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(Article begins on next page)

**Dietary inclusion of full-fat *Hermetia illucens* prepupae meal  
in practical diets for rainbow trout (*Oncorhynchus mykiss*):  
lipid metabolism and fillet quality investigations**

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**Abstract**

Insects are able to bio-convert organic by-products into a sustainable biomass for aquafeed formulation. Specifically, among several insect species, *Hermetia illucens* (H) is particularly interesting for its nutritious traits but, unfortunately, the lipidic fraction is poorly represented by polyunsaturated fatty acids n-3 and poses some limits in its application in aquafeed formulation.

The present study undertook an interdisciplinary approach to explore the effects of three experimental diets containing increasing levels of full-fat H meal (H0 diet based on fishmeal and purified protein-rich vegetable ingredients; H25 and H50 diets containing 25% or 50% of full-fat H

meal replacing fishmeal, respectively), on rainbow trout (*Oncorhynchus mykiss*) fed over a 98 days experimental period. The expression of genes related to lipid metabolism by RT-qPCR, liver histology, as well as the qualitative traits of fillets and fatty acid (FA) composition were investigated. Interestingly, *fads2* gene expression in pyloric caeca increased in fish fed diets containing the highest full-fat H meal inclusion ( $H_{50} > H_0$ ;  $p < 0.05$ ). Liver histological examinations showed normal morphological aspect even though hepatic FA profiles seemed to resemble those of the diets. However, liver docosahexaenoic acid did not significantly differ between the dietary groups and showed a mean value of 11.07 g FA methyl esters/100 g total FA methyl esters. Despite the FA profile of the three diets differed depending on the H meal inclusion level, biometrics, fillet physical traits, total lipids and the overall FA profile were not jeopardised, not even eicosapentaenoic and docosahexaenoic acids.

The overall results showed that the dietary full-fat H meal inclusion under study did not impair fish fillet quality, guaranteeing its nutritional value. Some effects on lipid metabolism were observed, as suggested by liver, pyloric caeca and mid intestine gene expression and liver FA profile. Future studies on the biological mechanisms behind the macroscopic traits of fish fed unprocessed insects are warmly encouraged.

**Key words:** black soldier fly, gene expression, qPCR, quality, DHA, docosahexaenoic acid.

## 1. Introduction

Sustainability has been set as a vital goal in every production process. Nowadays, insects have been deeply investigated as alternative protein source for aquafeed formulation (Lock et al., 2018; Nogales-Mérida et al., 2018; Sánchez-Muros et al., 2014), for their environmental, economic and societal benefits (Bosch et al., 2019; van Huis et al., 2013). Among others, the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae. H), is one the most studied and promising insect species

49 for aquafeed formulation, especially for its nutritional characteristics that meet most of the fish  
50 nutritional requirements (Barragan-Fonseca et al., 2017; Barroso et al., 2014; Henry et al., 2015).  
51 Nevertheless, insect lipids are primarily composed of saturated (SFA) and monounsaturated  
52 (MUFA) fatty acids (FAs), while polyunsaturated FAs n-3 (PUFAn-3) are scarce. This deficiency  
53 may affect fish welfare and the nutritional composition of the edible portion. In fact, fish are  
54 renowned for their high long-chain PUFAn-3 (LC PUFAn-3) content, which are mainly  
55 accumulated through the diet and, in some species, partly synthesised by endogenous production  
56 (especially in freshwater species) (Tocher, 2003). In humans, LC PUFAs, like eicosapentaenoic  
57 (EPA) and docosahexaenoic acids (DHA), are well known to reduce the risk of cardiovascular and  
58 inflammatory disorders and depression (Rosenlund et al., 2010) and thus one of the main goal of the  
59 aquaculture sector is to guarantee an adequate amount of these FAs in the final product. As a  
60 consequence, the aquaculture industry is continuously looking for “suitable, sustainable, and  
61 environmentally acceptable” dietary alternatives in order to reduce the use of conventional marine  
62 and vegetable sources and to guarantee fish physical, chemical and sensorial attributes. Recently,  
63 several feeding trials with different dietary inclusion levels of H meal have been performed on  
64 salmonids, showing that both digestibility and growth performance were not harshly affected  
65 (Cardinaletti et al., 2019; Lock et al., 2016; Renna et al., 2017). However, a potential effect of this  
66 new ingredient was evidenced on fillet qualitative traits (Belghit et al., 2018; Bruni et al., 2020;  
67 Mancini et al., 2018; Renna et al., 2017). To limit undesired outcomes on fillet FA composition,  
68 two approaches have been undertaken. On the one hand, prior to the administration to fish, insect  
69 larvae FA profile was tailored by rearing them on PUFAn-3-rich substrates, like *Ascophyllum*  
70 *nodosum* (Liland et al., 2017), microalgae (Truzzi et al., 2020) or fish material (Barroso et al., 2019,  
71 2017; St-Hilaire et al., 2007). The second approach considered a thorough modulation of the lipidic  
72 ingredients of fish feed by augmenting the dietary fish oil inclusion level (Belghit et al., 2019a).

73 The FA profile of the fish end-product is the macroscopic manifestation of complex metabolic  
74 processes, mainly involving intestine and liver, occurring between feed ingestion, digestion and  
75 lipid deposition in the fillet.

76 Nutrient absorption in fish takes place along the entire intestinal tract, usually decreasing along this  
77 same organ, in rainbow trout and Atlantic salmon (Bakke et al., 2010; NRC, 2011). The chyme  
78 contains emulsified lipids and lipid-soluble vitamins, which downstream are further emulsified by  
79 bile acids and consequently hydrolysed, absorbed and then re-esterified into triacylglycerols. The  
80 enzyme encoded by cluster of differentiation 36 (*cd36*) gene is involved in cellular FA uptake by  
81 enterocytes, hepatocytes and adipocytes (The UniProt Consortium, 2019). Pyloric caeca execute the  
82 absorption of several lipid components, such as free FAs, lysophospholipids and monoacylglycerols  
83 (Turchini et al., 2009); pyloric caeca are a site of *de novo* LC PUFA synthesis by means of a FA  
84 conversion pathway, involving elongase and desaturase enzymes, and are thought to play an  
85 important role in DHA synthesis (Bell et al., 2003). Scant investigations on mid intestine FA  
86 catabolism and *de novo* synthesis are found in the literature (Lazzarotto et al., 2018; Tacchi et al.,  
87 2012) , while more grounded information is available on the role of liver. Among the numerous  
88 functions, hepatocytes receive nutrients from the intestine through the circulatory stream, are  
89 involved in lipids storage (Bakke et al., 2010; NRC, 2011) and are the main district for the FA  
90 metabolism (Tocher, 2003).

91 FA metabolism is governed by a substantial number of genes [among others, the peroxisome  
92 proliferator-activated receptors (*ppars*), FA desaturases (*fads*<sub>s</sub>) and elongation of very long chain  
93 FAs (*elovl*<sub>s</sub>)] regulated by endogenous and exogenous conditions like dietary lipid quantity and  
94 quality (Tocher, 2003), beside a tissue-specific modulation (Morash et al., 2009). *Ppar*<sub>s</sub> are  
95 transcriptional regulators, potentially expressed in all tissues, acting as regulators in lipid storage,  
96 mobilisation and fat burning, other than in glucose homeostasis, respiration, morphogenesis and  
97 inflammatory response (Janani and Ranjitha Kumari, 2015). Desaturation and elongation also can  
98 take place in fish liver with a pathway similar to that of other vertebrates (Tocher, 2003). EPA and

99 DHA biosynthesis pathway starts with the essential FA C18:3n-3 and involves *fads* and *elovl*,  
100 working in turn until the production of C24:6n-3, that is finally  $\beta$ -oxidised to C22:6n-3, i.e., DHA  
101 (Tocher, 2003). Alternatively, Oboh et al. (2017) have recently pointed out that the *fads2* enzyme  
102 also exhibits  $\Delta 4$  activity in some fish species, but the authors did not specifically confirm these  
103 findings in salmonids. Nonetheless, salmonids have a more pronounced capacity of producing EPA  
104 and DHA in comparison to the other euryhaline or saltwater fish species, and this capacity is also  
105 modulated by the diet (Turchini et al., 2009).

106 The present study was part of the larger feeding trial detailed in Cardinaletti et al. (2019), which  
107 focused on growth performance, gastrointestinal integrity and stress of rainbow trout  
108 (*Oncorhynchus mykiss*) fed practical diets including increasing full-fat H meal levels. The  
109 additional value of the present study was to assess the effects of the same practical diets on  
110 the qualitative traits of rainbow trout fillets and on liver histology and FA composition. In  
111 addition, the expression of genes involved in lipid metabolism in liver, pyloric caeca and mid  
112 intestine, the main districts dedicated to FA absorption and metabolism, was investigated through  
113 real time PCR to question the physiological implications, behind fillet lipid constitution, when  
114 feeding a commercially important aquaculture fish species with this innovative ingredient.

115

## 116 **2. Material and methods**

### 117 **2.1. Ethical statement, diets, fish rearing and tissue sampling**

118 All procedures for animal handling and care were accomplished according to the guidelines of the  
119 European Union (Directive 2010/63/EU, 2010) and Italian law (D.L. 26/2014) and the experimental  
120 protocol was approved by the Ethical Committee of the University of Udine (Prot. N. 1/2018). The  
121 fish feeding trial was performed at the experimental facility of the Agricultural, Food,  
122 Environmental and Animal Sciences Department of the University of Udine (Italy) at the following  
123 conditions.

124 As previously described by Cardinaletti et al. (2019), three isonitrogenous, isolipidic, and  
125 isoenergetic diets were prepared (Table 1) in order to test one control diet containing fishmeal and  
126 purified protein-rich vegetable ingredients (H0) and two experimental diets where 25% (H25) or  
127 50% (H50) of the fishmeal had been replaced by full-fat H meal. Each diet was assigned to three  
128 fish groups made of 30 juvenile rainbow trout (*Oncorhynchus mykiss*) each, with an initial body  
129 weight of  $137.3 \pm 10.5$  g. Specimens were randomly allocated to nine 1 m<sup>3</sup> square fiberglass tanks  
130 and fed over 98 days as described in Cardinaletti et al. (2019). At the end of the feeding trial, after a  
131 10-hour fasting period to ensure that the intestinal tract was empty, fish were euthanised with MS-  
132 222 (300 mg/L) and the organs sampled as follows: from nine fish per dietary treatment, fillets were  
133 allocated to physical analyses, while liver, pyloric caeca and mid intestine (corresponding to the  
134 tract immediately behind the anterior segment to the ileorectal valve) were immediately excised, put  
135 in individual plastic tubes, frozen in liquid nitrogen and then stored at -80 °C for gene expression  
136 analyses. Subsamples of liver were quickly fixed in Bouin's solution (Merk Sigma Aldrich, Milan,  
137 Italy) for histological analysis; subsamples of liver and fillets were assigned to physical and  
138 chemical analyses.

139

## 140 **2.2. Gene expression analyses**

### 141 **RNA extraction and cDNA synthesis**

142 To enable the analysis of a number of genes, subsamples of liver (L), pyloric caeca (C) and mid  
143 intestine (M) samples were utilised to perform total RNA extraction using RNeasy® RT reagent  
144 (Sigma-Aldrich®, R4533) following the manufacturer's instructions. Total RNA extracted was  
145 eluted in 20 µL of RNase-free water (Qiagen). Final RNA concentration was determined by the  
146 NanoPhotometer® P-Class (Implen, München, Germany). RNA integrity was verified by GelRed™  
147 staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at -80 °C until

148 use. Finally, 2 µg of RNA were used for cDNA synthesis, employing the High Capacity cDNA  
149 Reverse Transcription Kit (Bio-Rad, Milan, Italy) following the manufacturer's instructions.

#### 150 **Real-Time qPCR**

151 Prior to qPCR reactions, all primer pairs were used in gradient reactions in order to determine the  
152 optimal annealing temperatures; control cDNA samples were pooled and used for this purpose.  
153 Then, PCR efficiency for each primer pair was determined using 10-fold serial dilutions of pooled  
154 liver, pooled pyloric caeca and pooled mid intestine cDNA samples.

155 qPCRs were performed in duplicate with SYBR® Green in an iQ5 iCycler thermal cycler (both from  
156 Bio-Rad, CA, USA). For each sample, reactions (10 µL) were set on a 96-well plate by mixing 1 µL  
157 cDNA diluted 1:20, 5 µL of 2× concentrated SYBR® Green as the fluorescent intercalating agent,  
158 0.2 µM forward primer, and 0.2 µM reverse primer. The thermal profile for all reactions was: 3 min  
159 at 95 °C, followed by 45 cycles of 10 s at 95 °C, 20 s at a variable temperature depending on the  
160 primer annealing temperature (Table 2) and 20 s at 72 °C. Fluorescence was monitored at the end of  
161 each cycle. In all cases, dissociation curve analysis showed a single pick.

162 Relative quantification of the expression of genes involved in fish lipid metabolism (*fads2*, *elovl1*,  
163 *elovl2*, *ppara*, *pparβ*, *pparδ*, *pparγ* and *cd36*) was performed using *arp* and *60S* as housekeeping  
164 genes to standardise the results (Table 2). The primer sequences were designed using Primer-  
165 BLAST tool available in NCBI (<http://www.ncbi.nlm.nih.gov/>). Data were analysed using the iQ5  
166 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5  
167 files (Bio-Rad, CA, USA). Modification of gene expression was reported in relation to controls.

168

### 169 **2.3. Liver analyses: hepatosomatic index, histology and fatty acid** 170 **characterisation**

171 Liver samples (n=9) were fixed by immersion in Bouin's solution (Sigma-Aldrich, Milan, Italy) and  
172 stored at 4 °C for 24h. Samples were washed three times with ethanol (70%) for ten minutes and



173 preserved in the same ethanol solution. Samples were then dehydrated in crescent ethanol solutions  
174 (80, 95 and 100%), washed with xylene and embedded in solid paraffin (Bio-Optica, Milan, Italy).  
175 Solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS, Nussloch, Germany)  
176 and 5 µm sections were stained with Mayer's haematoxylin and eosin Y (Sigma-Aldrich, Milan,  
177 Italy) according to Giorgini et al. (2018) and Randazzo et al. (2015). Sections were observed using  
178 a Zeiss Axio Imager.A2 (Oberkochen, Germany) microscope and images acquired by mean of a  
179 combined colour digital camera Axiocam 503 (Zeiss, Care Zeiss, Oberkochem, Germany).  
180 Furthermore, to ascertain the extent of fat accumulation in liver, a quantitative analysis was  
181 performed on a substantial number of histological sections from each experimental group in  
182 triplicate (n=9). Non-evaluable areas, such as blood vessels, were not considered. The percentage of  
183 fat fraction (PFF) on the total tissue areas was calculated using the ImageJ software setting a  
184 homogeneous threshold value as described in Zarantoniello et al. (2020).  
185 Other five livers per group were weighed in order to calculate the hepatosomatic index (HSI) as  
186 follows:

187 
$$\text{HSI} = [\text{liver weight/total body weight (g)}] \times 100.$$

188 Then, the same five livers were analysed for total lipid contents as well as FA profile, following the  
189 methods described by Folch et al. (1957) and Secci et al. (2018), respectively. The ratio of FA  
190 products:precursors was utilised to assess the desaturating and elongating activities, as proposed by  
191 Renaville et al. (2013).

192

#### 193 **2.4. Physical analyses, lipid composition and oxidative status of fillets**

194 Fillets from nine fish per diet were allocated to the physical analyses. The colour of the fillets was  
195 measured on triplicate positions (cranial, medial and caudal) on both fish sides with a CHROMA  
196 METER CR-200 (Konica Minolta, Singapore Japan) following the CIELab system (CIE, 1976) and  
197 recording L\* (lightness), a\* (redness index) and b\* (yellowness index) parameters. Colour values

198 were recorded and  $\Delta E$  between pairs of samples was calculated according to the following formula:

199 
$$\Delta E_{(\beta-\alpha)} = \left[ (L_{\beta}^* - L_{\alpha}^*)^2 + (a_{\beta}^* - a_{\alpha}^*)^2 + (b_{\beta}^* - b_{\alpha}^*)^2 \right]^{0.5}$$

200 where  $\alpha$  and  $\beta$  represent alternatively the mean colour values of H0, H25 or H50.

201 The values of pH and water holding capacity (WHC) were measured as well. The pH value was  
202 measured on triplicate fillet positions (cranial, medial and caudal) by a pH-meter SevenGo SG2™  
203 (Mettler-Toledo, Schwerzenbach, Switzerland). Afterwards, fillets were skinned, homogenised and  
204 utilised to determine WHC (Iaconisi et al., 2018) and chemical composition, as described below.

205 Total lipids were extracted from the fillets (n=5) following the method described by Folch et al.  
206 (1957), then they were gravimetrically quantified. The FA profile of each lipid extract was  
207 determined as FA methyl esters (FAME) using a Varian 430 gas chromatograph (Agilent, Palo  
208 Alto, CA, USA) set as described in Secci et al. (2018). Once obtained the FA profile, the ratio of  
209 FA products:precursors was utilised to assess the desaturating and elongating activities of fillets, as  
210 proposed by Renaville et al. (2013).

211 The following nutritional indices were also calculated:

212  
213 Atherogenicity index (AI) (Ulbricht and Southgate, 1991):

214 
$$\frac{C12:0 + (4 \times C14:0) + C16:0}{MUFA + PUFA_{n-6} + PUFA_{n-3}}$$

215  
216 Thrombogenicity index (TI) (Ulbricht and Southgate, 1991):

217 
$$\frac{C14:0 + C16:0 + C18:0}{(0.5 \times MUFA) + (0.5 \times PUFA_{n-6}) + (3 \times PUFA_{n-3}) + \left(\frac{n-3}{n-6}\right)}$$

218  
219 - hypocholesterolaemic/Hypercholesterolaemic FA ratio (h/H) (Santos-Silva et al., 2002):

220 
$$\frac{C18:1_{n-9} + C18:2_{n-6} + C18:3_{n-3} + C20:4_{n-6} + C20:5_{n-3} + C22:5_{n-3} + C22:6_{n-3}}{C14:0 + C16:0}$$

221 - PUFAn-3/PUFAn-6 ratio

222

223 Finally, 2 g of homogenised fillet were utilised to determine the secondary lipid oxidation products  
224 (thiobarbituric acid reactive substances, TBARS) according to Vyncke (1970). The absorbance at  
225 532 nm was read with a 50 Scan spectrophotometer equipped with Cary Win UV software (Varian,  
226 Palo Alto, CA, USA) on two technical replicates for each sample.

227

## 228 2.5. Statistical analysis

229 The statistical software package Prism6 (GraphPad Software, La Jolla, California, USA,  
230 www.graphpad.com) was used to analyse q-PCR data with a one-way ANOVA. Significance was  
231 set at  $p \leq 0.05$ ; residuals df and F values are also reported. Results are presented as means  $\pm$  standard  
232 deviation.

233 Data about PFF, chemical composition, physical traits, nutritional indices and TBARS content were  
234 assessed for normality (Shapiro-Wilk test) and homoscedasticity (Levene's test). If normality and  
235 homoscedasticity were not met, a boxcox transformation was performed. Then, a one-way ANOVA  
236 followed by a Tukey's test were performed using the free software environment R (R Core Team,  
237 2018), with significance set at  $p \leq 0.05$ ; residuals df and F values are also reported. Results are  
238 presented as means and pooled standard error of the mean (SEM), if not otherwise stated.

239

## 240 3. Results

### 241 3.1. Gene expression

242 The gene expression of *cd36*, *ppar*, and some genes related to LC PUFA biosynthetic pathways was  
243 analysed (Figure 1a-h). Following the dietary H meal inclusion, pyloric caeca *cd36* gene expression  
244 increased, while mid intestine *ppara*, liver *ppar $\delta$*  and the *ppar $\beta$*  gene expression showed faint  
245 decreasing trends; *ppar $\gamma$*  seemed to be moderately stable across the three different dietary regimes;

10

**Commentato [11]:** Io df e f li aggiungerei solo dove c e diff  
statisticamente significativa.  
Poi sentiamo anche gli altri

246 no significant differences were detected ( $p>0.05$ ;  $df=9, 9, 8$ ;  $F=0.5636, 0.7667, 1.536$ ; L, C and M,  
247 respectively). The *fads2* gene expression in the pyloric caeca significantly increased in fish fed the  
248 highest dietary H meal inclusion (H50 vs H0;  $p<0.05$ ;  $df=9$ ;  $F=4.468$ ). For the same target gene, a  
249 similar trend ( $p>0.05$ ;  $df=8$ ;  $F=1.695$ ) was also observed in the mid intestine (M). Finally, no  
250 significant differences in the expression of both *elov11* and *elov12* were observed in the different  
251 tissues under study (liver, pyloric caeca and mid intestine).

Commentato [LB2]: Anche qui dove  $p>0.05$ ?

Commentato [LB3]: Anche qui dove  $p>0.05$ ?

### 253 3.2. Analyses on livers

254 The calculated HSI lined up at 1.08, 1.04 and 1.13% ( $p>0.05$ ;  $df=12$ ;  $F=0.29$ ) for H0, H25 and H50  
255 dietary groups, respectively.

Commentato [LB4]: Anche qui dove  $p>0.05$ ?

256 The analysis of liver parenchyma did not allow to observe obvious abnormal morphological aspect.  
257 Conversely, results evidenced a variable degree of lipid accumulation in the liver of the three  
258 experimental groups (Figure 2). The most appreciable visual differences were observed in H50  
259 group (Figure 2c) in comparison to H0 and H25 (Figures 2a and 2b, respectively). These results  
260 were confirmed by the statistical quantification of the PFF, which showed a significantly ( $p<0.05$ ;  
261  $df=2$ ;  $F=2.14$ ) higher liver fat accumulation in H50 ( $54.41\pm1.5\%$ ) respect to both H0 ( $43.2\pm0.9\%$ )  
262 and H25 ( $42.49\pm1.5\%$ ).

Commentato [BR5]: Se togliamo la figura, dobbiamo togliere anche questa frase

Commentato [LB6]: Basilio puoi aggiungere tu?

Commentato [BR7]: fatto

263 Although by means of Folch et al. (1957)'s extraction method no differences in the hepatic total  
264 lipid content between dietary treatments were observed ( $p>0.05$ ;  $df=27$ ;  $F=2.045$ ), it enabled FA  
265 profile detection, which was significantly influenced by the diets (Table 3). In particular, the fatty  
266 acid C18:2n-6 was the highest in the livers of fish fed H25 diet and the lowest in H0 ( $p<0.01$ ;  
267  $df=27$ ;  $F=7.748$ ). With the exception of EPA, all the 20 carbon atom-long FAs were significantly  
268 higher in H50 dietary treatment than in the H0 group. DHA did not significantly differ between  
269 groups and showed a mean value of 11.07 g FAME/100 g total FAME. Overall, SFA were  
270 abundantly present in all the dietary treatments (47.25 g FAME/100 g total FAME) and they were

Commentato [LB8]: Anche qui dove  $p>0.05$ ?

not significantly different between the three dietary treatments, whilst both MUFA and PUFA-6 increased with the increasing inclusion of H meal ( $p<0.05$ ;  $df=27$ ;  $F=3.906$ ;  $p<0.01$ ;  $df=27$ ;  $F=8.207$ , respectively) in the diet, and PUFA-3 tended to show an opposite trend. The PUFA-3/PUFA-6 ratio was significantly lower ( $p<0.001$ ;  $df=27$ ;  $F=18.21$ ) in the liver of fish fed H50 and H25 diets compared to H0 livers.

The products: precursors ratios did not highlight any significant difference ( $p>0.05$ ).

**Commentato [LB9]:** Aggiungo df e F anche qui dove  $p>0.05$ ?

### 3.4. Analyses on fillets

As shown in Table 4 the total lipids and the FA profile of fillets were not significantly affected by the different dietary regimes, except for SFAs. Indeed, total SFAs, C12:0 and C14:0 increased with the increasing dietary inclusion of H meal ( $p<0.01$ ;  $df=12$ ;  $F=7.35$ ;  $p<0.001$ ;  $df=12$ ;  $F=28.2$ ;  $p<0.01$ ;  $df=12$ ;  $F=11.35$ , respectively), while C16:0 showed an opposite trend ( $p<0.01$ ;  $df=12$ ;  $F=8.478$ ). The oleic acid (C18:1n-9) showed a decreasing trend ( $p>0.05$ ) following the increasing dietary H meal inclusion. Independently to the dietary composition, fillet C18:2n-6 showed a mean value of 11.41 g FAME/100 g total FAME between groups. The sum of EPA+DHA was in average 14.92 g FAME/100 g total FAME. Products:precursors ratios showed that C18:4n-3/C18:3n-3 ratio was higher in H25 and H50 than in H0 group.

**Commentato [LB10]:** Aggiungo df e F anche qui dove  $p>0.05$ ?

As displayed in Table 5, TI and the n-3/n-6 ratio were not significantly affected by the tested dietary treatments, whilst the AI was higher in H50 than in H25 and H0 groups ( $p<0.001$ ;  $df=12$ ;  $F=23.21$ ). The h/H ratio showed the lowest ( $p<0.05$ ;  $df=12$ ;  $F=4.306$ ) value recorded in the fillets from fish fed the H50 diet and the highest one in the H25 dietary group, with H0 showing intermediate values. As shown in Figure 3, the levels of malondialdehyde-equivalents of the fillets from fish fed the H25 and H50 diets presented lower values than H0 fillets ( $p<0.05$ ;  $df=24$ ;  $F=16.59$ ). Fillets exhibited similar physical traits between dietary treatments (Table 6). The yellowness index ( $b^*$ ) was the only parameter affected by the diet, reaching the lowest value in fillets from fish fed

H25 diet and the highest one in H0 diet ( $p<0.05$ ;  $df=27$ ;  $F=4.649$ ).  $\Delta E$  calculations showed the following results: H0 vs H25: 2.28, H0 vs H50: 1.23, H25 vs H50: 1.37.

## 4. Discussion

Rainbow trout is an economically important species worldwide and an important freshwater species in the European Countries, with Turkey, Chile, Norway, Peru, China and Italy being the first six producers in the world, accounting for 60% of the global production (FAO, 2019). Feed plays a major role for the further virtuous and sustainable development of aquaculture, and each single ingredient performs as a main actor in the definition of the end-product quality. The evaluation of fish quality is a *sine qua non* both for business outcome and for human nutrition and health. In fact, aquaculture products are particularly renowned for their high LC PUFA-3 content, mainly drawn by the fish from the diet and, in the case of Salmonids and freshwater species, from a relatively small endogenous production (Tocher, 2003). Among the LC PUFAs, EPA and DHA are the most valued FAs for their benefits on human health (Rosenlund et al., 2010).

*H. illucens*, one of the most promising insects for being an innovative, sustainable and nutritious ingredient for aquafeed formulation, is unfortunately scant of LC PUFAs (Barroso et al., 2014). Typically, fish fed on insects mirror the FA profile of the administered diets (Belghit et al., 2019b; Iaconisi et al., 2018; Renna et al., 2017) and for this reason, in some feeding trials, dietary fish oil was included in the diet when H meal was used as ingredient (Belghit et al., 2019a; Sealey et al., 2011). Cardinaletti et al. (2019) reared rainbow trout on diets including increasing full-fat H meal levels. Consequently, by increasing the full fat h meal, the fish oil in the diets decreased, representing a good example of environmental sustainability. However, by increasing H meal in the diets, a decreasing trend in the dietary PUFAs and a parallel increase in SFAs was observed.

Specific FAs have different physiological functions in fish: energy production, membrane structure and functionality, eicosanoid production, transcriptional control and lipid homeostasis (Tocher,

**Commentato [I11]:** Puoi fondere queste frasi per dire quanto suggerito sopra

**Commentato [I12]:** Leonardo credo che l'aggiunta di olio di pesce serva a rendere isolipidica la dieta. Una volta nota la quantità di lipidi della h farina in uso credo si faccia un semplice calcolo per bilanciare i lipidi  
La cambierei sentiamo il parere di Udine se è il caso di cambiare la frase

2003). Specifically, energy in fish is preferentially obtained from C16:0 and MUFA (e.g. C18:1n-9, C20:1n-9, C22:1n-11), seldom from EPA and only in particular cases from DHA; short-chain FAs may be used as energy source, too. As regards membrane structure and functionality, FA length and degree of unsaturation plays a key role in membrane biophysical properties such as fluidity. Eicosanoids are bioactive molecules involved in blood clotting, immune response and inflammatory response, renal and neural function, cardiovascular tone, and reproduction. They originate from C20:3n-6, C20:4n-6 and EPA and the products of EPA are generally less biologically active than the ones of C20:4n-6; also, EPA and C20:3n-6 compete with C20:4n-6. Finally, FAs and particularly PUFAs play a role in transcriptional control of their own homeostasis (Tocher, 2003).

Rainbow trout has a malleable lipid metabolism. Liver and intestine are the main contributors to the digestive physiology and to the end-product nutritional characteristics. Specifically, intestine and liver regulate dietary lipid absorption, storage and *de novo* synthesis (Tocher, 2003). Several laboratory techniques allowing the assessment of fish physiological response to new diets exist. While biometric indices and histology are able to detect possible alterations in fish organs, molecular biology offers a quick and precocious vision into fish metabolism. For instance, gene expression can be helpful to precociously detect fish physiological responses to this new ingredient (Cardinaletti et al., 2019; Li et al., 2020; Zarantoniello et al., 2020, 2018). Although specimens in Cardinaletti et al. (2019) and in the present paper came from the same growth trial, the former work focused on growth performance, gastrointestinal integrity and stress, and showed a non-significant downward flection of biometrics measurements and no significant differences in growth biomarkers (*igf1*, *mstn1a*) analysed in the liver. The present study combined the use of chemical analysis to assess fillet qualitative aspects and the use of molecular biology tools to investigate some of the lipid metabolism mechanisms behind.

Although no significant differences were noticed when it comes to *cd36* and *ppar* gene expression, interesting trends were noticed. A slight increase in pyloric caeca *cd36* gene expression was noticed

Commentato [I13]: Una referenza

Commentato [I14]: referenza

Commentato [I15]: referenza

upon the increase of dietary H meal inclusion. In a similar manner, Li et al. (2019) described an increased expression of *cd36* in the proximal intestine of pre-smolt Atlantic salmon fed a diet containing H meal, having simultaneously raised dietary fish oil content and diminished rapeseed oil. Underlining that *cd36* is a multifunctional receptor binding ligands involved in different physiological processes (The UniProt Consortium, 2019) and that the function of *cd36* in rainbow trout has not been deeply studied yet, the observed increasing trend cannot be directly attributed to H meal only, because the interaction with the other ingredients could have played a major role on *cd36* modulation.

Also *ppar<sub>s</sub>* regulate different signalling pathways related to lipid metabolism (Kortner et al., 2013). Belghit et al. (2018) compared the effect on Atlantic salmon liver gene expression of replacing 85% of dietary protein with H larvae meal, and/or replacing all the vegetable oils with two different H oils. Differently from the present study, Belghit et al. (2018) found that *ppara* was not affected by the different diets and that *ppar $\gamma$*  was downregulated in the diets containing H meal, irrespective of the dietary oily source. Although their diets contained the same amount of LC PUFA, the H meal diets contained a slightly higher amount of total lipids than that of the ones lacking insect. This element might possibly have contributed to the modulation of *ppar<sub>s</sub>* in a different way than that of the present study, as *ppar<sub>s</sub>* are regulated by both the FA type and their overall amount (Tocher, 2003).

It should be remarked that pyloric caeca are a significant site of DHA synthesis in rainbow trout (Bell et al., 2003). In the present study, pyloric caeca *elovl2* expression tended to increase with the increasing amount of the dietary H meal inclusion. In addition, an increased *fads2* expression was noted in pyloric caeca and possibly mid intestine; their production could have contributed to the uniform DHA content in livers and fillets belonging to the three different dietary groups, overcoming the lack in the diets containing insect. Moreover, the investigation on FA products:precursors ratios hinted that a higher deposition of C18:4n-3 in fillets of fish fed H meal was realised, suggesting that elongase and desaturase enzymes were active. A direct enzymatic



activity assessment would be a useful tool to evaluate the real outcome of gene expression and to explain the endogenous mechanisms of PUFA biosynthesis.

As fillet DHA content surprisingly did not reflect dietary content, it seemed clear that endogenous elongase and desaturase enzymes changed the dietary FA profile and improved DHA deposition in the muscle. Zarantoniello et al. (2019, 2018) used zebrafish as experimental model to test the effects of diets equivalent to those tested in the present study. Similarly to the present study, the authors observed an increased *elovl2* gene expression in the 50% group after 21 days of feeding trial (Zarantoniello et al., 2018); after six months of feeding, in the same dietary treatment, *fads2* gene expression was upregulated (Zarantoniello et al., 2019). The authors also found a significant decrease in EPA after six months, while the DHA content in zebrafish belonging to the 0 or 25% groups was even and significantly higher than the content of 50% zebrafish (Zarantoniello et al., 2019, 2018). Usually, elongase and desaturase genes are upregulated when fish are fed vegetable oil-based diets, possibly for the deprivation of LC PUFA or the increased content of C18:3n-3, as reviewed by Tocher (2015). A similar mechanism could explain our findings.

The HSI commonly indicates the general status of metabolic activity and energy reserves. Some previous studies (Belforti et al., 2015; Sealey et al., 2011) evidenced a lower HSI in rainbow trout fed with *Tenebrio molitor* or H meal, in comparison to trout fed fishmeal-based control diets. The lack of a significant effect of the experimental diets here tested on rainbow trout HSI represents a promising result. In spite of this, hepatic histological analyses, performed to evaluate lipid accumulation or steatosis, showed a significantly higher PFF in H50 livers in comparison to those of the other two groups. Belghit et al. (2019a) did not find different size distribution of hepatic lipid droplets of Atlantic salmon fed diets with fishmeal or where fishmeal had been replaced by H meal, nor the hepatic triacylglycerol concentrations were dissimilar; the administered diets were isolipidic and contained very similar amounts of the different FA classes, specifically, SFA and PUFA n-3 were to some extent higher while MUFA and PUFA n-6 were slightly lower in the diet containing H meal in comparison to the control diet. On the contrary, Zarantoniello et al. (2019, 2018) fed

zebrafish isolipidic diets equivalent to those tested here and showed that liver of the H25 and H50 groups accumulated a higher amount of lipids than the H0 group. An associations between high n-6/n-3 ratio and steatosis was found (Zarantoniello et al., 2020, 2019, 2018). Besides, comparing H0 and H50 diets we point out that SFA increased from 33.75 to 48.24% of total FAMES, while PUFA<sub>n</sub>-3 fell from 22.01 to 10.11% of total FAMES. Reviews on rodents show that PUFA<sub>n</sub>-3-rich diets reduce intrahepatic triglyceride content and steatosis, while diets poor in PUFA<sub>n</sub>-3 fuel steatosis (Di Minno et al., 2012). Also, saturated free FAs possibly have an impact on lipotoxicity, which is thought to be fought by increasing intrahepatic triglyceride accumulation, a method to dispose of excess FAs (Leamy et al., 2013).

As revealed by gas-chromatography, total SFA content in liver was not different between the dietary treatments although C12:0 and C14:0 significantly increased in H25 and H50 dietary groups, counterbalanced by the decrease in C16:0. On the other hand, Belghit et al. (2018) noticed a decreased liver triacylglycerol and C12:0 content (~1.5% of total FAs, regardless of the administered diet) when freshwater Atlantic salmon was fed diets containing H derivatives and a high fish oil level, in comparison to the control diet. It can be thus assumed that fish of the present study accumulated C12:0 rather than oxidising it. As concerns FAs of the n-6 series, their presence in the livers mainly derived from endogenous production as they could be found in little amounts in the experimental diets. Since no different *elovl1* and *elovl2* expression was observed in the liver of the three groups and the calculated FA products:precursors indices resulted unaffected, the equal level of DHA in liver of the three groups might suggest that DHA was accumulated in the liver after a synthesis in pyloric caeca or mid intestine by elongase and desaturase enzymes. Data on the overall upregulation of *fads2* and *elovl2* in H25 and H50 pyloric caeca seemed to support this hypothesis.

As molecular markers produce changes in the composition of the end-product, quality-oriented parameters of fillets, such as their nutritional value and oxidative status, were investigated. The main results are encouraging and partly contradicting some earlier findings. In fact, only the

increase in fillet SFA content parallel to the increasing H meal inclusion levels agreed with the findings of similar studies (Borgogno et al., 2017; Iaconisi et al., 2018; Stadtlander et al., 2017; Zarantoniello et al., 2019). Conversely, and surprisingly, all the other lipid classes, primarily the PUFA<sub>n-3</sub> as well as DHA amounts, did not reflect the dietary content, thus resulting in a well-balanced FA profile of the fillets regardless the dietary treatment.

Standing on the improved DHA deposition in the muscle of H-fed specimens and the parallel increased pyloric caeca *elovl2* and *fads2* expression as well as the possible increased mid intestine *fads2* expression, it seemed clear that endogenous elongase and desaturase enzymes were effective in modifying the dietary FA profile. The question to be answered is why the present results substantially differed from the available literature. Indeed, although Zarantoniello et al. (2019, 2018) observed an increased *elovl2* and *fads2* gene expression in zebrafish fed an H50 diet totally equivalent to that tested here compared to H0 and H25 groups, the DHA content in zebrafish belonging to the H0 or H25 groups were significantly higher than the content of H50 zebrafish after 180 days of feeding trial. Contrariwise, the DHA content was significantly higher in the muscle of juvenile Jian carp (*Cyprinus carpio* var. Jian) fed a diet where H larvae oil replaced soybean oil (at 25, 50, 75 or 100% levels) than in the muscle of the control group (Li et al., 2016). Despite the authors did not explain the possible causes of this unexpected effect, it is of interest that H oil could play a key-role in promoting elongase and desaturase activity in rainbow trout. In view of the contrasting findings mentioned, despite elongase and desaturase gene upregulation is usually associated to severe deprivation of dietary LC PUFA (Tocher, 2015), the equal amount of fillet PUFA<sub>n-3</sub> content in contrast to the decrease dietary PUFA<sub>n-3</sub> content cannot be easily explained. Other factors must play a role in muscle lipid storage constitution, like fish age, specific content of single dietary FAs, free FAs, etc. Further investigation should address these factors, as well as analyse the direct elongase and desaturase enzyme activity and serum biochemical indices.

The nutritional and oxidative status of the fillets were assessed calculating AI, TI, h/H, n-3/n-6 ratios and TBARS content. The overall fillet FA profile was mirrored in the nutritional indices. For

instance, both AI and h/H were worsened in H50 group due to the high SFA content, while the stability of the other lipid classes (especially PUFA<sub>n-3</sub>) can be the cause of the rather stable TI and n-3/n-6 ratio values. Our findings did not agree with the available literature. Indeed, fillets of rainbow trout fed 0, 25 or 50% substitution levels of fishmeal with partially defatted H meal showed that PUFA/SFA ratio, AI, TI and n-3/n-6 ratio were negatively affected by insect inclusion in the diet (Renna et al., 2017). To conclude, the positive results obtained here pave the way to new opportunities for the study and use of full-fat H larvae meal.

A limited number of studies analysed the TBARS content in fish fed diets containing insect meal. Similarly to the present results, Secci et al. (2019) found a decreasing trend following the increasing H meal inclusion. This finding could be primarily attributed to the higher SFA content in the fillets belonging to H25 and H50 groups. However, at a closer look, recent works underlined that dietary H meal seemed to ameliorate the antioxidant defence of fish by increasing serum catalase activity (Li et al., 2017) or glutathione S-transferase, ethoxyresorufin O-deethylase and total glutathione in rainbow trout liver and kidney (Elia et al., 2018).

Finally, results on the fillet physical traits, agreeing with most of the results from similar studies using defatted insect meals (Iaconisi et al., 2018; Mancini et al., 2018), showed that pH, WHC, lightness index and redness index were not affected by the different dietary treatments, while yellowness index was significantly lower in H25 in comparison to H0. Colour variations are difficult to interpret as they depend on the presence of dietary pigments (especially vegetable pigments), on rancidity in the case of the stored products, on the punctual readings of the colourimeter. Therefore, it is difficult to explain unambiguously the reason of the increased yellowness index (b\*) in H25 fillets. The  $\Delta E$  result worth considering is the couple H0 vs H25, which was 2.28. This value is considered just above the threshold enabling unexperienced observers noticing colour differences (Mokrzycki and Tatol, 2011). Nevertheless, following Sharma (2003)'s indications, a  $\Delta E=2.28$  is below the limit enabling an unexperienced observer noticing the

476 differences. Presumably, a test with trained panellists could cast light on the actual perceived  
477 difference.

478

## 479 **5. Conclusions**

480 The dietary full-fat H meal influenced rainbow trout lipid metabolism, as shown by qPCR gene  
481 expression analysis and liver histological examinations. Nonetheless, the final qualitative traits of  
482 the fillets were not negatively affected, and a nutritious final product was guaranteed. In the light of  
483 the differences between previous literature and the present positive results, further studies on the  
484 biological mechanisms behind the macroscopic traits of fish fed insects are warmly encouraged, for  
485 instance by directly assessing elongase and desaturase enzyme activities and serum biochemical  
486 indices, as well as by discerning the impact of other possible factors, as fish age, specific content of  
487 single dietary FAs, free FAs, etc.

488

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493

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711 **Table 1.** Ingredients (g/kg), proximate composition (g/100 g), total lipids (g/100 g) and fatty acid  
712 profile (% of total FAMES) of the experimental diets (from Cardinaletti et al. (2019) and further  
713 deepened in the present study).

	Diet		
	H0	H25	H50
<b>Ingredients</b>			
Chile prime fish meal <sup>1</sup>	420	315	210
Protein-rich vegetable ingredients <sup>2</sup>	110	156	200
H meal <sup>3</sup>	-	105	210
Wheat flour <sup>4</sup>	290	268	255
Fish oil	70	40	28
Palm oil	70	75	56
Mineral <sup>5</sup> and Vitamin <sup>6</sup> supplements	20	20	20
Binder	20	20	20
L-Methionine	-	1	1
<b>Proximate composition<sup>7</sup></b>			
Moisture	4.24±0.03	5.49±0.03	5.31±0.18
Crude Protein, CP	40.27±0.45	39.98±0.37	40.16±0.39
Ether Extract, EE	18.63±0.27	18.56±0.14	17.68±0.20
Ash	14.30±0.28	14.20±0.23	14.13±0.31
Gross Energy (MJ/kg)	22.10±0.11	22.30±0.03	21.28±0.06
Total lipids	19.76±0.09	18.94±0.27	19.08±0.51
<b>Fatty acids<sup>7</sup></b>			
C10:0	tr	0.36±0.03	0.74±0.04
C12:0	0.12±0.05	6.49±0.68	13.05±1.37
C13:0	tr	tr	tr
C14:0	2.89±0.16	4.02±0.31	5.68±1.05
C15:0	0.42±0.09	0.43±0.08	0.54±0.26
C16:0	23.63±1.63	24.11±2.81	21.62±1.21
C16:1n-9	0.11±0.04	0.13±0.05	0.18±0.02
C16:1n-7	3.24±0.36	3.88±0.91	5.25±0.84
C17:0	0.47±0.11	0.48±0.04	0.59±0.13
C18:0	5.57±0.74	5.44±1.11	5.08±0.23
C18:1n-9	26.25±0.55	27.85±1.65	24.97±1.76
C18:1n-7	1.29±0.38	0.99±0.16	0.98±0.31
C18:2n-6	10.11±1.53	8.95±1.46	8.03±1.49
C18:3n-3	1.59±0.13	1.13±0.23	0.95±0.35
C20:0	0.38±0.07	0.42±0.14	0.45±0.07
C20:1n-9	0.95±0.35	0.86±0.22	0.94±0.20
C20:2n-6	0.17±0.01	0.10±0.001	tr
C20:3n-6	0.12±0.04	tr	tr
C20:4n-6	0.73±0.74	0.49±0.13	0.41±0.06
C20:3n-3	0.15±0.07	0.10±0.14	tr
C20:5n-3, EPA	6.85±0.24	4.24±0.49	3.12±0.74
C22:0	0.19±0.03	0.27±0.01	0.36±0.07
C22:1n-9	0.56±0.13	0.39±0.05	0.35±0.08
C22:6n-3, DHA	13.42±0.89	8.24±0.36	5.97±0.72
C24:1n-9	0.47±0.38	0.37±0.08	0.38±0.08

SFA	33.76±0.14	42.13±4.51	48.24±1.67
MUFA	32.87±1.23	34.46±0.61	33.04±1.45
PUFAn-3	22.01±0.45	13.71±0.22	10.11±0.33
PUFAn-6	11.33±2.20	9.68±1.56	8.62±1.50
n-3/n-6	1.99±0.43	1.44±0.25	1.19±0.17

714 <sup>1</sup> Bioceval GmbH & Co. KG Cuxhaven, Germany.

715 <sup>2</sup> Protein-rich vegetable ingredients: blend of pea protein concentrate (Lombarda trading srl, Casalbuttano & Uniti,  
716 Cremona, Italy) and wheat gluten meal (Sacchetto SpA, Turin, Italy) in 1:1 ratio.

717 <sup>3</sup> SmartBugs srl (Treviso, Italy).

718 <sup>4</sup> Consorzio Agrario (Pordenone, Italy).

719 <sup>5</sup> Mineral supplement composition (% mix):  $\text{CaHPO}_4 \times 2\text{H}_2\text{O}$ , 78.9;  $\text{MgO}$ , 2.725;  $\text{KCl}$ , 0.005;  $\text{NaCl}$ , 17.65;  $\text{FeCO}_3$ ,  
720 0.335;  $\text{ZnSO}_4 \times \text{H}_2\text{O}$ , 0.197;  $\text{MnSO}_4 \times \text{H}_2\text{O}$ , 0.094;  $\text{CuSO}_4 \times 5\text{H}_2\text{O}$ , 0.027;  $\text{Na}_2\text{SeO}_3$ , 0.067.

721 <sup>6</sup> Vitamin supplement composition (% mix): thiamine HCL Vit B1, 0.16; riboflavin Vit B2, 0.39; pyridoxine HCL Vit B6,  
722 0.21; cyanocobalamin B12, 0.21; niacin Vit PP, 2.12; calcium pantothenate, 0.63; folic acid, 0.10; biotin Vit H, 1.05;  
723 myoinositol, 3.15; stay C Roche, 4.51; tocopherol Vit E, 3.15; menadione Vit K3, 0.24; Vit A (2500 UI/kg diet), 0.026;  
724 Vit D3 (2400 UI/kg diet), 0.05; choline chloride, 83.99.

725 <sup>7</sup> Values reported as mean of triplicate analyses.

726 *tr*: fatty acids below of 0.1% of total FAMES.

727  $\text{SFA} = \text{C10:0} + \text{C12:0} + \text{C13:0} + \text{C14:0} + \text{C15:0} + \text{C16:0} + \text{C17:0} + \text{C18:0} + \text{C20:0} + \text{C21:0} + \text{C22:0} + \text{C24:0}$ .

728  $\text{MUFA} = \text{C16:1n-9} + \text{C16:1n-7} + \text{C18:1n-9} + \text{C18:1n-7} + \text{C20:1n-9} + \text{C22:1n-9} + \text{C24:1n-9}$ .

729  $\text{PUFAn-3} = \text{C18:3n-3} + \text{C20:3n-3} + \text{C20:5n-3} + \text{C22:6n-3}$ .

730  $\text{PUFAn-6} = \text{C18:2n-6} + \text{C18:3n-6} + \text{C20:2n-6} + \text{C20:3n-6} + \text{C20:4n-6}$ .

731



732 **Table 2.** Primer pair sequences and annealing temperature conditions for genes used in real-time  
 733 PCR.

Gene	3' primer sequence		Annealing temperature (°C)
	Forward	Reverse	
<i>cd36</i>	TCAAGCGTTGTCTGTAGTGAGT	CCCAGTAGCGTAAATTGCACA	58.2
<i>ppara</i>	AGTCGAGTAACGGCTCTGAAGG	CCGACACTCCAGGTTGAGAGA	60.0
<i>pparβ</i>	ATCAGCAGGAGAAGGGGAGTAG	GGAGACGATGTCTGGGACAGAT	58.2
<i>pparδ</i>	TCCTGTTTCCTGTGAGTGGGA	CCAGTCAGCACATTGCCATTTC	56.0
<i>pparγ</i>	GCCCTTATCGCCTTCTCAGT	AGAGCTGGCGTCTGTGTAAG	56.0
<i>fads2</i>	GCCCTACCATCACCAACACC	AAACTCATCGACCACGCCAG	60.0
<i>elovl1</i>	TTGCCCAAGCAGGATACCAA	ATTTCATGCGTCTTGGGTGTTT	59.2
<i>elovl2</i>	TGGATGGGTCCCAGAGATGA	AGAAGGACAAGATCGTGAGGC	56.0
<i>arp</i>	GAAAATCATCCAATTGCTGGA	CTTCCCACGCAAGGACAGA	60.0
<i>60S</i>	AGCCACCAGTATGCTAACCAG	TGTGATTGCACATTGACAAAA	60.0

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**Table 3.** Total lipids (g/100 g liver), fatty acid profile (% total FAMES) and products:precursors ratios (grey background) in livers of rainbow trout fed experimental diets including increasing levels of full-fat *Hermetia illucens* prepupae meal (0%, H0; 25%, H25; 50%, H50).

	Diet			SEM <sup>1</sup>	<i>p</i> -value <sup>2</sup>
	H0	H25	H50		
<b>Total lipids</b>	11.79	10.53	11.66	0.34	<i>ns</i>
C12:0	0.13 <sup>c</sup>	1.41 <sup>b</sup>	3.16 <sup>a</sup>	0.24	***
C14:0	1.52 <sup>c</sup>	2.25 <sup>b</sup>	3.75 <sup>a</sup>	0.18	***
C16:0	32.99 <sup>a</sup>	29.09 <sup>b</sup>	25.81 <sup>b</sup>	1.02	**
C16:1n-7	1.28	1.52	1.51	0.05	<i>ns</i>
C18:0	14.10	13.02	13.65	0.24	<i>ns</i>
C18:1n-9	21.43 <sup>b</sup>	24.05 <sup>a</sup>	22.44 <sup>ab</sup>	0.48	*
C18:1n-7	2.00	2.08	1.97	0.04	<i>ns</i>
C18:2n-6	4.63 <sup>b</sup>	5.82 <sup>a</sup>	5.12 <sup>ab</sup>	0.16	**
C20:1n-9	2.33 <sup>b</sup>	2.86 <sup>ab</sup>	3.25 <sup>a</sup>	0.13	**
C20:2n-6	0.94 <sup>b</sup>	1.29 <sup>ab</sup>	1.42 <sup>a</sup>	0.07	**
C20:3n-6	0.56 <sup>b</sup>	0.93 <sup>a</sup>	1.04 <sup>a</sup>	0.05	***
C20:4n-6	1.44 <sup>b</sup>	1.88 <sup>a</sup>	1.86 <sup>a</sup>	0.07	**
C20:5n-3, EPA	1.15 <sup>a</sup>	0.94 <sup>b</sup>	0.73 <sup>c</sup>	0.04	***
C22:6n-3, DHA	11.55	11.26	10.41	0.29	<i>ns</i>
SFA	50.00	44.30	47.45	1.04	<i>ns</i>
MUFA	28.21 <sup>b</sup>	31.94 <sup>a</sup>	30.72 <sup>ab</sup>	0.68	*
PUFAn-6	7.79 <sup>b</sup>	10.15 <sup>a</sup>	9.66 <sup>a</sup>	0.31	**
PUFAn-3	13.62	13.27	11.89	0.35	<i>ns</i>
EPA+DHA	12.70	12.20	11.13	0.32	<i>ns</i>
SFA/UFA <sup>3</sup>	1.00	0.83	0.91	0.03	<i>ns</i>
n-3/n-6	1.78 <sup>a</sup>	1.32 <sup>b</sup>	1.23 <sup>b</sup>	0.06	***
C18:4n-3/C18:3n-3	0.36	0.39	0.34	0.05	<i>ns</i>
C20:5n-3/C18:3n-3	3.22	2.67	2.79	0.14	<i>ns</i>
C22:6n-3/C18:3n-3	32.93	32.65	40.53	2.06	<i>ns</i>
C20:4n-6/C18:2n-6	0.31	0.32	0.36	0.01	<i>ns</i>

The following FAs were used for calculating the classes of FAs but they are not listed because below 1% of total FAME: C14:1n-5, C15:0, C16:1n-9, C16:2n-4, C17:0, C16:3n-4, C17:1, C18:2n-4, C18:3n-6, C18:3n-4, C18:3n-3, C18:4n-3, C20:0, C20:1n-11, C20:1n-7, C20:3n-3, C20:4n-3, C22:0, C22:1n-11, C22:1n-9, C22:5n-3.

<sup>1</sup>SEM: standard error of the mean

<sup>2</sup>ns: not significant ( $p > 0.05$ ); a, b, c as superscript letters indicate significantly different means at  $p < 0.05$  (\*);  $p < 0.01$  (\*\*);  $p < 0.001$  (\*\*\*)

<sup>3</sup>UFA: unsaturated fatty acids.

**Table 4.** Total lipids (g/100 g fillet), fatty acid profile (% of total FAMES) and products:precursors ratios (gray background) in fillets of rainbow trout fed experimental diets including increasing levels of full-fat *Hermetia illucens* prepupae meal (0%, H0; 25%, H25; 50%, H50).

	Diet			SE	<i>p</i> -value <sup>2</sup>
	H0	H25	H50	M <sup>1</sup>	
<b>Total lipids</b>	5.13	4.42	4.87	0.36	<i>ns</i>
C12:0	0.26 <sup>c</sup>	2.15 <sup>b</sup>	5.00 <sup>a</sup>	0.57	***
C13:0	7.23	8.52	9.27	0.86	<i>ns</i>
C14:0	1.68 <sup>b</sup>	1.94 <sup>b</sup>	2.66 <sup>a</sup>	0.14	**
C16:0	15.47 <sup>a</sup>	13.51 <sup>b</sup>	13.89 <sup>b</sup>	0.30	**
C16:1n-7	2.13	2.10	2.15	0.08	<i>ns</i>
C18:0	3.59	3.44	3.49	0.05	<i>ns</i>
C18:1n-9	25.35	24.69	23.79	0.83	<i>ns</i>
C18:1n-7	2.32	2.16	1.88	0.10	<i>ns</i>
C18:2n-6	11.79	11.69	10.77	0.45	<i>ns</i>
C18:3n-3	2.07	1.85	1.66	0.08	<i>ns</i>
C20:1n-11	1.45 <sup>a</sup>	1.24 <sup>ab</sup>	1.15 <sup>b</sup>	0.05	*
C20:4n-6	0.85	1.11	1.10	0.05	<i>p</i> =0.0568
C20:5n-3, EPA	2.58	2.38	2.19	0.09	<i>ns</i>
C22:6n-3, DHA	12.70	13.03	11.87	0.71	<i>ns</i>
SFA	28.46 <sup>b</sup>	30.44 <sup>b</sup>	35.09 <sup>a</sup>	0.90	**
MUFA	29.77	30.95	29.66	1.01	<i>ns</i>
PUFAn-6	13.11	14.50	13.61	0.41	<i>ns</i>
PUFAn-3	18.48	19.76	17.97	0.75	<i>ns</i>
EPA+DHA	15.28	15.41	14.06	0.77	<i>ns</i>
SFA/UFA <sup>3</sup>	0.45 <sup>b</sup>	0.47 <sup>ab</sup>	0.58 <sup>a</sup>	0.02	*
C18:4n-3/C18:3n-3	0.21 <sup>b</sup>	0.29 <sup>a</sup>	0.29 <sup>a</sup>	0.01	**
C20:5n-3/C18:3n-3	1.26	1.33	1.37	0.08	<i>ns</i>
C22:6n-3/C18:3n-3	6.25	7.50	7.43	0.64	<i>ns</i>
C20:4n-6/C18:2n-6	0.07	0.10	0.10	0.01	<i>ns</i>

The following FAs were used for calculating the classes of FAs but they are not listed because below 1% of total FAME: C14:1, C15:0, C16:2n-4, C17:0, C16:3n-4, C17:1, C16:4n-1, C18:2n-6 trans, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-3, C18:4n-1, C20:0, C20:1n-11, C20:1n-9, C20:2n-6, C20:3n-6, C20:3n-3, C20:4n-3, C22:0, C22:1n-11, C22:1n-9, C22:4n-6, C21:5n-3, C22:5n-3, C24:0, C24:1.

<sup>1</sup>SEM: Standard error of the mean

<sup>2</sup>*ns*: not significant (*p*>0.05); a, b, c as superscript letters indicate significantly different means at *p*<0.05 (\*); *p*<0.01 (\*\*); *p*<0.001 (\*\*\*)

<sup>3</sup>UFA: unsaturated fatty acids.

**Table 5.** Nutritional indices in fillets of rainbow trout fed experimental diets including increasing levels of full-fat *Hermetia illucens* prepupae meal (0%, H0; 25%, H25; 50%, H50).

	Diet			SEM <sup>1</sup>	<i>p</i> -value <sup>2</sup>
	H0	H25	H50		
AI	0.34 <sup>b</sup>	0.36 <sup>b</sup>	0.48 <sup>a</sup>	0.019	***
TI	0.25	0.23	0.26	0.008	<i>ns</i>
h/H	3.30 <sup>ab</sup>	3.63 <sup>a</sup>	3.18 <sup>b</sup>	0.080	*
n-3/n-6	1.41	1.42	1.35	0.089	<i>ns</i>

<sup>1</sup>SEM: standard error of the mean.

<sup>2</sup>*ns*: not significant (*p*>0.05); a, b as superscript letters indicate significantly different means at *p*<0.05 (\*); *p*<0.001 (\*\*\*).

AI: atherogenicity index; TI: thrombogenicity index; h/H: hypocholesterolaemic/Hypercholesterolaemic fatty acid ratio.

**Table 6.** Fillet physical parameters of rainbow trout fed experimental diets including increasing levels of full-fat *Hermetia illucens* prepupae meal (0%, H0; 25%, H25; 50%, H50).

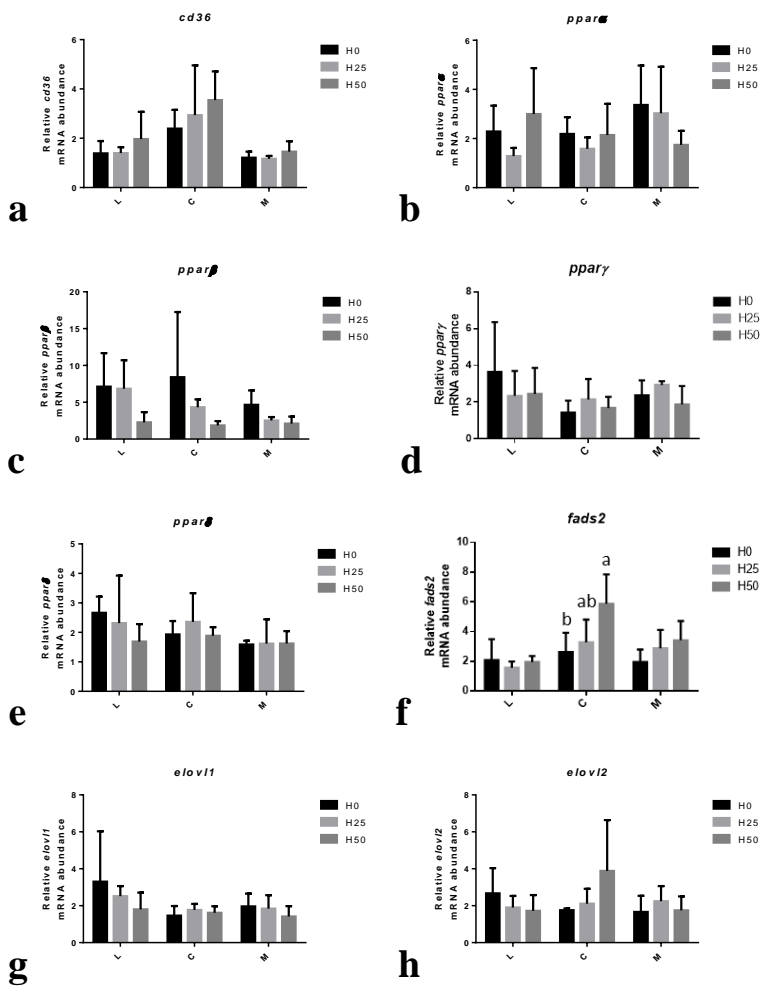
	Diet			SEM <sup>1</sup>	p-value <sup>2</sup>
	H0	H25	H50		
pH	6.59	6.67	6.59	0.02	ns
WHC <sup>3</sup> , %	91.66	93.89	91.82	0.52	ns
Colour					
L*	48.42	46.79	47.61	0.43	ns
a*	0.31	1.08	0.32	0.18	ns
b*	5.57 <sup>a</sup>	4.21 <sup>b</sup>	4.43 <sup>ab</sup>	0.21	*

<sup>1</sup>SEM: standard error of the mean

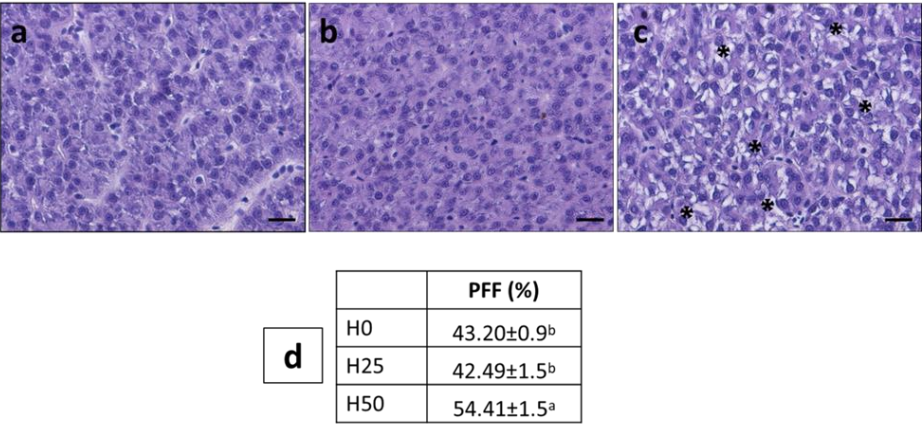
<sup>2</sup>ns: not significant ( $p>0.05$ ); a, b as superscript letters indicate significantly different means at  $p<0.05$  (\*).

<sup>3</sup>WHC: water holding capacity.

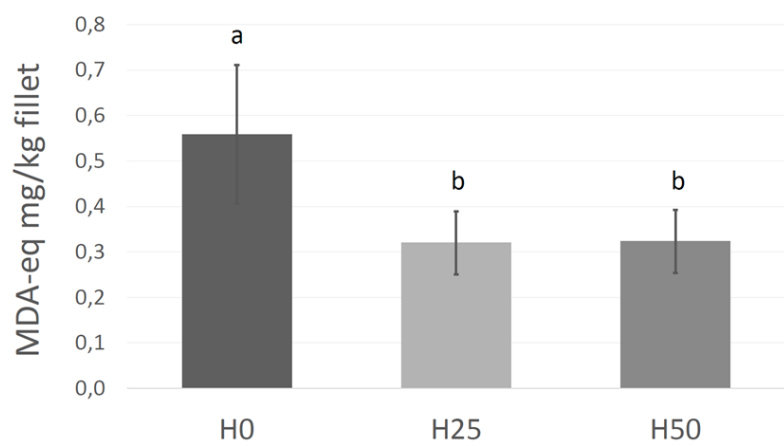
788 **Figure 1.** Relative mRNA abundances of genes related to the lipid metabolism along the  
 789 gastrointestinal tract (L, liver; C, pyloric caeca; M, mid intestine). a: *cd36*; b to e: *ppar*s; f: *fads2*; g  
 790 and h: *elovl*s. Bars indicate standard deviation.



793 **Figure 2.** Liver histology of rainbow trout fed experimental diets including increasing levels of full-  
 794 fat *Hermetia illucens* prepupae meal: 0%, H0 (a); 25%, H25 (b) and 50%, H50 (c). Percentage of fat  
 795 fraction (PFF) evaluation (d). Asterisks in figure c indicate fat accumulation in the liver  
 796 parenchyma stained with periodic acid of Schiff (PAS). Scale bars: 10  $\mu$ m. Data about fat  
 797 accumulation are reported as mean and standard deviation of percentage of fat on sections.



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 802 **Figure 3.** Fillet TBARS content of rainbow trout fed experimental diets including increasing levels  
 803 of full-fat *Hermetia illucens* prepupae meal (0%, H0; 25%, H25; 50%, H50). Values are expressed  
 804 as mg of malondialdehyde-equivalent/kg of fillet (MDA-eq/kg fillet). Bars indicate standard  
 805 deviation.



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