <sup>a</sup>	0.98 $\pm$ 0.12 <sup>b</sup>	0.75 $\pm$ 0.07 <sup>c</sup>	0.58 $\pm$ 0.06 <sup>d</sup>	0.043	<0.001	1.09 $\pm$ 0.09
C22:6n3	12.91 $\pm$ 1.2 <sup>a</sup>	11.35 $\pm$ 1.12 <sup>b</sup>	10.04 $\pm$ 0.60 <sup>b</sup>	7.66 $\pm$ 0.98 <sup>c</sup>	0.385	<0.001	12.46 $\pm$ 0.59
SFA	30.43 $\pm$ 2.54 <sup>d</sup>	35.37 $\pm$ 2.38 <sup>c</sup>	39.47 $\pm$ 1.38 <sup>b</sup>	44.62 $\pm$ 2.55 <sup>a</sup>	1.012	<0.001	33.39 $\pm$ 1.39
MUFA	43.74 $\pm$ 0.98 <sup>a</sup>	41.44 $\pm$ 0.89 <sup>b</sup>	39.25 $\pm$ 0.61 <sup>c</sup>	37.34 $\pm$ 1.01 <sup>d</sup>	0.455	<0.001	42.31 $\pm$ 0.90
PUFA	25.82 $\pm$ 1.94 <sup>a</sup>	23.18 $\pm$ 1.77 <sup>b</sup>	21.28 $\pm$ 1.00 <sup>b</sup>	18.04 $\pm$ 1.70 <sup>c</sup>	0.579	<0.001	24.30 $\pm$ 1.67
n-3	17.23 $\pm$ 1.58 <sup>a</sup>	15.22 $\pm$ 1.46 <sup>b</sup>	13.34 $\pm$ 0.78 <sup>c</sup>	10.40 $\pm$ 1.35 <sup>d</sup>	0.504	<0.001	16.80 $\pm$ 1.57
n-6	8.59 $\pm$ 0.40 <sup>a</sup>	7.97 $\pm$ 0.45 <sup>b</sup>	7.94 $\pm$ 0.33 <sup>b</sup>	7.64 $\pm$ 0.37 <sup>b</sup>	0.090	<0.001	7.50 $\pm$ 0.26
n-3/n-6	2.00 $\pm$ 0.12 <sup>a</sup>	1.91 $\pm$ 0.14 <sup>a</sup>	1.68 $\pm$ 0.09 <sup>b</sup>	1.36 $\pm$ 0.12 <sup>c</sup>	0.049	<0.001	2.24 $\pm$ 0.20

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of *H. illucens* larva meal, respectively; BIO is a commercial diet (BioMar Inicio, Brande, Denmark); SD, standard deviation, SEM, standard error of the mean; SFA saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids. Different letters within a row indicate significant difference ( $p \leq 0.05$ ). \* Statistical analysis did not include BIO; nd = not detected.

### 3.5. Haematological Analyses

Hemoglobin (Hb) concentration, HCT, RBBC, MCV, MCH and MCHC showed no differences among the feeding groups (Table 8).

**Table 8.** Hematological parameters of Eurasian perch fed experimental and commercial control diet (mean  $\pm$  SD, n = 9).

Items	Unit	CON	H20	H40	H60	SEM	p-Value	BIO *
Hb	(g/L)	51.5 $\pm$ 2.9	51.4 $\pm$ 2.6	50.5 $\pm$ 3.6	51.0 $\pm$ 2.8	0.426	0.987	51.8 $\pm$ 2.8
HCT	(L/L)	32.4 $\pm$ 6.9	31.9 $\pm$ 10.2	30.4 $\pm$ 6.1	28.8 $\pm$ 9.3	1.180	0.829	32.7 $\pm$ 7.5
RBBC	(T/L)	1.90 $\pm$ 0.3	1.75 $\pm$ 0.4	1.58 $\pm$ 0.2	1.81 $\pm$ 0.6	0.060	0.466	1.89 $\pm$ 0.4
MCV	(fl)	173.1 $\pm$ 43.4	185.5 $\pm$ 46.7	191.5 $\pm$ 25.8	165.9 $\pm$ 41.5	5.534	0.616	174.1 $\pm$ 25.4
MCH	(pg)	27.6 $\pm$ 4.5	32.5 $\pm$ 4.5	32.4 $\pm$ 5.0	33.8 $\pm$ 4.9	1.767	0.296	28.7 $\pm$ 6.8
MCHC	(g/L)	0.17 $\pm$ 0.04	0.18 $\pm$ 0.07	0.17 $\pm$ 0.04	0.20 $\pm$ 0.08	0.009	0.931	0.17 $\pm$ 0.05

Groups CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion, of *H. illucens* meal, respectively; BIO is a commercial diet (BioMar Inicio, Brande, Denmark). SD, standard deviation, Hb, hemoglobin concentration; HCT, hematocrit; RBBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; SEM, standard error mean; \* Statistical analysis did not include BIO.

### 3.6. Economic Analysis and Environmental Sustainability of Feeds

The FIFO index decreased proportionally with increased insect meal proportions, reaching 3.04 (CON), 2.17 (H20), 1.56 (H40) and 1.18 (H60). The *H. illucens* meal diets differed significantly with respect to ECR and EPI (Table 9), with cost increasing concurrent with *H. illucens* meal replacement. The inclusion of insect meal led to an overall increase of environmental sustainability parameters GWP, EU and LD and a reduction in freshwater demand.

**Table 9.** Economic and environmental sustainability parameters of European perch production using feeds differing in insect meal inclusion level (mean  $\pm$  SD, n = 3).

Items	CON	H20	H40	H60	SEM	p-Value	BIO *
FIFO	3.04 $\pm$ 0.21 <sup>a</sup>	2.17 $\pm$ 0.12 <sup>b</sup>	1.56 $\pm$ 0.07 <sup>c</sup>	1.18 $\pm$ 0.06 <sup>d</sup>	0.214	<0.01	-
GWP (kg CO <sub>2</sub> -eq)	1.81	2.64	3.48	4.32	-	-	-
EU (GJ/MT)	15.35	24.80	34.26	43.71	-	-	-
LD (ha/MT)	0.06	0.08	0.11	0.13	-	-	-
WD (m <sup>3</sup> /MT)	376	304	232	161	-	-	-
ECR	1.4 $\pm$ 0.10 <sup>c</sup>	1.71 $\pm$ 0.10 <sup>c</sup>	2.13 $\pm$ 0.10 <sup>b</sup>	3.13 $\pm$ 0.17 <sup>a</sup>	0.198	<0.01	2.62 $\pm$ 0.34
EPI	0.36 $\pm$ 0.01 <sup>a</sup>	0.36 $\pm$ 0.01 <sup>a</sup>	0.34 $\pm$ 0.01 <sup>a</sup>	0.26 $\pm$ 0.02 <sup>b</sup>	0.012	<0.04	0.35 $\pm$ 0.04

CON, H20, H40 and H60 represent 0%, 20%, 40% and 60% inclusion of *H. illucens* meal, respectively; BIO is a commercial diet (BioMar Inicio, Brande, Denmark). SD, standard deviation, FIFO, fish-in fish-out ratio; ECR, economic conversion ratio; EPI, economic profit index, GWP, global warming potential; EU, energy use; LD, land demand; WD, water demand; Different letters within a row indicate significant difference ( $p \leq 0.05$ ).

\* The statistical analysis did not include BIO.

## 4. Discussion

Intensive culture of Eurasian perch is still a young industry in Europe with the main producers being Ireland, France, Poland, Belgium and Denmark. Insects have been proposed as an efficient and high-quality alternative protein source for poultry [37,38], swine [11,39] and carnivorous fish [13,15–17,20,40] and interest in use of insect meals in perch diets is high. Insects are a viable source of protein and lipids [9,10] and a typical component of Eurasian perch natural diet. Nogales-Mérida [9], confirmed insects as an excellent source of several vitamins and minerals including iron, potassium, calcium and magnesium. Use of *H. illucens* insect meal is consistent with production of perch as an organic product, as insect meal can be produced locally on a variety of substrates [41,42].

The present study represents the first reported use of defatted black soldier fly *H. illucens* larva meal as an alternative feed ingredient for Eurasian perch reared in intensive culture. Bußler et al. [43] demonstrated that *H. illucens* is an appropriate insect species for insect meal production. It has a well-balanced essential amino acid profile, an average protein content of 55% DM and ~35% fat DM, which may be reduced to 5–9% by defatting, making it more digestible. However, complete FM replacement by insect meal has not been shown feasible. Henry et al. [44], reported that the maximum dietary replacement of FM by *H. illucens* meal ranges from 6 to 25%, depending on fish species, with higher inclusion levels reducing growth performance. Sealey et al. [45], reported up to 50% *H. illucens* inclusion without negative effects on growth of rainbow trout. Our study showed that there is no significant effect up to 400 g/kg of *H. illucens* in the perch diet on body weight or specific growth rate. Similar results were demonstrated by Renna et al. [15], where partially defatted *H. illucens* larva meal up to 40% of inclusion level was used in rainbow trout diet without negative effects on survival rate, growth performance, condition factor, somatic indices, physical quality or gut morphology. Magalhaes et al. [17], replaced 45% of the FM in diet of juvenile European seabass with up to 19.5% *H. illucens* meal corresponding to 22.5% protein without adverse effects on growth performance and feed utilization. Kroeckel et al. [20], reported that inclusion higher than 33% of defatted *H. illucens* larvae decreased protein digestibility, feed acceptance and growth performance of juvenile turbot. Lock et al. [12], showed that drying slightly defatted *H. illucens* meal (255 g/kg DM) at low temperature is the most suitable procedure and produced a good alternative feed for Atlantic salmon growth.

Proximate composition of fish is driven by endogenous (size, life cycle stage) as well as exogenous factors (water quality, feed) [46]. To minimize bias, we reared European perch under similar conditions. We found no significant differences in DM, CP and EE in whole-fish homogenate among tested *H. illucens* diets. This is in line with Gasco et al. [47], who found no significant difference in DM and CP content of European sea bass fed mealworm *Tenebrio molitor* at different diet proportions. Contrary results were obtained in rainbow trout fed *T. molitor*, in which increasing the proportion of insect meal triggered significant decreases in DM, CP and EE [48], while increased enriched *H. illucens* prepupae content resulted in decline in DM and EE [45].

Reduction in DM and EE content may result from decreased nutrient availability [15], depending on insect species [23,42] or on its culture substrate [37,39]. Culture substrate also substantially affects insect ash content [49,50]. Although body ash content has been reported similar among fish consuming various insect meal diets [20,51], we found a significant difference among our diet groups, with the highest ash content in H60, while lower ash levels were observed in CON, H20 and H40 groups. This is in contrast to the proximate analysis of tested diets per se, in which the ash content decreased with increasing *H. illucens* inclusion. Kirchgessner and Schwarz [52] and Shearer [46] reported no effect of crude dietary ash on ash content of fish body, provided sufficient levels of essential elements are present. This suggests that the partially defatted *H. illucens* meal used in our study may lack some essential element or elements, although this complex mechanism is largely unexplored and needs further study. The GE content decreased significantly with increased *H. illucens* larva meal inclusion, reflecting the non-significant decrease in both CP and EE with higher *H. illucens* larva content.

We found total n-3 and n-6 fatty acid in Eurasian perch to decrease significantly with higher levels of *H. illucens* larva meal in the diet, reflecting lower fish oil content, with the n-3/n-6 ratio being inversely related to *H. illucens* inclusion. This is in agreement with findings of Borgogno et al. [51] and Renna et al. [15], who reported significant reduction of n-3/n-6 ratio in rainbow trout fed with *H. illucens* larva meal. The opposite effect was observed in Atlantic salmon fed *H. illucens* meal [13]. The differences among studies could be related to diet composition. In the present study, as well as those of Borgogno et al. [53] and Renna et al. [15], fish oil was used as a fat source, while Belghit et al. [54], used large quantities of rapeseed oil, which contain high level of n-6 polyunsaturated fatty acids contributing to maintain constant the n-3/n-6 ratio between insect meal based diets.

These comparisons underscore differences among insect species and culture media. We found increased *H. illucens* proportions to be associated with significantly higher SFA content in fish homogenate, reflecting that partially defatted *H. illucens* meal is rich in SFAs (lauric acid C12:0, myristic acid C14:0 and palmitic acid C16:0), while *T. molitor* larva meal is rich in MUFAs and n-6 PUFAs. A similar trend was observed in studies of Jian carp [55] and rainbow trout [13], fed *H. illucens* larva meal. The positive effect on HSI observed in the present study could be related to reduction of lipid storage in liver, as was demonstrated in Atlantic salmon [54].

Hematological parameters, essential tools in evaluation of fish welfare related to stress and immune status [56–58], are highly influenced by feeding regime [59]. Studies of FM substitutes such as cottonseed [60], soybean [61,62], housefly (*Musca domestica*) maggot [63] and cricket (*Gryllus bimaculatus*) [64], showed no significant effect of tested meals on hematological parameters of fish of various species. This reinforces our suggestion that dietary *H. illucens* larva meal does not impact welfare of Eurasian perch but further investigations of diet formulations and feeding strategies are needed to collect additional data for this new area of study and to obtain more comprehensive results on fish growth rate.

The fish-in fish-out ratio is a practical indicator of environmental sustainability [31]. This index uses a global average wet weight (whole fish) to fishmeal yield of 22.5% and wet weight to fish oil yield of 5%. A ratio >1 indicates net removal of fish globally. We found the FIFO ratio to be substantially reduced with increasing proportions of insect meal and that FIFO could be decreased by 49% in perch fed an insect-based diet without affecting growth. This downward trend is in agreement with forecast of Tacon and Marc [65].



Increasing *H. illucens* larva meal proportions in commercial fish feeds could lead to higher energy and land use and increased greenhouse gas production. A lower impact was found for freshwater use. Insect meal inclusion level, which does not affect growth parameters, led to a 144% increase in greenhouse gas production, 123% increase in energy demand and 77% increase in land use. Fresh water use was decreased by 38% compared to control. These findings suggest ongoing monitoring of agricultural resources and related socio-economic and environmental impact during the shift in resource demands from the oceans onto the land.

Future studies should be focused on fine-tuning for optimal insect meal inclusion in the range of 40% to 60%, as well as evaluation of diets with a higher contribution of plant-based protein in combination with insect meal. Long-term studies of rearing fish to a higher market size (>200 g), in combination with sensory and texture analysis of the final product, should be carried out to explore full potential of insect-based diets for perch.

When the inclusion level was >60%, growth was significantly reduced compared with the control group, suggesting that incorporation of up to 40% *H. illucens* larva meal in the feed formulation for perch is feasible and can reduce reliance on marine resources. However, even if presents limitations, such as production cost and increased impact in some environment-related parameters, the partial replacement of fishmeal by insect protein will be more important in the future as getting enough amount of fishmeal will be difficult and culture of insects like a *H. illucens* using waste food means to convert non-resources to important protein resources is a promising solution to cope this problem.

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## **CHAPTER 9**

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**GENERAL DISCUSSION**

**ENGLISH SUMMARY**

**CZECH SUMMARY**

**ACKNOWLEDGEMENTS**

**LIST OF PUBLICATIONS**

**TRAINING AND SUPERVISION PLAN DURING STUDY**

***CURRICULUM VITAE***

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## General discussion

### 1.1. Overview of production performance and environmental impacts of aquaculture species fed dietary insect meal

Evaluating innovative feed ingredients for aquaculture species necessitates thorough examinations of nutritional content, growth performance, and economic and environmental aspects (Hua, 2021; Naylor et al., 2009; Pelletier et al., 2018). Although insect meals have been recognized as the most promising alternative protein in aquafeed, only the first two categories have received the greatest attention (Gasco et al., 2019; Henry et al., 2015; Hua, 2021; Liland et al., 2021; Makkar et al., 2014; Nogales Mérida et al., 2019). Reviews on the nutritional properties of insect meals have covered a wide variety of insect taxa, processing techniques, and life phases, and it is widely agreed that such diversity drives the variation in nutrient contents, e.g., protein, lipid, amino acids, fatty acids, among others (Basto et al., 2020; Liu et al., 2017; Nogales Mérida et al., 2019). Recent narrative and meta-analysis studies, evaluating the growth performance of aquatic animals fed insect meals, have deemed insect meals a generic concept without regard for their variation (Hua, 2021; Liland et al., 2021). These works have also focussed on a limited number of insect candidates, such as black soldier fly (*Hermetia illucens*), yellow mealworm (*Tenebrio molitor*), and housefly (*Musca domestica*), despite the fact that a wide range of insect meals has been used as a fishmeal replacement in fish diets (Henry et al., 2015).

Our work (Tran et al., 2022b; Chapter 2), employing a meta-analysis approach, highlighted the potential of several insect meals in terms of the growth performance of fed organisms. We also found a substantial variation in the specific growth rate, feed conversion, and protein efficiency ratio of fish fed insect meals with different processing methods and life stages of insect meals. Many biotic and abiotic factors, e.g., temperature, trophic levels, and fishmeal/fish oil inclusion levels, were discovered to drive the influence of insect meals on fish performance. The role of low dietary insect-derived chitin in supporting the performance of some fish species was investigated; the excessive supply of this non-protein nitrogen causes an inverse effect. Our study also shed light on the future selection of insect meals concerning the production performance of aquaculture species; these include defatted (*T. molitor*), full-fat silkworm (*Bombyx mori*), defatted (*H. illucens*), full-fat fly (*Chrysomya megacephala*), cricket (*Gryllus bimaculatus*), grasshoppers (*Oxya fuscovittata*) and (*Zonocerus variegatus*) and full-fat moth (*Cirina butyrospermi*). Taking into account past research, our study delivered a comprehensive assessment of the influence of diverse insect forms on fish production, offering a shortlist of ingredients for future aquafeed manufacture.

Aquafeed production has been highlighted as a primary driver of the environmental implications of aquaculture systems (Bohnes et al., 2019). Previous research has shown that alternative aquafeed sources must satisfy a number of requirements in order to provide superior environmental advantages, including reducing the use of marine fishery resources, decreasing nutrient loss at the farm, and lowering environmental and life cycle impacts (Papatryphon et al., 2004; Pelletier et al., 2018; Pelletier and Tyedmers, 2007). Some research concentrated on the environmental effects of insect meal manufacturing and insect-containing diet for aquatic animal species. Specifically, insect meals exhibited higher environmental impacts than fishmeal but equivalent to or lower than terrestrial protein ingredients, notably for land use (Maiolo et al., 2020; Salomone et al., 2017; Smetana et al., 2019; van Zanten et al., 2015). In a broader sense, our study (Tran et al., 2022a; Chapter 3) demonstrated the lower environmental performance of insect meal production compared to a variety of conventional and novel ingredients, except for fishmeal. The emphasis should be drawn to global warming

potential, in which insect meals remained comparable with other plant proteins but required far less land. The insect, therefore, could be a preferable candidate for future aquafeed in satisfying the ever-growing aquaculture industry in a finite natural resource. Technological advancements, potentially through scaling up the insect rearing facility and optimizing feed substrates, have the great potential to lessen the environmental burden of insect meals (Halloran et al., 2016; Smetana et al., 2021).

Fecal loss is a critical concern when incorporating novel ingredients in aquafeed. Weththasinghe et al. (2021) demonstrated that dietary full-fat fly (*H. illucens*) significantly increased fecal nitrogen excretion but had no effect on that of phosphorus. This study highlighted the importance of considering a broader perspective, in addition to physiological parameters, while introducing insect meals into fish diets. By retrieving data from many published papers, we (Tran et al., 2022a; Chapter 3) validated past work, demonstrating that the great majority of insect-containing diets generated more nitrogenous solid waste than the control. Our research, on the other hand, highlighted the environmental benefits of incorporating insect meals in aquafeed, regarding lower fecal phosphorus loss.

Aquaculture is expected to significantly contribute to global seafood demand by 2050, and to tap this, aquaculture needs to lower its reliance on marine fish resources by reducing the use of wild-derived fishmeal and fish oil (Béné et al., 2015; Naylor et al., 2009). Fish-in fish-out, measuring the amount of marine fish input required to produce farmed fish, is a valuable metric that indicates the dependence of aquaculture on the marine ecosystem (Tacon and Metian, 2008). The use of insect meals of fly (*H. illucens*) (Rawski et al., 2020) and cricket (*Acheta domesticus*) (Estévez et al., 2022) steadily reduced fishmeal, and to a lesser extent, fish oil, in fish diets, thereby lowering fish-in fish-out ratio for the production of Siberian sturgeon (*Acipenser baerii*) and meagre (*Argyrosomus regius*), respectively. On a global scale, this ratio was 0.28 for main fed-aquaculture species in 2017, a significant decrease from the previous 20-year record (Naylor et al., 2021). Recent advances in economic allocation have suggested shifting from conventional fish-in fish-out to a more precise concept known as economic fish-in fish-out (Kok et al., 2020). Based on this principle, our metadata (Tran et al., 2022a; Chapter 3) consolidated previously indicated findings that dietary insect meals significantly reduced economic fish-in fish-out for all fish taxa.

Overall, when considering the growth production of fish in conjunction with the environmental consequence of incorporating insect meals in aquafeed, dietary insect meals, at the plausible thresholds, support fish performance and reduce aquaculture's reliance on finite fisheries resources but entail burdens associated with nitrogen waste output and environmental impacts from life cycle assessment approach. Along with selecting appropriate insect species for aquafeed input, it is prudent to consider insect-containing feed in a specific aquaculture system, e.g., integrated multi-trophic aquaculture, pond aquaculture, and recirculated aquaculture system, where solid waste can be managed or used for extractive species (Naylor et al., 2021). Furthermore, insects could play a vital role in a closed-loop circular economy and environmental impact reduction of our food system since insects have a high ability to convert low-value substrates to high-quality feedstock (Cappelozza et al., 2019; Jagtap et al., 2021; Lalander and Vinnerås, 2022; Ojha et al., 2020).

Our studies (Tran et al., 2022a,b; Chapters 2 and 3) further stressed the importance of the complementarity among feed resources in aquafeed to satisfy the nutritional requirement of targeted fish species and ensure environmental sustainability. Given that a single alternative protein ingredient will not be able to satisfy the protein demand of aquafeed, blends of insect meals and other feed ingredients have been recently developed and shown promising results in terms of acceptance by fed organisms and environmental aspects (Bruni et al., 2021; Chaklader et al., 2021; Estévez et al., 2022; Pulido-Rodriguez et al., 2021).

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## 1.2. Insect meals (*Tenebrio molitor* and *Hermetia illucens*) fed Eurasian perch (*Perca fluviatilis*)

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Until recently, among seven insect meals authorized to use in aquafeed in Europe, fly (*H. illucens*) and mealworm (*T. molitor*) have received the most interest from academia (Hua et al., 2019). To further explore the possibility of using these insect meals in potential aquaculture species, perch (*P. fluviatilis*), we have layout two separate feeding trials, Chapter 4 (Tran et al., 2022c) and Chapter 8 (Stejskal et al., 2020). The recent studies employing the meta-analysis approach have demonstrated that the plausible inclusion level of insect meal while sustaining growth performance of fed aquatic species is insect-species-specific (Hua, 2021; Liland et al., 2021). This is also the case for European perch (*P. fluviatilis*) fed dietary insect meals. Tilami et al. (2020) documented that including 10% of an insect meal blend, house cricket (*Acheta domesticus*) and superworm (*Zophobas morio*), as a 25% substitution for fishmeal in diets for perch did not support growth performance when compared to the insect-free diet, as evidenced by the specific growth rate. In contrast, Chapter 4 (Tran et al., 2022c) indicated that dietary yellow mealworm (*T. molitor*) at as low as 6.8% sustained perch growth compared to the control diet; however, oversupplying this insect caused a negative effect. Therefore, it was concluded that 6.8% of defatted mealworm or 25% of fishmeal substitution is appropriate for perch diets. Furthermore, incorporating black soldier fly (*H. illucens*) (Chapter 8; Stejskal et al., 2020) suggested that 40% of *H. illucens* can be used successfully in diets for perch. These Chapters (4 and 8) highlighted the high feeding rate of perch with fly- and mealworm-containing diets, implying that these insect meals are highly palatable for fish. Unlikely, cricket and superworm were attributed to unpalatable flavour, bitterness, sourness, or odor for perch (Tilami et al., 2020).

Hua (2021) drew attention to *T. molitor*, suggesting that fish are well tolerated with a high dietary level of this mealworm, whereas the plausible inclusion of black soldier fly for fish is 29%, beyond this level, growth performance is depressed. However, our findings (Chapters 4 and 8) revealed otherwise. It is widely accepted that the threshold to which dietary insect meals continue to support the growth performance of fed organisms relative to the control diet is highly dependent on a variety of factors, not just insect species themselves. Our study (Chapter 3) suggested that these factors included the magnitude of insect defatting, life-stage, anti-nutrients (chitin), and nutrient balance designed in experiments diets. For instance, despite the fact that experimental diets were formulated on an isonitrogenous, isolipidic, and isoenergetic basis, the deficiency of some essential amino acids and fatty acids, together with increasing levels of non-protein nitrogen in insect-based diets, depressed nutrient digestibility, thus growth performance of perch in our studies. It is worth noting that although the chitin level in *H. illucens*-containing diets (0.98–3.15% dry basis; Chapter 8) is relatively higher than that in *T. molitor* diets (0.34–1.01% dry basis, Chapter 4), the growth performance of perch favoured the former over the latter. This implies that chitin is not the only factor compromising fish growth, which agrees with a prior study (Hua, 2021).

Perch fed increasing dietary mealworm (Chapter 4) evidenced stress status, as illustrated by stress-indicated aspartate aminotransferase in blood serum, whereas fish fed black soldier fly exhibited no change in haematological parameters (Chapter 8). The taurine deficiency may be taken into account, along with nutrient shortfalls and the presence of chitin in both studies. Taurine plays a vital role in the growth and degree of susceptibility to diseases of many fish species (Salze and Davis, 2015). According to a recent study (Basto et al., 2020), defatted mealworm had less taurine than non-defatted mealworms, while that is otherwise for soldier fly. This phenomenon could explain the low toleration of perch fed dietary defatted mealworm.

Despite the poor fish performance, the substantial replacement of fishmeal by both insect meals greatly lowered fish-in fish-out ratio, implying that fewer marine forage fish are required to produce farmed perch. A similar pattern was observed in Siberian sturgeon (Rawski et al., 2020) and meagre (Estévez et al., 2022). The environmental advantages of using substantial dietary insect meals were also reported for total solid waste, phosphorus solid waste (Chapter 4), and water demand (Chapter 8), which were comparable to or even less than insect-free diets. A similar amount of phosphorus waste generated in control and insect-containing diets was also reported for Atlantic salmon (*Salmo salar*) fed dietary full-fatted *H. illucens* (Weththasinghe et al., 2021).

As far as the economic aspect of insect-fed fish is concerned, Chapter 8 found that increasing dietary *H. illucens* in perch diets raised the economic conversion ratio but decreased the economic profit index, which was ascribed to the higher price of insect meal (3.5 €·kg<sup>-1</sup>) vs. fishmeal (1.48 €·kg<sup>-1</sup>). The introduction of insect meal (*T. molitor*) in seabass farming also raised concerns about feeding costs due to the high cost of insect meal (2.50–5.0 €·kg<sup>-1</sup>) (Arru et al., 2019). Our study (Chapter 8) highlighted that in order to gain the benefits from perch farming, the inclusion levels of insect meal should be limited to 20%. The vast majority of insect rearing facilities are still in their infancy, with only a limited volumes produced (Allaboutfeed, 2022), resulting in a high cost of production and thus high sale price (Dobermann et al., 2017). Therefore, it is suggested that increasing mechanization, scaling up the facility, and using low-value feed substrates could significantly reduce production costs (Niyonsaba et al., 2021).

Recent research has focused on additional benefits of including insect meals in the aquaculture diet, such as immunological improvement (Henry et al., 2018b) and gut microbiota modulation (Huyben et al., 2019), both of which are important indicators of farmed aquatic animals' welfare (Toni et al., 2019). In this sense, we performed swimming performance and metabolism of perch fed dietary *T. molitor* to examine whether this dietary treatment alters fish welfare-related indicators (Chapter 7). The results revealed that the addition of insect meal at the expense of fishmeal did not impair the swimming capacity of perch, but a variety of alterations in physiological indices, notably blood biochemistry, were necessary to sustain the swimming performance. It is also worth noting from this research from this study (Chapter 7) that perch prefer high water flow compared to numerous other fish species. This brings important implications for the technical design of the perch farming system in terms of establishing current velocity, as knowledge on such aspects remains limited (Polcar et al., 2015).

The physiological responses of aquaculture fish fed dietary insect meals are typically attributed to the context of whole feed formula. In fact, multiple feed ingredients in a diet are digested and assimilated to construct tissues, thereby underpinning such responses. In this sense, we (Chapter 5) employed stable isotope analysis and mixing model, which has been increasingly used in aquaculture nutrition to estimate the proportional contribution of feed ingredients to fish tissue construction (Enyidi et al., 2013; Gamboa-Delgado et al., 2008; Gamboa-Delgado et al., 2014; Gamboa-Delgado et al., 2016; Gamboa-Delgado and Le Vay, 2009; Gamboa-Delgado et al., 2020; Gamboa-Delgado et al., 2013; Gamboa Delgado, 2021; Liu et al., 2021). The Chapter provided an insight into complementarity among feed ingredients in the presence or absence of insect meal *T. molitor*, which occurred in different tissues, muscle, liver, and blood. In general, insect meal was shown to have a more significant function in the liver and blood, whereas its contribution to the muscle is lower than its dietary availability. The inclusion of soybean ingredients was dominant in all diets, and as a consequence, their contribution to all tissues was utmost. As future aquafeed requires the input of multiple protein sources to fulfil growing demand (Tacon, 2020), evaluating the role

and the nutritional complementarity among these sources in fish development, using stable isotopes and the mixing model, could provide beneficial information toward better feed formulas in satisfying the nutrient requirements of targeted fish species.

Overall, our research (Chapters 4, 5, 7, and 8) suggested the feasibility of incorporating partially defatted *H. illucens* and defatted *T. molitor* in diets for Eurasian perch (*P. fluviatilis*). However, bottlenecks associated with nutrient deficiency, chitin, and the cost of insect meals have hampered their substantial inclusion.

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### 1.3. Dietary black soldier fly (*Hermetia illucens*) for pikeperch (*Sander lucioperca*)

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Among percid fish in Europe, pikeperch (*S. lucioperca*) has piqued the greatest interest from academia and stakeholders as a promising candidate for freshwater aquaculture in Europe (FAO, 2020; Policar et al., 2019). The recent introduction of alternative protein sources from microorganisms in pikeperch diets has brought issues related to pikeperch's ability to digest cell wall in these novel ingredients and to tolerate plant-based feed (Schafberg et al., 2018; Schafberg et al., 2021). Furthermore, due to their sensitiveness to stress induced by the aquaculture system and nutrition, this species requires extreme care (Lund et al., 2012; Schafberg et al., 2018).

Promisingly, our study (Chapter 6; Tran et al., 2021) underlined that defatted *H. illucens* could replace up to 50% fishmeal, equivalent to an inclusion level of 18%, in diets for juvenile pikeperch without compromising weight gain and feed conversion ratio; higher levels violated both variables. Furthermore, dietary *H. illucens* enhanced antioxidative enzymes and favourable gut microbiota in pikeperch, implying an immunological boosting effect.

As previously stated, pikeperch is extremely sensitive to non-digestible components in the diet, being cell wall presented in microbial ingredients. In spite of this, insect-derived chitin appears to be tolerated at a low dietary level by pikeperch. It, therefore, could be anticipated from Chapter 6 that dietary chitin of as low as 1% could sustain physiological indices of pikeperch relative to a chitin-free diet. Such low dietary chitin has been ascribed to enhancing antioxidative enzymes and beneficial gut microbiome (Gasco et al., 2021; Henry et al., 2018a), which may further support our findings. Although chitinase is evidenced to be present in the gut of first-exogenous-feeding pikeperch and aids chitin digestion (Lahnsteiner, 2017), increasing chitin levels did not accompany elevation of chitinase in many fish (Kono et al., 1987). Excessive chitin may reduce energy availability and nutrient digestibility for fish, resulting in depressed growth performance (Ringø et al., 2012). This is most likely true for fish fed 100% fishmeal substituted by *H. illucens* at the corresponding chitin level of 1.93% in our study (Chapter 6). It is proposed that the application of exogenous chitinase or enzyme-degraded probiotics might enhance the substantial use of insect meal in pikeperch diets.

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### 1.4. Conclusions

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The present Ph.D. thesis provided a holistic evaluation of the use of insect meals in aquaculture fish diets with regard to growth production, environmental impacts, and the effect of dietary insect meals, black soldier fly (*H. illucens*), and yellow mealworm (*T. molitor*) on aquaculture-oriented percid fish, Eurasian perch (*P. fluviatilis*) and pikeperch (*S. lucioperca*), using broad physiological and environmental indices. Considering a wide range of insect meals in general, it was proposed that only a subset of insect meals might deliver possible results towards better acceptance by fed species and environmental benefits. Such preferable insect species

could be defatted larval yellow mealworm (*T. molitor*), full-fat pupal silkworm (*Bombyx mori*), and to a lesser extent, larval fly (*Chrysomya megacephala*), defatted prepupal black soldier fly (*H. illucens*), cricket (*G. bimaculatus*), grasshoppers (*O. fuscovittata*), and (*Z. variegatus*) and full-fat moth (*C. butyrospermi*). While introducing insect meals into aquaculture diets, there are still trade-offs between the advantage of reducing forage fish use and phosphorus waste load versus the burden associated with nitrogenous waste and environmental impacts. This necessitates more research into optimizing insect-containing feed formulas, insect farm infrastructure, and appropriate farming strategies for insect-fed fish.

Regarding insect-fed percid fish, we concluded that partially defatted *H. illucens* was preferable over highly defatted *T. molitor* in diets for Eurasian perch (*P. fluviatilis*), with plausible inclusion levels varying widely between both insects, being 40% and 6.8%, respectively. The substantial inclusion of *T. molitor* was thought to cause significant stress yet had little effect on the abundance and diversity of gut microbiota in Eurasian perch. In addition, as compared to the control diet, such inclusion imposed a greater environmental consequence. Concerning pikeperch (*S. lucioperca*), dietary *H. illucens* also showed promising results. Accordingly, an 18% inclusion or a 50% fishmeal substitution of/by highly-defatted black soldier fly was deemed appropriate. This insect form likewise delivered positive results for pikeperch in terms of health enhancement.

Stable isotope analysis and Bayesian mixing models were useful in determining the role of insect meal *T. molitor*, fishmeal, soybean, and corn meal in perch tissue development. The increasing inclusion level of insect meals did not significantly improve its contribution to fish muscle, liver, and blood but did reduce that of fishmeal. These tools provided insight into nutrient complementarity within diet formulation.

Overall, this doctoral dissertation laid the groundwork for further research into the possible use of insect meals in the diet of aquaculture species, particularly percid fish.

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### 1.5. Further scrutiny

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From a systematic standpoint, more research should be warranted to further investigate the benefits and drawbacks of using a diverse range of insect meals in aquafeeds, with an emphasis on, but not limited to, fillet quality, immune response, gut microbiota, among others, through systematic review and meta-analysis.

Aquaculture production of percid fish in Europe is rising, accompanying growing demand for feed input. It is strategic to discover additional innovative feed ingredients, such as other authorized insect meals, single-cell proteins, and animal-processed protein, to diversify feed formulations and ensure cost-effectiveness if the price of any feed ingredient fluctuates. The combination of ingredients based on nutritional complementarity in feed for percid fish could be a potential approach to sustain the physiological indices of fed organisms and the environmental advantages.

Given that substantial inclusion of insect meals in percid fish diets brings certain environmental benefits but compromises fish performance, balancing the nutritional composition of insect-containing diets relative to the control, such as amino acids, fatty acids and minerals, and supplementing dietary chitin-digestion boosters could be an effective way to promote successful use of insect meals.

In the context of the aquaculture production system, in which aquafeed plays an important role, further research into suitable systems to handle the nitrogenous load from fish fed insect-containing diets is warranted. Furthermore, the excrements from fish fed insect meals might be valuable secondary products, rather than waste, for different purposes, such as

organic fertilizers, since such products contain specific amounts of undigested chitin, on which beneficial microbes can thrive.

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## English summary

**Insect meal as feed source in nutrition of percids, Eurasian perch (*Perca fluviatilis*) and pikeperch (*Sander lucioperca*)**

Tran Quang Hung

The ever-expansion of aquaculture requires increasing aquafeed inputs, which have traditionally relied on protein and lipid from marine fisheries resources. Among a variety of novel protein sources proposed to the aquafeed ingredient shortlist, insect meals appear to be the most promising ingredients for fulfilling the future protein demand for aquafeed. Despite the fact that such protein-rich ingredients have been extensively used in diets for aquaculture species throughout academia, an overview of various insect meal forms, including different insect taxonomies, life-stages, and processing methods, on growth performance and environmental impacts remains limited. Furthermore, as aquaculture expands to a broader range of fish species, research into alternative protein sources in diets for such emerging species becomes increasingly important.

The present Ph.D. thesis employed 1) meta-analysis and systematic approach to investigate the effect of insect meals in diets for aquaculture fish species on production performance and environmental consequences, and 2) *in-vivo* feeding trials involved frequent-used insect meals, black soldier fly (*Hermetia illucens*) and yellow mealworm (*Tenebrio molitor*) in diets for potential aquaculture-oriented percid fishes, Eurasian perch (*Perca fluviatilis*) and pikeperch (*Sander lucioperca*), concerning a wide range of parameters.

Chapters 2 and 3 provided a comprehensive overview of growth performance indices and environmental impacts of aquaculture species fed dietary insect meals. Accordingly, defatted larval yellow mealworm (*T. molitor*) and full-fat pupal silkworm (*Bombyx mori*) are the most promising insects in supporting the specific growth rate of aquatic animals. Chapter 2 also suggested a list of well-known and underexploited insects, which could sustain fish growth at a specific plausible inclusion, including defatted (*H. illucens*), full-fat fly (*Chrysomya megacephala*), cricket (*Gryllus bimaculatus*), grasshoppers (*Oxya fuscovittata*) and (*Zonocerus variegatus*) and full-fat moth (*Cirina butyrospermi*). Chapter 3 revealed that feeding insect meal-containing diets, in comparison to insect-free diets, to aquaculture species reduced the demand for marine-derived forage fish required for farmed fish output, as evidenced by the economic fish-in fish-out ratio and phosphorus waste load but had negative impacts on nitrogen waste and environmental impacts categories, including global warming potential, energy use, land use, water use, acidification, and eutrophication. As demonstrated in Chapter 3, the inclusion of housefly (*Musca domestica*) at the expense of fishmeal and some plant ingredients in aquatic animal diets considerably decreased land use. These studies suggested the importance of combining feed ingredients to fill the production gap for future aquafeed, meet the nutrient requirement of targeted fish, and sustain environmental benefits.

Our works demonstrated that dietary black soldier fly (*H. illucens*) and yellow mealworm (*T. molitor*) were well accepted by both percid fishes, Eurasian perch (*P. fluviatilis*) and pikeperch (*S. lucioperca*), concerning growth performance. Chapters 4, 6 and 8 revealed that Eurasian perch tolerated dietary *H. illucens* better than *T. molitor*, since the former threshold was 40% compared to 6.8% of the latter; nevertheless, the former was accepted by pikeperch at 18%. Chapter 6 evidenced the effects associated with antioxidative enzyme and gut microbiome modulation of highly defatted *H. illucens* fed to pikeperch. Adding to this, Chapter 7 confirmed that dietary *T. molitor* did not alter the swimming capacity and metabolism of Eurasian perch, which hinted at fish welfare assurance. The introduction of *T. molitor* into

Eurasian perch diets faces challenges regarding physiological responses and environmental consequences, with substantial levels causing stress to fish and pressuring environmental indices. Our study (Chapter 5) revealed that the stable isotope and mixing model might be used to predict food sources and estimate the proportional contribution of feed components to perch's tissue. Such tools are also helpful to provide insight into complementarity among ingredients in feed formulation.

Further works to investigate various alternative protein sources for percid fish are warranted to ensure the sector's future growth. Beyond the use of single alternative in aquafeed, such as underexploited insect meals authorized to use in aquafeed in Europe, single-cell proteins, macroalgae, among others, combining among ingredients based on nutritional complementarity would pave the way for aquafeed's future success in an era of finite resources.

## Czech summary

**Hmyzí moučka jako zdroj krmiv ve výživě okounovitých ryb, okouna říčního (*Perca fluviatilis*) a candáta obecného (*Sander lucioperca*)**

Tran Quang Hung

Světová produkce akvakultury kontinuálně roste a s tím se zvyšuje potřeba surovin pro výrobu krmiv pro vodní živočichy. Suroviny tradičně pocházely z mořských zdrojů bílkovin a lipidů, jejichž vyšší spotřeba není trvale udržitelná. Hledají se proto nové zdroje bílkovin pro akvakulturu jako např. různé hmyzí moučky, které se zdají být slibnými ingrediencemi pro zajištění budoucí poptávky po bílkovinách pro krmiva v akvakultuře. Navzdory skutečnosti, že tyto ingredience bohaté na bílkoviny byly používány v krmivech pro různé druhy akvakulturně chovaných ryb po celém světě, přehled využití různých forem hmyzí moučky, včetně různých hmyzích taxonů, jejich vývojových stádií a metod zpracování, pokud jde o růst ryb a dopady na životní prostředí, zůstává omezený. Kromě toho se rozšiřuje škála akvakulturně chovaných druhů ryb, výzkum alternativních zdrojů bílkovin v krmivech tak nabývá na významu i pro tyto druhy ryb.

V této dizertační práci byla použita: 1) metaanalýza a systematický přístup k přezkoumání vlivu hmyzích mouček v krmivech pro akvakulturní druhy okounovitých ryb s ohledem na produkční výkonnost a důsledky pro životní prostředí a 2) *in vivo* krmné experimenty zahrnující hmyzí moučky z bráněnký (*Hermetia illucens*) a larev potemníka moučného (*Tenebrio molitor*) v krmivech pro akvakulturně významné okounovité ryby, okouna říčního (*Perca fluviatilis*) a candáta obecného (*Sander lucioperca*), se zřetelem na široké spektrum parametrů.

Kapitoly 2 a 3 poskytují komplexní přehled růstové výkonnosti a dopadů na životní prostředí u různých druhů akvakulturně chovaných ryb krmených dietami s obsahem hmyzích mouček. Z přehledu je patrné, že odtučněná moučka z larev *T. molitor* a plnotučná moučka z bource morušového (*Bombyx mori*) jsou nejslibnějšími hmyzími surovinami pro podporu specifické rychlosti růstu vodních živočichů. Kapitola 2 navrhla seznam známých, avšak pro potřeby krmiv zatím nedostatečně využívaných druhů hmyzu, které by mohly pomoci udržet růst ryb, a to odtučněné moučky *H. illucens*, plnotučné moučky bzučivky (*Chrysomya megacephala*), cvrčka dvojskvrnného (*Gryllus bimaculatus*), kobylek (*Oxya fuscovittata*, *Zonocerus variegatus*) a martináče (*Cirina butyrospermi*). Kapitola 3 odhalila, že podávání krmiv obsahujících hmyzí moučku ve srovnání s dietou bez obsahu hmyzu různým druhům ryb snížilo poptávku po krmných rybách pocházejících z odlovu v mořích, jak dokládá ekonomický poměr ryb z odlovu v mořích (eFIFO) a zatížení prostředí fosforem. Nicméně používání krmiv s obsahem hmyzích mouček mělo negativní dopady na množství dusíku produkovaného akvakulturou a na některé kategorie dopadů na životní prostředí, včetně potenciálu globálního oteplování, spotřeby energie, potřebu půdy, spotřebu vody, acidifikaci a eutrofizaci. Jak je naznačeno v kapitole 3, zahrnutí mouchy domácí (*Musca domestica*) na úkor rybí moučky a některých rostlinných složek do krmiv vodních živočichů značně snížilo potřebu půdy. Studie naznačuje, že je důležité vhodně kombinovat suroviny pro druhy ryb v akvakultuře, aby se naplnila vznikající potřeba pro akvakulturní krmiva, byly splněny požadavky chovaných ryb na živiny a byl udržen přínos těchto krmiv pro životní prostředí.

Naše práce prokázala, že krmiva s obsahem *H. illucens* a *T. molitor* byla dobře přijímána okounovitými rybami – okounem říčním a candátem obecným i s ohledem na růstovou výkonnost. Kapitoly 4, 6 a 8 odhalily, že okoun říční toleroval moučku z *H. illucens* v krmivech více než moučku z *T. molitor*, protože u prvního druhu byla optimální úroveň 40 % ve srovnání s 6,8 % u druhého druhu. Nicméně *H. illucens* v krmivu akceptoval candát obecný

při zastoupení na úrovni 18 %. Kapitola 6 prokázala účinky spojené s modulací antioxidantních enzymů a střevního mikrobiomu za použití vysoce odtučněné moučky z *H. illucens* v krmivech pro candáta obecného. Kromě toho kapitola 7 potvrdila, že obsah *T. molitor* v krmivu nezměnil kapacitu plavání a metabolismus okouna říčního, což naznačuje zajištění dobrých životních podmínek ryb při použití takových krmiv. Zavedení moučky *T. molitor* do krmiv okounů říčních čelí výzvám týkajícím se fyziologických reakcí a environmentálních důsledků, přičemž zvýšené úrovně zastoupení způsobují rybám stres a negativně ovlivňují environmentální ukazatele. Naše studie (kapitola 5) odhalila, že „mixing“ modely stabilních izotopů lze použít k predikci zdrojů potravy a odhadu proporcionálního příspěvku složek krmiva k výstavbě tkání okouna říčního. Tyto metody jsou také užitečné při poskytování náhledu na komplementaritu mezi složkami krmiv.

Další výzkum různých alternativních zdrojů bílkovin pro okounovité ryby je potřeba pro zajištění budoucího růstu odvětví akvakultury. Kromě použití jednotlivých surovin v krmivech pro akvakulturu, jako jsou doposud nedostatečně využívané hmyzí moučky druhů povolených k použití v Evropě, jednobuněčné proteiny či makrořasy, by kombinování těchto ingrediencí založené na komplementaritě výživy mělo připravit řešení pro budoucí úspěšnou produkci krmiv v éře omezených zdrojů a expandující akvakultury.

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## List of publications

### Peer-reviewed journals with IF

- Tran, H.Q.**, Nguyen, T.T., Prokešová, M., Gebauer, T., Doan, H.V., Stejskal, V., 2022. Systematic review and meta-analysis of production performance of aquaculture species fed dietary insect meals. *Reviews in Aquaculture* 14 (3): 1637–1655. (IF 2021 = 10.618)
- Tran, H.Q.**, Van Doan, H., Stejskal, V., 2022. Environmental consequences of using insect meal as an ingredient in aquafeeds: A systematic view. *Reviews in Aquaculture* 14: 237–251. (IF 2021 = 10.618)
- Tran, H.Q.**, Prokešová, M., Zare, M., Matoušek, J., Ferrocino, I., Gasco, L., Stejskal, V., 2022. Production performance, nutrient digestibility, serum biochemistry, fillet composition, intestinal microbiota and environmental impacts of European perch (*Perca fluviatilis*) fed defatted mealworm (*Tenebrio molitor*). *Aquaculture* 547: 737499. (IF 2021 = 5.135)
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- Tran, H.Q.**, Prokešová, M., Zare, M., Gebauer, T., Elia, A.C., Colombino, E., Ferrocino, I., Caimi, C., Gai, F., Gasco, L. and Stejskal, V., 2021. How does pikeperch (*Sander lucioperca*) respond to dietary insect meal (*Hermetia illucens*)? Investigation on gut microbiota, histomorphology, and antioxidant biomarkers. *Frontiers in Marine Science* 8: 681942 (IF 2020 = 4.912)
- Tran, H.Q.**, Kiljunen, M., Doan, H.V., Stejskal, V., 2021. European perch (*Perca fluviatilis*) fed dietary insect meal (*Tenebrio molitor*): From a stable isotope perspective. *Aquaculture* 545: 737265. (IF 2020 = 4.242)
- Tran, H.Q.**, Van Doan, H. and Stejskal, V., 2021. Does dietary *Tenebrio molitor* affect swimming capacity, energy use, and physiological responses of European perch *Perca fluviatilis*? *Aquaculture* 539: 736610. (IF 2020 = 4.242)
- Zare, M., **Tran, H.Q.**, Prokešová, M., Stejskal, V., 2021. Effects of garlic *Allium sativum* powder on nutrient digestibility, haematology, and immune and stress responses in Eurasian perch *Perca fluviatilis* juveniles. *Animals* 11: 2735. (IF 2020 = 2.752)
- Hien, V.D., Lumsangkul, C., Hoseinifar, S.H., **Tran, Q.H.**, Stejskal, V., Ringø, E., Dawood, M.A.O., Esteban, M.Á., 2020. Administration of watermelon rind powder to Nile tilapia (*Oreochromis niloticus*) culture under biofloc system: Effect on growth performance, innate immune response, and disease resistance. *Aquaculture* 528: 735574. (IF 2019 = 3.224)
- Piamsomboon, P., Jaresitthikunchai, J., **Tran, H.Q.**, Roytrakul, S., Wongtavatchai, J., 2020. Identification of bacterial pathogens in cultured fish with a custom peptide database constructed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). *BMC Veterinary Research* 16: 52. (IF 2018 = 1.792)

- Stejskal, V., **Tran, H.Q.**, Prokešová, M., Gebauer, T., Giang, P.T., Gai, F., Gasco, L., 2020. Partially defatted *Hermetia illucens* larva meal in diet of Eurasian perch (*Perca fluviatilis*) juveniles. *Animals* 10: 1876. (IF 2019 = 2.323)
- Van Doan, H., Hoseinifar, S.H., **Tran, H.Q.**, Lumsangkul, C., Jaturasitha, S., El-Haroun, E. and Paolucci, M., 2020. Dietary inclusion of chestnut (*Castanea sativa*) polyphenols to Nile tilapia reared in biofloc technology: Impacts on growth, immunity, and disease resistance against *Streptococcus agalactiae*. *Fish and Shellfish Immunology* 105: 319–326. (IF 2020 = 4.581)

### Abstracts and conference proceedings

- Tran, H.Q.**, Kiljunen, M., Doan, H.V., Stejskal, V., 2022. Natural stable isotopes of various insect meals and their contribution to tissue construction of European perch (*Perca fluviatilis*). In Book of abstracts "XX International symposium on fish nutrition and feeding towards precision fish nutrition and feeding", 5–9<sup>th</sup> June 2022, Sorrento, Italy
- Stejskal, V., **Tran, H.Q.**, Zare, M., Prokešová, M., Gebauer, T., Ferrocino, I., Caimi, C., Gai, F., Gasco, L., 2021. The effect of *Hermetia ilucens* insect meal inclusion on intestine microflora in pikeperch *Sander lucioperca*. In Book of abstracts "Aquaculture Europe 2021", 4–7<sup>th</sup> October 2021, Madeira, Portugal, p. 1246
- Tran, H.Q.**, Kiljunen, M., Doan, H.V., Stejskal, V., 2021. European perch (*Perca fluviatilis*) fed dietary insect meal (*Tenebrio molitor*): From a stable isotope perspective. In Book of abstracts "Aquaculture Europe 2021", 4–7<sup>th</sup> October 2021, Madeira, Portugal, p. 1295
- Zare, M., **Tran, H.Q.**, Prokešová, M., Stejskal, V., 2021. Effects of garlic *Allium sativum* powder on nutrients, haematology, and immune and stress response in Eurasian perch *Perca fluviatilis* juveniles. In Book of abstracts "Aquaculture Europe 2021", 4–7<sup>th</sup> October 2021, Madeira, Portugal, p. 1386
- Prokešová, M., Bušová, M., Korytár, T., Zare, M., **Tran, H.Q.**, Stejskal, V., 2019. Effects of humic substances on growth performance and health status of juvenile *Clarias gariepinus* (Burchell, 1822). In Book of abstracts "Aquaculture Europe 2019", 7–10<sup>th</sup> October 2021, Berlin, Germany

Training and supervision plan during study	
<b>Name</b>	Tran Quang Hung
<b>Research department</b>	2018–2022 – Laboratory of Controlled Reproduction and Intensive Fish Culture of FFPW
<b>Supervisor</b>	Assoc. Prof. Vlastimil Stejskal
<b>Period</b>	3 <sup>rd</sup> September 2018 until 15 <sup>th</sup> September 2022
Ph.D. courses	Year
Basic of scientific communication	2019
Pond aquaculture	2019
Ichthyology and fish taxonomy	2019
Applied hydrobiology	2020
Biostatistics	2020
English language	2020
Scientific seminars	Year
Annual Ph.D. seminars organized by FFPW	2019 2020 2021
Ph.D. seminars organized by USB	2022
International conferences	Year
<b>International conferences</b>	
<b>Tran, H.Q.</b> , Kiljunen, M., Doan, H.V., Stejskal, V., 2022. Natural stable isotopes of various insect meals and their contribution to tissue construction of European perch ( <i>Perca fluviatilis</i> ). In Book of abstracts "XX International symposium on fish nutrition and feeding towards precision fish nutrition and feeding", 5–9th June 2022, Sorrento, Italy	2022
Stejskal, V., <b>Tran, H.Q.</b> , Zare, M., Prokešová, M., Gebauer, T., Ferrocino, I., Caimi, C., Gai, F., Gasco, L., 2021. The effect of <i>Hermetia ilucens</i> insect meal inclusion on intestine microflora in pikeperch <i>Sander lucioperca</i> . In Book of abstracts "Aquaculture Europe 2021", 4–7th October 2021, Madeira, Portugal, p. 1246	2021
<b>Tran, H.Q.</b> , Kiljunen, M., Doan, H.V., Stejskal, V., 2021. European perch ( <i>Perca fluviatilis</i> ) fed dietary insect meal ( <i>Tenebrio molitor</i> ): From a stable isotope perspective. In Book of abstracts "Aquaculture Europe 2021", 4–7th October 2021, Madeira, Portugal, p. 1295	2021
Zare, M., <b>Tran, H.Q.</b> , Prokešová, M., Stejskal, V., 2021. Effects of garlic <i>Allium sativum</i> powder on nutrients, haematology, and immune and stress response in Eurasian perch <i>Perca fluviatilis</i> juveniles. In Book of abstracts "Aquaculture Europe 2021", 4–7th October 2021, Madeira, Portugal, p. 1386	2021
Prokešová, M., Bušová, M., Korytár, T., Zare, M., <b>Tran, H.Q.</b> , Stejskal, V., 2019. Effects of humic substances on growth performance and health status of juvenile <i>Clarias gariepinus</i> (Burchell, 1822). In Book of abstracts "Aquaculture Europe 2019", 7–10th October 2021, Berlin, Germany	2019
Foreign stays during Ph.D. study at FFPW	Year
Dr. Mikko Kiljunen. University of Jyväskylä, Department of Biological and Environmental Science, Finland. Stable isotope analysis and its applications in fish nutrition (2 months)	2020
Prof. Laura Gasco and Assoc. Prof. Ilario Ferrocino. University of Torino, Department of Agricultural, Forest and Food Sciences. Italy. Gut microbiota analysis in fish (2 months)	2022

Pedagogical activities	Year
• Leading of summer school project: Swimming capacity of asp ( <i>Aspius aspius</i> ) and nutritional composition of seven potential insect meals in fish nutrition	2021
• Lecturing and laboratory tutoring for bachelor and master students from Fishery discipline at FFPW, USB in range of 50 teaching hours	2019–2021
• Thesis consultation for student at FFPW, USB: Critical swimming speed in intensively cultured European perch ( <i>Perca fluviatilis</i> L.): Influence of fish size, production system and repeated testing	2019
• Thesis consultation for student at FFPW, USB: Využití hmyzí moučky z potměníka moučného ( <i>Tenebrio molitor</i> ) v krmivech pro okouna říčního ( <i>Perca fluviatilis</i> L.)	2021

## Curriculum vitae

### PERSONAL INFORMATION

**Name:** Hung Quang  
**Surname:** Tran  
**Title:** M.Sc.  
**Born:** 21<sup>st</sup> February 1986, Nam Dinh, Vietnam  
**Nationality:** Vietnamese  
**Languages:** English (B2 level – FCE certificate),  
 Vietnamese  
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### EDUCATION

**2018 – present** Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic  
**2013–2015** M.Sc., Faculty of Agriculture, Department of Animal and Aquatic Science, Chiang Mai, Thailand  
**2004–2008** B.Sc., Faculty of Fisheries, Vietnam National University of Agriculture, Hanoi, Vietnam  
**1992–2004** Primary, Secondary and High Schools, Hai Hau district, Nam Dinh province, Vietnam

### TRAINING COURSES

18–22/11/2019 Fish Nutrition and Feed, INRA, France (In-person)  
 14–23/06/2021 Fish Nutrition and Feeding, IAMZ, Zaragoza, Spain (Online)

### RESEARCH STAY

21/5-28/7/2022 Prof. Laura Gasco and Assoc. Prof. Ilario Ferrocino, University of Torino, Department of Agricultural, Forest and Food Sciences, Italy  
 1/3–30/4/2020 Dr. Mikko Kiljunen. University of Jyväskylä, Department of Biological and Environmental Science, Finland

### COLLABORATIONS

January 2022 to present Dr. Thomas Jansen, Bern university of Applied Science, Switzerland  
 Insect meal and alternative feed sources in percids nutrition  
 2021 to present Michel van Spankeren, Protix, the Netherlands  
 Insect meal in fish nutrition  
 2021 to present Dr. Fabio Brambrilla, Naturealleve, Italy  
 Rainbow trout and African catfish nutrition  
 2020 to present Dr. Marek Šmejkal, Institute of Hydrobiology CAS, Czech Republic  
 Swimming physiology of asp, crucian carp and gibel carp  
 2020 to present Dr. Mikko Kiljunen, University of Jyväskylä, Finland  
 Stable isotope technique  
 2019 to present Assoc. Prof. Francesco Gai, National Research Council, Italy  
 Insect meal in fish nutrition

- 2019 to present Prof. Martin Flajšhans, Laboratory of Molecular, Cellular and Quantitative Genetics, Czech Republic  
Swimming physiology and respirometry in sturgeon relative to its ploidy level
- 2019 to present Prof. Laura Gasco and Assoc. Prof. Ilario Ferrocino, University of Torino, Italy  
Novel ingredients in aquafeeds
- 2018 to present Assoc. Prof. Hien Van Doan, Chiang Mai University, Thailand  
Immunology in fish