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DOUTORAMENTO EM CIÊNCIA ANIMAL - NUTRIÇÃO

Insects as protein source for European sea bass (*Dicentrarchus labrax*)

Ana Basto

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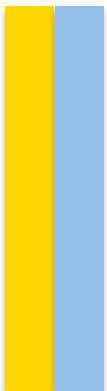

Ana Filipe Basto dos Santos

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Tese de Candidatura ao grau de Doutor em Ciência Animal, Especialidade em Nutrição; Programa Doutoral da Universidade do Porto (Instituto de Ciências Biomédicas Abel Salazar)

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Thesis for applying to a Doctor degree in Animal Science, Specialty in Nutrition; Doctoral Programme of University of Porto (Institute of Biomedical Sciences Abel Salazar – School of Medicine and Biomedical Sciences)

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Diretivas Legais

No cumprimento do disposto no Decreto-Lei no 204/2018 de 23 de Outubro, declara-se que o autor desta Tese participou na conceção e na execução do trabalho experimental que esteve na origem dos resultados apresentados, bem como na sua interpretação e na redação dos respetivos manuscritos. Nesta tese incluem-se 5 artigos científicos publicados e 1 artigo científico submetidos em revistas internacionais resultantes de uma parte dos resultados obtidos no trabalho experimental, referenciados como:

Basto, A., Matos, E., Valente, L.M.P., 2020. Nutritional value of different insect larvae meals as protein sources for European sea bass (*Dicentrarchus labrax*) juveniles. Aquaculture, 521. https://doi.org/10.1016/j.aquaculture.2020.735085.

Basto, A., Calduch, J., Oliveira, B., Petit, L., Sá, T., Maia, M.R.G., Cabral-Fonseca, S., Matos, E., Pérez-Sánchez, J., Valente, L.M.P., 2021. The use of defatted *Tenebrio molitor* larvae meal as a main protein source is supported in European sea bass (*Dicentrarchus labrax*) by data on growth performance, lipid metabolism and flesh quality. Front. Physiol. 12. https://doi.org/10.3389/fphys.2021.659567.

Basto, A., Valente, L.M.P., Conde-Sieira, M., Soengas, J.L., 2021. Central regulation of food intake is not affected by inclusion of defatted *Tenebrio molitor* larvae meal in diets for European sea bass (*Dicentrarchus labrax*). Aquaculture, 544. https://doi.org/10.1016/j.aquaculture.2021.737088.

Basto, A., Valente, L.M.P., Soengas, J.L., Conde-Sieira, M., 2022. Partial and total fishmeal replacement by defatted *Tenebrio molitor* larvae meal does not alter short- and mid-term regulation of food intake in European sea bass (*Dicentrarchus labrax*). Aquaculture, 560. https://doi.org/10.1016/j.aquaculture.2022.738604.

Basto, A., Marques, A., Silva, A., Sá, T., Sousa, V., Oliveira, M.B.P.P., Aires, T., Valente, L.M.P., 2022. Nutritional, organoleptic and sensory quality of market-sized European sea bass (*Dicentrarchus labrax*) fed defatted *Tenebrio molitor* larvae meal as main protein source. Aquaculture, 566. https://doi.org/10.1016/j.aquaculture.2022.739210.

Basto, A., Sousa, V., Valente, L.M.P., Soengas, J.L., Conde-Sieira, M., 2022. Total fishmeal replacement by defatted *Tenebrio molitor* larvae meal induces alterations in intermediary metabolism of European sea bass (*Dicentrarchus labrax*). J. Anim. Sci., 101 (1-11). https://doi.org/0.1093/jas/skad040.

Legal Details

In compliance with what is stated in Decree-Law no. 204/2018 of 23 October 23, it is hereby declared that the author of this thesis participated in the creation and execution of the experimental work leading to the results shown, as well as in their interpretation and the writing of respective manuscripts. This thesis includes 5 scientific papers published and 1 submitted for publication in international journals from part of the results obtained in the experimental work, referenced to as:

Basto, A., Matos, E., Valente, L.M.P., 2020. Nutritional value of different insect larvae meals as protein sources for European sea bass (*Dicentrarchus labrax*) juveniles. Aquaculture, 521. https://doi.org/10.1016/j.aquaculture.2020.735085.

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List of Abbreviations and Acronyms

(In the case of enzymes both protein and gene transcripts are mentioned)

18s	Ribosomal RNA 18s
a*	Redness
AA	Amino acid
Aat1/*gpt*	Alanine transaminase (EC 2.6.1.2)
Acly/*acly*	Adenosine triphosphate citrate lyase (EC 4.1.3.8)
acox	Peroxisomal acyl-coenzyme A oxidase
actb	β-actin
ADC	Apparent digestibility coefficient
ADF	Acid detergent fibre
AgRP/*agrp2*	Agouti-related protein
AI	Atherogenicity index
Ala	Alanine
ALA	α-linolenic acid
apob100	Apolipoprotein B-100
Arg	Arginine
Asn	Aspargine
Asp	Aspartate
Ast1/*got1*	Aspartate aminotransferase (EC 2.6.1.1)
atgl	Adipose triglyceride lipase
ATP	Adenosine triphosphate
b*	Yellowness
BBM	Brush border membrane
C*	Chroma
C_{18}	18-carbons
capn1	Calpain 1
capn2	Calpain 2
capn3	Calpain 3
CART/*cartpt2*	Cocaine- and amphetamine-related transcript protein (-like 2)
cd36	Platelet glycoprotein
CEAA	Conditionally essential amino acid
CF	Crude fat
CO_2	Carbon dioxide
CO_2 eq.	Carbon dioxide equivalent
CoA	Coenzyme A
CoA-SH	Coenzyme A with sulphydryl functional group
coxi	Cytochrome c oxidase subunit I
CP	Crude protein

cpst	Calpastatin
Cpt1a/*cpt1L*	Carnitine palmitoyltransferase 1 (EC 2.3.1.21)
Cpt2a	Carnitine palmitoyltransferase 2
cs	Citrate synthase
CTTAD	Coefficient of total tract apparent digestibility
cyb	Cytochrome b
Cyp7a1/*cyp7a1*	Cholesterol 7α-hydroxylase
Cys	Cysteine
d-	Defatted
DE	Digestible energy
DHA	Docosahexaenoic acid
DI	Distal intestine
DM	Dry matter
DP	Digestible protein
EAA	Essential amino acid
EC	European Commission
eef1a1	Elongation factor 1α
EFSA	European Food Safety Authority
Elovl	Protein for elongation of very long-chain fatty acids
elovl1	Elongation of very long chain fatty acids 1
elovl2	Elongation of very long chain fatty acids 2
elovl4	Elongation of very long chain fatty acids 4
elovl5	Elongation of very long chain fatty acids 5
elovl6	Elongation of very long chain fatty acids 6
EPA	Eicosapentaenoic acid
EUMOFA	European Market Observatory for Fisheries and Aquaculture Products
EUR	Euros
FA	Fatty acid
FAA	Free amino acid
Fad	Fatty acyl desaturase
FAD	Flavin adenine dinucleotide
Fad5	Fatty acid desaturase 5
Fad6	Fatty acid desaturase 6
fads2	Fatty acid desaturase 2
FAO	Food and Agriculture Organization
Fas/*fasn*	Fatty acid synthetase (EC 2.3.1.85)
Fbpase/*fbp1*	Fructose-1,6-bisphosphatase (EC 3.1.3.11)
FBW	Final body weight
FCR	Feed conversion ratio
FFA	Free fatty acid
fgf4	Fibroblast growth factor 4

fgf6	Fibroblast growth factor 6
FI	Farinha de inseto
FM	Fishmeal
FO	Fish oil
FP	Farinha de peixe
fst	Follistatin
G6pase/*g6pc1*	Glucose-6-phosphatase (EC 3.1.3.9)
G6pdh/*g6pd*	Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)
Gck/*gck*	Glucokinase (EC 2.7.1.2)
Gdh/*glud1*	Glutamate dehydrogenase (EC 1.4.1.2)
GE	Gross energy
GH/IGF	Growth-hormone/insulin-growth-factors
GHG	Greenhouse gases
ghr-i	Growth hormone receptor-type I
ghr-ii	Growth hormone receptor-type II
GIT	Gastrointestinal tract
Gln	Glutamine
GLUT	Glucose transport protein
Gly	Glycine
Gpase/*pygl*	Glycogen phosphorylase (EC 2.4.1.1)
Gsase/*gys2*	Glycogen synthase (EC 2.4.1.11)
GWP	Global warm potential
h/H	Hypo/hypercholesterolemic ratio
H^+	Hydrogen proton
H_2O	Water
hadh	Hydroxyacyl-Coenzyme A dehydrogenase
HDL	High-density lipoprotein
HI	*Hermetia illucens*
His	Histidine
Hk/*hk1*	Hexokinase (EC 2.7.1.1)
hl	Hepatic lipase
Hº	Hue angle
HSI	Hepatosomatic index
hsl	Hormone-sensitive lipase
HUFA	Highly unsaturated fatty acids
I	Intestine
IBW	Initial body weight
IDL	Intermediate-density lipoprotein
igfbp1a	Insulin-like binding-protein 1b
igfbp3a	Insulin-like binding-protein 3a
igfbp4	Insulin-like binding-protein 4

igfbp6b	Insulin-like binding-protein 6b
igf-i	Insulin-like growth factor I
igf-ii	Insulin-like growth factor II
igfpb2b	Insulin-like binding-protein 2b
igfpb5b	Insulin-like binding-protein 5b
Ile	Isoleucine
IM	Insect meal
IPIFF	International Platform of Insects for Food and Feed
kg	Kilograms
Kp	Nitrogen-to-protein conversion factor
L	Liver
L*	Lightness
LA	Linoleic acid
LCA	Life cycle assessment
LC-PUFA	Long-chain polyunsaturated fatty acid
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
Leu	Leucine
LM	*Locusta migratoria*
lpl	Lipoprotein lipase
LPO	Lipid peroxidation
lxrα	Liver X receptorα
Lys	Lysine
M	Muscle
mafbx	Muscle atrophy F-box
Me	Malic enzyme
Met	Methionine
MI	Mid-intestine
mrf4	Myogenic regulatory factor 4
mstn	Myostatin
MUFA	Monounsaturated fatty acids
murf1	Muscle RING-finger protein-1
myf5	Myogenic factor 5
mymk	Myomaker
myod1	Myoblast determination protein 1
myod2	Myoblast determination protein 2
myog	Myogenin
N	Nitrogen
Na^+	Sodium cation
$NADP^+$	Nicotinamide adenine dinucleotide phosphate oxidized form)
$NADP^+$-Idh	NADP+-dependent isocitrate dehydrogenase

NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
nd5	NADH dehydrogenase subunit 5
NDF	Neural detergent fibre
NEAA	Non-essential amino acid
NPY/npy	Neuropeptide Y
O_2	Oxygen
OA	Oleic acid
OP	Óleo de peixe
P	Phosphorus
PA	Palmitic acid
PAP	Processed animal protein
PC	Pyloric caeca
Pepck/*pck1*	Phosphoenolpyruvate carboxykinase (EC 4.1.1.32)
PI3K/AKT/TOR	Phosphoinositide 3-kinases/protein kinase B/target of rapamycin
PepT1	Peptide transporter 1
PepT2	Peptide transporter 2
Pfk1	Phosphofructokinase-1
Phe	Phenylalanine
Pi	Proximal intestine
Pk/*pklr*	Pyruvate-kinase (EC 2.7.1.40)
Pl	Plasma
POMC/*pomca*	Pro-opio melanocortin (a)
PP	Plant protein
pparα	Peroxisome proliferator-activated receptor-α
pparβ	Peroxisome proliferator-activated receptor-β
pparγ	Peroxisome proliferator-activated receptor-γ
pparδ	Peroxisome proliferator-activated receptor-δ
PPP	Pentose phosphate pathway
Pro	Proline
Psat	Phosphoserine aminotransferase
PUFA	Polyunsaturated fatty acid
S	Stomach
SBM	Soybean meal
scd1b	Stearoyl-CoA desaturase 1b
sdhc	Succinate dehydrogenase cytochrome b560 subunit
SFA	Saturated fatty acid
SGLT	Sodium-dependent glucose transporter
SGR	Specific growth rate
sirt1	Sirtuin 1
sirt2	Sirtuin 2
srebp1	Sterol regulatory element-binding protein 1

SWOT	Strengths, Weaknesses, Opportunities, and Threats
TAG	Triacylglyceride
Tau	Taurine
TCA	Tricarboxylic acid
Thr	Threonine
TI	Thrombogenecity index
TM	*Tenebrio molitor*
Trp	Tryptophan
Tyr	Tyrosine
ucp1	Mitochondrial respiratory uncoupling protein 1
ucp3	Mitochondrial respiratory uncoupling protein 3
Val	Valine
VFI	Voluntary Feed Intake
VLDL	Very-low-density lipoprotein
VSI	Viscerosomatic index
WB	Whole-body
WHC	Water-holding capacity

Resumo

A aquacultura é o setor de produção de alimentos de origem animal com a maior taxa crescimento em todo o mundo, tendo, portanto, um papel de extrema importância no combate à fome e desnutrição. Os alimentos compostos para peixes representam um dos maiores custos de produção numa aquacultura (50-70%), sendo a sua qualidade crucial para o crescimento, reprodução e manutenção do estado de saúde dos animais. Apesar do contributo da farinha e óleo de peixe (FP e OP, respetivamente) produzidos a partir de subprodutos de peixe (cabeças, vísceras, pele, espinhas e escamas) ter crescido nos últimos anos, a sua produção (particularmente da FP) está ainda muito dependente do processamento de pequenas espécies de peixes pelágicos obtidos através da pesca. Os desembarques de peixes provenientes de populações biologicamente sustentáveis têm vindo a aumentar, no entanto, mais de 33% das poluções naturais permanecem sobreexploradas. Desta forma, para garantir a capacidade de resposta e sustentabilidade da aquacultura nos próximos anos, é crucial reduzir a sua dependência de FP e OP derivados de pequenos peixes pelágicos, e promover uma utilização estratégica destes ingredientes. Os insetos têm sido apontados como um dos substitutos da FP mais promissores quando comparados com outras novas fontes de proteína, tais como micro- e macro-algas, bactérias e leveduras. No entanto, a viabilidade do uso de farinha de insetos (FI), como substituto da FP em formulações de alimentos compostos para o robalo Europeu (*Dicentrarchus labrax*), permanece pouco explorada. Neste sentido, formulou-se a hipótese de que a inclusão de uma FI numa dieta equilibrada para esta espécie poderia substituir a FP, sem afetar o consumo de ração, a digestibilidade e o metabolismo, resultando num elevado crescimento e valor nutricional dos peixes.

No capítulo 2, foi analisada a composição nutricional e biodisponibilidade de quatro FI autorizadas na União Europeia e facilmente encontradas no mercado, obtidas a partir de larvas de *Hermetia illucens* (HI) e *Tenebrio molitor* (TM); testaram-se farinhas com o teor total de gordura (HI e TM, respetivamente) e já desengorduradas (*d*HI e *d*TM, respetivamente). Adicionalmente, foi também analisada uma FI obtida a partir da espécie *Locusta migratoria* (LM). Os resultados obtidos demonstraram que o LM foi o ingrediente com o menor potencial para incorporação em rações para peixes como substituto da FP, devido ao seu baixo teor em proteína (24% da matéria seca), um perfil carecido em certos aminoácidos essenciais (AAE) e elevado teor de fibra bruta. Por outro lado, a *d*TM foi a única FI com maior teor de proteína (68,9% vs. 60,2%) do que uma farinha de anchova de elevada qualidade. Além disso, a *d*TM também apresentou alto teor de proteína e AA digestíveis (>89%), demonstrando seu potencial elevado como fonte de proteína em dietas para robalo Europeu. Nos capítulos 3 e 5, avaliou-se a palatabilidade

da *d*TM quando incluída em rações para robalo Europeu. Foi avaliado pela primeira vez o impacto de níveis crescentes de inclusão de *d*TM na ingestão de alimento. Este impacto foi avaliado através do estudo de mecanismos subjacentes à regulação do apetite, exibidos a nível central, a curto, médio e longo prazo. Os resultados destes estudos revelaram que a substituição parcial e total de FP por FI não afetou a expressão dos principais genes envolvidos na resposta orexigénica e anorexigénica (estimulação e inibição do apetite, respetivamente). No entanto, o consumo voluntário de alimento diminuiu com a utilização de dietas onde 30% da proteína total era proveniente da *d*TM (50% de substituição de FP), durante 16 semanas, mas sem comprometer o crescimento.

Nos capítulos 4, 6 e 7, o impacto da substituição parcial e total de FP por *d*TM na utilização de nutrientes foi avaliado de forma multidisciplinar, através da digestibilidade e balanço de nutrientes, metabolismo e crescimento dos peixes. Apesar da digestibilidade da proteína ter diminuído nos peixes alimentados com dietas onde 30-100% da proteína provinha da *d*TM (50-100% de substituição da FP), os valores do coeficiente de digestibilidade aparente foram elevados (89-92%). Foi observado um comportamento compensatório de retenção de azoto e diminuição das suas perdas, resultando em ganhos de azoto e proteína semelhantes entre as várias dietas. Além disso, foi claramente demonstrado que quando 59 ou 100% da proteína da dieta provém da *d*TM (substituindo totalmente a FP), o desempenho de crescimento e a eficiência alimentar do robalo Europeu mantém-se após 10-16 semanas de alimentação. De facto, foi também observado um mecanismo compensatório de crescimento (menor expressão de genes responsáveis pela atrofia muscular, acompanhada de maior expressão de genes responsáveis pela fusão dos mioblastos durante a miogénese) quando a FP foi totalmente substituída por *d*TM. Por outro lado, a substituição total da FP pela *d*TM claramente induziu um aumento da eficiência da retenção dos lípidos e redução das suas perdas, independentemente do nível de contribuição da *d*TM para a proteína total, resultando num aumento do ganho e conteúdo de lípidos nas carcaças (capítulos 4 e 7). Foi também demonstrado que a substituição total da FP pela *d*TM induziu a síntese *de novo* de ácidos gordos e inibiu a sua β-oxidação, resultando em elevados níveis de ácidos gordos não esterificados e triglicéridos no plasma (capítulo 6), o que a longo prazo pode comprometer a saúde dos animais e a sua qualidade para consumo humano. Na generalidade, estes resultados provam que a utilização de elevados níveis de proteína provenientes da dTM para substituir totalmente a FP, afetam acentuadamente o metabolismo lípido do robalo Europeu.

Por fim, avaliou-se o impacto da inclusão de *d*TM na qualidade nutricional, organolética e sensorial do robalo (capítulos 4 e 7). Os resultados demonstraram que

níveis elevados de proteína provenientes da *d*TM (até 100%; substituição total da FP) resultaram em níveis de ácido eicosapentaenoico e docosahexaenoico (EPA+DHA) acima dos valores recomendados pela EFSA (250 mg 100 g^{-1} peso fresco) para prevenir o risco de doenças cardiovasculares em humanos. No entanto, quando mais de 30% da proteína (50% de substituição da FP) provém da *d*TM, alguns índices de qualidade relativos a certos ácidos gordos ficam comprometidos. A substituição parcial e total de FP por *d*TM não induziu impactos relevantes nas características organoléticas do robalo; os consumidores aceitaram muito bem as postas cozidas de todos os peixes, associando aqueles alimentados com *d*TM a uma textura mais suculenta.

Em suma, com a presente tese foi claramente demonstrado que o robalo Europeu é capaz de usar de forma eficiente formulações com altos níveis de proteína fornecida pela *d*TM (até 59%), substituindo totalmente a FP. Estas dietas testadas durante 16 semanas em condições controladas incluíram 12,5-14% de OP de alta qualidade e foram suplementadas com doses adequadas de aminoácidos sintéticos. No entanto, e apesar de terem sido observados mecanismos fisiológicos compensatórios, os resultados sugerem que, a longo prazo, níveis tão elevados de proteína derivada da *d*TM podem comprometer a saúde dos animais e o seu valor nutricional para consumo humano. Além disso, atualmente a disponibilidade e os preços de mercado da FI ainda são proibitivos. Assim, neste momento, formulações com níveis mais baixos de proteína derivada de *d*TM (< 30%) parecem ser mais apropriadas para a indústria de alimentos compostos para peixes.

Summary

Aquaculture is the fastest-growing animal-food production sector worldwide, and thus plays an important role in combating hunger and addressing malnutrition. Aquafeed is one of the foremost expenses of the aquaculture industry (50-70% of variable costs) and its quality is crucial for the appropriate growth, reproduction, and health status of fish. Despite the increasing share of fishmeal (FM) and fish oil (FO) from fish by-products (i.e., heads, viscera, skin, bones, and scales) over the last years, the production of these commodities (particularly FM) is still highly dependent on the processing of whole small pelagic fish species obtained from fisheries. Landings from biologically sustainable fish stocks have been increasing, but over 33% of fish stocks remain overexploited. Thus, to ensure the responsiveness and sustainability of the aquaculture industry over the upcoming years, it is imperative to reduce its dependence on FM and FO derived from small pelagic forage fish and promote the strategic use of these ingredients. Insects have been identified as one of the most promising alternatives to FM when compared to other novel protein sources (e.g., micro- and macroalgae, bacteria, and yeast). However, the feasibility of using insect meal (IM) as an FM substitute in aquafeed formulations for European sea bass, remains largely underexplored. In this connection, this thesis hypothesized that if an IM was included in a well-balanced diet, FM could be replaced in diets for this species without affecting feed intake, digestibility, and metabolism, resulting in high fish growth and flesh nutritional value for human consumption.

In chapter 2, the nutritional composition and bioavailability of four easily accessible IM, authorized in the European market, *Hermetia illucens* (HI) and *Tenebrio molitor* larvae meal, both full fat (HI and TM, respectively) and defatted (*d*HI and *d*TM, respectively) were analysed. Additionally, an IM obtained from *Locusta migratoria* (LM) was also characterized for comparison. The obtained results demonstrated that LM was the test ingredient with the lowest potential to be incorporated into aquafeeds as an FM substitute, due to its low protein content (24% on a dry matter (DM) basis), poor essential AA (EAA) profile and high crude fibre levels. On the other hand, *d*TM was the only IM with a higher protein content (68.9% *vs* 60.2%) than a high-quality anchovy meal. Besides, *d*TM also had high digestible protein content and a high amount of digestible EAA (>89%), revealing its high potential as protein source in diets for European sea bass. The palatability of *d*TM was further evaluated in chapters 3 and 5. For the first time, the impact of increasing dietary levels of *d*TM on fish voluntary feed intake (VFI) was tested, tackling the underlying regulation mechanisms displayed at the central level in the short-, mid-, and long-term. These studies revealed that neither the partial nor the total FM replacement by *d*TM affected the expression of key neuropeptides involved in orexigenic or anorexigenic

responses (stimulation and inhibition of appetite, respectively). However, VFI decreased after 16 weeks of feeding when *d*TM provided 30% of the dietary protein (50% FM replacement), but without compromising growth.

In chapters 4, 6, and 7 nutrient utilization was assessed in fish fed diets with a partial or total FM replacement by *d*TM. Despite protein digestibility decreased in European sea bass fed diets where *d*TM provided 30-100% of the dietary protein (50-100% FM replacement), their ADC values were still high (89-92%). A compensatory nitrogen retention behaviour and reduced nitrogen losses were observed, resulting in similar nitrogen gain and whole-body protein content. Besides, it was clearly demonstrated that when *d*TM contributed with 59 or 100% of the dietary protein (totally replacing FM), European sea bass growth performance and feed efficiency were not impaired after 10-16 weeks of feeding. Indeed, it was observed a compensatory growth mechanism at the transcriptional level (*i.e*., down-regulation of muscle atrophy F-box and up-regulation of myoblast fusion factor transcripts) when FM was totally replaced by *d*TM. On the other hand, it was demonstrated that total FM replacement by *d*TM, improved lipid retention efficiency and reduced losses, regardless of the level of IM contribution to dietary protein. This trade-off resulted in increased lipids gain and augmented whole-body lipid content (chapters 4 and 7). It was also clearly demonstrated that total FM replacement by *d*TM induced *de novo* synthesis of fatty acids and inhibited β-oxidation, resulting in high levels of plasmatic non-esterified fatty acids and triacylglycerides, which in the long-term may compromise fish health and flesh quality for human consumption. Altogether, these results prove that high levels of dietary protein provided by *d*TM to totally replace FM strongly affect lipids metabolism in European sea bass.

Finally, the impact of dietary inclusion of *d*TM on the nutritional, organoleptic, and sensorial quality of European sea bass was evaluated (chapters 4 and 7). The results demonstrated that high levels of dietary protein provided by *d*TM (up to 100%; total FM replacement) resulted in muscle eicosapentaenoic *plus* docosahexaenoic acid (EPA+DHA) levels above the minimum recommended by EFSA (250 mg 100 g^{-1} wet weight) to prevent the risk of cardiovascular diseases in humans. However, when more than 30% of the dietary protein (50% FM replacement) was provided by *d*TM, some fatty acids quality indexes were impaired. Consumers accepted very well the cooked slabs of European sea bass fed *d*TM and even associated them with a juicier texture, which is considered one of the most important quality attributes of fish for consumers

Overall, with the present thesis it was clearly demonstrated that European sea bass can efficiently use dietary formulations with 53 to 59% of the total protein provided by *d*TM (i.e., totally devoid of FM) combined with 12.5-14% inclusion of high-quality FO and

proper supplementation with synthetic amino acids, for 16 weeks under controlled conditions. However, despite some compensatory mechanisms have been observed, results suggest that such high levels of dietary protein derived from *d*TM may compromise fish health and their muscle nutritional quality for human consumption in the long-term. Moreover, the current availability and market prices of IM are still prohibitive. Therefore, at present, dietary formulations with low levels of protein derived from *d*TM (<30%) are deemed more appropriate for the aquafeed/aquaculture industry.

Chapter 1.

General Introduction

1.1. Need for new raw materials in aquaculture diets

In a world where more than 800 million people still suffer from hunger, 3 billion people cannot afford healthy diets, and the global population is expected to exceed 9 billion people by 2050, feeding the planet while safeguarding its natural resources for future generations is a challenge of paramount importance (FAO, 2022). Although the aquaculture sector has grown at a slower rate in the last two years (3.3% in 2018-2019 and 2.6% in 2019-2020 in contrast to an average of 4.6% per year during the period 2010–2018), it remains the fastest growing animal-food production sector worldwide and thus plays an important role in combating hunger and addressing malnutrition. The most recent data from FAO (2022) reported that in 2020, more than half (i.e., 56%; 88 million tonnes) of the aquatic food available for human consumption already came from aquaculture production. This share is forecasted to increase by 22%, reaching 106 million tonnes by 2030 (FAO, 2022). The expansion of the aquaculture sector necessarily implies an increase in aquafeeds production. Fishmeal (FM) and fish oil (FO) are highly valuable protein and lipids sources, respectively. Despite the share of FM and FO produced from fish by-products (i.e., heads, viscera, skin, bones, and scales) has grown over the last years, the production of these commodities (particularly FM) is still highly dependent on the processing of whole small pelagic fish species obtained from fisheries (Figure 1.1). In 2020, over 16 million tonnes of small pelagic fish species were reduced into FM and FO and according to Cottrell et al. (2020), the demand for such fish species would increase by 25% to over 24 million tonnes by 2030. But according to FAO (2022) forecast, capture fisheries are expected to only increase 6% (5 million tonnes) by 2030. In fact, despite the number of landings of biologically sustainable fish stocks has been increasing, over 33% of fish stocks remain overexploited (FAO, 2022). Thus, to ensure the responsiveness and sustainability of the aquaculture industry over the upcoming years, it is imperative to reduce its dependence on FM and FO derived from small pelagic forage fish and increase the use of these ingredients only strategically (Naylor et al., 2021).

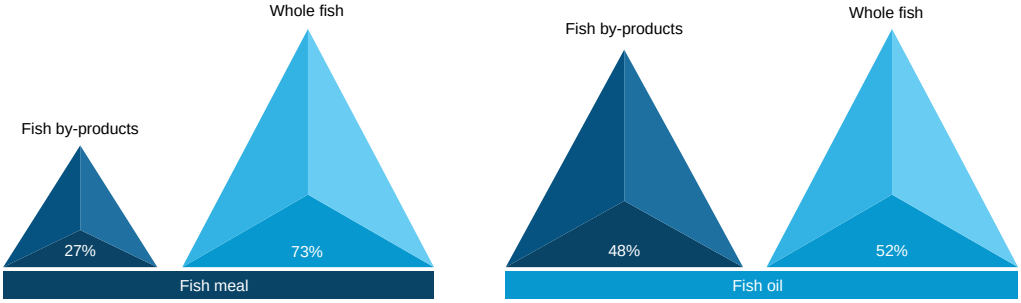

Figure 1.1. Share of whole small pelagic fish species and fish by-products used for the production of fishmeal and fish oil. Adapted from FAO, 2022a.

Over the last decades, there has been intensive research on the use of different alternative ingredients to FM, but notable attention has been given to plant protein sources (PP). Indeed, SBM and a range of cereal, oilseed, pulse, and various grain legume seed products are already widely used in aquafeeds for herbivorous, omnivorous, and carnivorous fish species (Glencross et al., 2019; Jia et al., 2022). However, high inclusion levels of such protein sources, particularly when used in diets for carnivorous fish species, not only can negatively affect the growth performance and health status of fish but also compromise their nutritional value for human consumption (Glencross et al., 2019). Besides, Newton and Little (2017) and Newton et al. (2023) demonstrated that PP (i.e., wheat gluten and soybean and pea protein concentrates) have a higher environmental footprint than marine ingredients, related to unsustainable deforestation, high greenhouse gas emissions, and high energy requirements during processing. Altogether, these issues have pressured both the industrial and scientific communities to identify other raw materials as alternative ingredients to FM. Using insects as a source of nutrients is not a novel concept, edible insects have been part of human diets in Latin America, Africa, and Asia for centuries (Raheem et al., 2019). However, in Europe, the interest in insect protein has raised since the inclusion of this ingredient in aquafeed was authorized by the European Union (EU) in 2017 (European Commission (EC) Regulation No 893/2017 of the European Parliament and the Council of the EU; European Commission, 2017). According to Cottrell et al. (2020), insect protein has been identified as the most promising alternative to FM when compared to other novel protein sources, such as micro- and macroalgae, bacteria, and yeast. Insects have a high protein content (up to 75%) and can also be valuable sources of healthy compounds (Nogales-Mérida et al., 2018; Hua et al., 2019; Gasco et al., 2020). Insects production requires less water, less land, and less production time when compared to PP sources. Insects grow fast and reproduce easily, have a low feed conversion ratio (FCR), and some species may be raised on bio-wastes contributing to a circular economy (Maulu et al., 2022). When local underutilized vegetables are used as feed substrates to farm insects, not only the food-waste challenge is tackled, but a local feed production system is also created (Modahl and Brekke, 2022).

1.2. Insects

1.2.1. Nutrient profile

The nutrient profile of insects varies greatly from species to species, but even insects of the same species can have different nutritional profiles, depending on the developmental stage, rearing substrates, and processing method that is considered (Finke, 2013; Finke, 2015). The protein content of insects ranges between 25% and 75% on a dry matter (DM) basis, but most species contain around 60% of protein (DM basis) (Oonincx and Finke, 2021). Since amino acids (AA) are the building blocks of proteins, their sum corresponds to the true protein value of a matrix. However, protein content is commonly estimated by multiplying nitrogen content by the nitrogen-to-protein conversion factor (Kp) of 6.25, which may result in protein overestimation due to the presence of other nitrogen-containing compounds in insects, such as chitin. Based on the AA composition of *Alphitobius diaperinus, Hermetia illucens,* and *Tenebrio molitor*, Janssen et al. (2017) suggested the use of an alternative Kp of 4.76 for whole larvae and a Kp of 5.60 for insect-derived processed animal proteins (PAP). Belghit et al. (2019a) obtained similar Kp values (4.21–5.05) for 18 different PAP samples derived from *A. diaperinus* (3), *Acheta domesticus* (2) *H. illucens* (8) and *T. molitor* (5). However, based on available data on AA content from 20 insect samples (including 13 species and different developmental stages) Oonincx and Finke (2021) obtained Kp values varying from 4.56 to 6.45. So, results are far from being consensual among studies and/or species. The AA analysis is the best method to accurately determine insects' protein content, but when it is not possible to use it, maintaining a Kp of 6.25 can facilitate comparisons among studies (Oonincx and Finke, 2021). AA profile of four commonly raised insect species is compared with a high-quality referenced anchovy meal (5–01-985; NRC, 2011) in Table 1.1. Despite the great variability of AA profile within each insect species, *A. diaperinus* is the insect species with the closest AA profile to that of anchovy meal. In general, the most abundant AA are alanine (Ala), aspartate (Asp), and glutamate (Glu), whereas histidine (His), methionine (Met), Cysteine (Cys), and tryptophane (Trp) are present in a lower quantity. Lysine (Lys) and Met are usually the most limiting AA in PP-based diets for different fish species. For instance, SBM has 2.5-3.1% DM and 0.7-0.0.8% DM of Lys and Met, respectively (5-04-604/612; NRC, 2011). Although IM may not be extremely rich in Lys and Met when compared to anchovy meal (Table 1.1), this protein source seems to be a more valuable FM alternative than SBM.

Table 1.1. Amino acid profile (% dry matter) of four commonly raised insect species and a referenced anchovy meal (5–01-985; NRC, 2011). Adapted from Nogales-Mérida et al., 2018.

	A. diaperinus **(larvae)**	***H. illucens*** **(larvae/ pupae)**	***T. molitor*** **(larvae)**	***M. domestica*** **(larvae/ pupae)**	**Anchovy meal**
Arginine	4.1-5.0	1.9-3.2	2.0-4.4	2.4-6.1	4.0
Histidine	1.3-1.6	1.1-1.6	1.1-1.9	1.2-3.0	1.7
Isoleucine	2.4-3.1	1.7-2.4	1.4-2.9	1.1-3.1	3.3
Leucine	4.3-6.7	2.4-3.7	2.8-5.6	2.2-6.4	5.4
Lysine	3.5-4.4	2.1-3.7	1.9-3.6	1.7-5.0	5.6
Methionine	0.9-1.4	0.7-1.2	0.5-1.0	0.5-4.0	2.1
Phenylalanine	1.9-2.3	1.4-2.4	1.5-2.1	1.3-3.8	2.9
Threonine	2.2-2.7	1.5-2.2	1.6-2.4	0.9-3.3	3.1
Tryptophane	0.4-0.5	0.5-0.8	0.4-0.7	0.7-4.1	0.8
Valine	3.3-4.2	2.2-3.8	2.7-4.3	1.9-5.0	3.8
Σ EAA	***24.3-31.9***	***15.5-25.5***	***15.9-28.9***	***13.9-43.8***	***31.9***
Alanine	5.4-7.1	2.4-4.4	3.2-5.3	1.5-4.6	*na*
Aspartate	4.7-6.0	3.2-5.3	3.1-4.9	0.6-6.5	*na*
Cysteine	0.6-0.7	*vest*-1.4	0.4-1.3	0.1-1.7	0.7
Glutamate	6.9-8.3	3.9-6.4	2.1-6.9	*nd* -8.4	*na*
Glycine	3.2-4.3	1.9-3.0	2.0-5.9	1.0-3.4	*na*
Proline	3.6-4.3	2.1-5.6	2.2-4.3	1.6-3.3	*na*
Serine	2.4-3.7	1.5-2.7	1.9-2.8	0.9-2.8	*na*
Tyrosine	3.1-4.4	2.1-3.4	1.9-3.8	1.3-4.1	2.3
Taurine	nd-4.6	vest-0.4	vest	0.3-0.6	na
Σ NEAA	***29.9-43.4***	***17.1-32.6***	***16.8-35.2***	***7.3-35.4***	***na***
True Protein (Σ AA)	***29.9-75.3***	***32.6-58.1***	***32.7-64.1***	***21.2-79.2***	***na***

AA, amino acids; EAA, essential amino acids; na, not available data; nd, not detectable amino acid; NEAA, non-essential amino acids; vest, vestigial amount of amino acid (< 0.1% dry matter).

The lipid content of insects is also highly variable ranging from 10% to 70% (DM basis). Besides, due to low energy expenditure, industrially produced insects seem to have higher lipid content than those collected from the wild (Oonincx and Finke, 2021). The fatty acid (FA) profile of the four insect species abovementioned is presented in Table 1.2. Insects' FA profile is mainly composed of saturated fatty acids (SFA), particularly palmitic acid (PA; C16:0) and stearic acid (SA; C18:0), followed by monounsaturated fatty acids (MUFA), composed predominantly of oleic acid (OA; C18:1n-9), and the polyunsaturated fatty acids (PUFA), linoleic (LA; C18:2n-6) and α-linolenic (ALA; 18:3n-3) acids. The main nutritional limitation of insects, likewise other terrestrial protein sources, is their vestigial content, or even absence, of eicosapentaenoic (EPA; C20:5n-3) and docosahexaenoic acids (DHA; C22:6n-3), resulting in high n-6/n-3 ratios. Several studies have demonstrated that the FA profile of insects reflects the FA profile of their rearing substrates (Oonincx et al., 2020; Fitriana et al., 2022; Rodrigues et al., 2022a) and can hence be modulated. Rodrigues et al. (2022b) reported that *H. illucens* can successfully use expired fish feed as a feeding substrate, resulting in a decreased n-6/n-3 ratio.

Moreover, Romero-Lorente et al. (2022) demonstrated that it is possible to reduce the n-6/n-3 ratio when *T. molitor* larvae are reared on fresh fish by-products, due to increased EPA and DHA content in insect biomass.

Table 1.2. Fatty acid profile (% total fatty acids) of four commonly raised insect species. Adapted from Nogales-Mérida et al., 2018.

	A. diaperinus **(larvae)**	***H. illucens*** **(larvae/ prepupae)**	***T. molitor*** **(larvae)**	***M. domestica*** **(pupae)**
C12:0	0.2-0.3	23.6-60.9	0.1-0.4	*nd*
C14:0	1.6-1.7	5.1-9.5	0.4-4.5	2.1-4.5
C16:0	23.7-24.8	8.7-19.8	16.1-23.6	21.4-38.1
C18:0	4.6-6.8	1.0-6.5	2.4-7.9	2.4-4.4
Σ SFA	31.3-32.4	51.3-81.8	20.0-33.7	26.2-40.4
C16:1n-7	2.4-2.8	2.0-7.6	0.2-2.9	29.6
C18:1n-9	26.6-30.2	5.7-22.7	36.3-57.6	22.2-49.0
Σ MUFA	29.0-33.0	8.1-29.0	2.0-59.4	22.2-73.2
C18:2n-6	nd-31.8	4.5-19.3	15.5-31.9	2.8-36.3
C18:3n-3	nd-1.7	nd-1.6	0.1-1.8	0.1-2.7
C20:4n-6	nd	0.1-2.1	nd-0.1	nd-0.1
C20:5n-3	0.5-0.8	nd[b]-3.5	0.1-0.2	vest-0.2
C22:6n-3	nd	vest-1.7	0.1-0.2	vest
Σ PUFA	34-34.3	6.8-19.7	15.5-33.7	nd-39.2
Σ n-3 PUFA	2.2-2.5	0.1-6.2	nd-1.8	nd-7.3
Σ n-6 PUFA	31.8	4.5-20.7	15.5-1.9	nd-36.3
Σ n-3 PUFA / Σ n-6 PUFA	vest-0.1	vest-1.1	vest-0.1	vest-0.1

MUFA, monounsaturated fatty acids; nd, not detectable fatty acid; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; vest, vestigial amount of fatty acid (< 0.1% g 100 g^{-1} total fatty acids).

Likewise proteins and lipids, the carbohydrate content of insects varies greatly from species to species, ranging from 1% to 20% of DM (Tran et al., 2015). The different types of insects' carbohydrates have not been extensively studied and remain unknown for most insect species. However, Son et al. (2021) recently demonstrated that among the 11.5% (DM basis) of crude carbohydrates present in *T. molitor* larvae, only 3.2% (DM basis) are total soluble sugars, including glucose, fructose, and sucrose. On the other hand, Son et al. (2021) concluded that *T. molitor* larvae do not contain galactose, maltose, or lactose. Through the difference between crude carbohydrates and total soluble sugar, it was predicted that the carbohydrates present in *T. molitor* larvae are mainly constituted by insoluble fractions (Son et al., 2021). Chitin is an insoluble carbohydrate commonly found in insects, and Son et al. (2021) demonstrated that almost half (4.7% of DM) of the total carbohydrates present in *T. molitor* larvae are in the form of chitin. Over the last few years, several efforts have been made to develop a direct and accurate method for the quantification of insects' chitin (Hahn et al., 2020). However, the indirect method of insects' chitin estimation through acid detergent fibre (ADF) analysis developed by Van Soest et al. (1991) (Figure 1.2) still is the most used method due to the absence of lignin

in insects, and the structural similarity between insects' chitin (β-(1-4) *N*-acetyl-D-glucosamine) and plants' cellulose (β-(1-4)-D-glucopyranose) (Koutsos et al., 2019). Finke (2007) demonstrated that this method results in an overestimation of insects' chitin content because their ADF residue contains significant amounts of AA, and therefore, to estimate chitin more accurately it is still necessary to adjust ADF for its AA content (Finke, 2007; Marono et al., 2015).

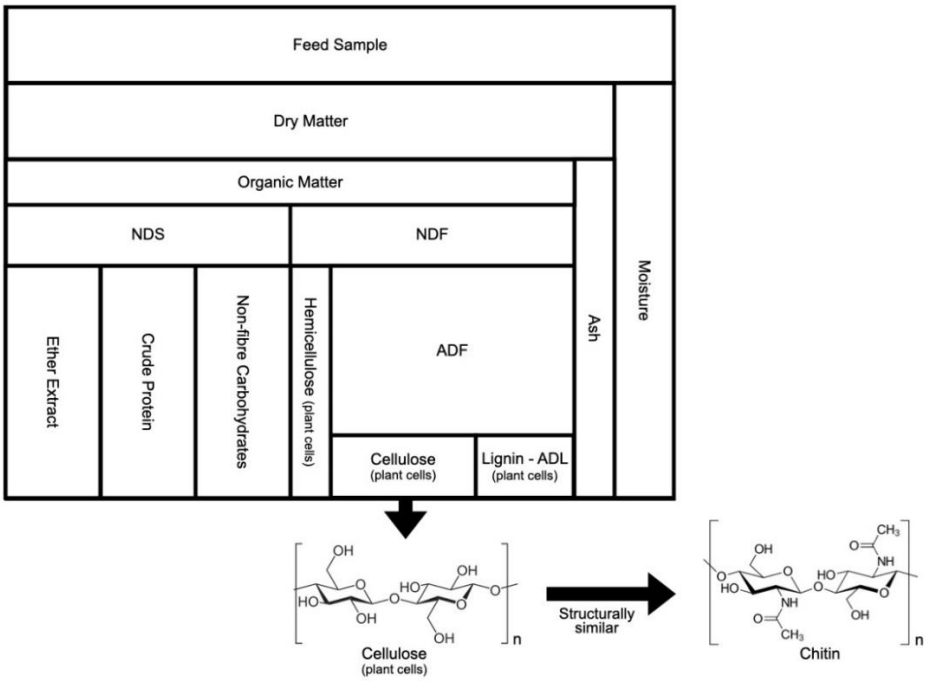

Figure 1.2. Scheme of chemical constituents of feed samples according to Van Soest et al, 1991. ADF, acid detergent fibre; ADL, acid detergent lignin; NDF, neutral detergent fibre; NDS, neutral detergent solubles.

Some insect species are rich in minerals and vitamins. Insects are good sources of highly bioavailable phosphorus, contrarily to plants, where this mineral is present as phytate that is poorly used by fish and other vertebrates (Oonincx and de Boer, 2012). Insects also contain reasonable amounts of magnesium, potassium, iron, zinc, copper, manganese, and selenium (Oonincx and Finke, 2021), but generally low levels of calcium, as their exoskeleton mainly consists of protein and chitin. However, in *H. illucens* the amount of calcium can reach up to 1.6% (as feed basis) due to its mineralised exoskeleton (Koutsos et al., 2019).

Data on the vitamin content of insects is limited, but some studies have reported the presence of vitamins A, B, D, and E in some species (Finke, 2002; Finke, 2013; Finke, 2015). Alike vertebrates, insects can convert dietary carotenoids into vitamin A. However, in the case of commercially produced insects, the presence of low quantities of such carotenoids in the rearing substrates results in low amounts of vitamin A (Oonincx and Finke, 2021). On the other hand, some studies demonstrated that insects are a very good

source of riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folic acid, and cyanocobalamin (*i.e.*, vitamin B_2, B_3, B_5, B_6, B_7, B_9, B_{12}, respectively) (Finke, 2002; Finke, 2013; Finke, 2015). For a long time, insects were considered to contain low levels of vitamin D, but it was recently revealed that likewise vertebrates, some insect species (e.g., *T. molitor*) can synthesise vitamin D_3 *de novo* when exposed to UV-B, reaching over 6,000 IU kg^{-1} (DM basis) (Oonincx et al., 2018).

1.2.2. Insect production sector: status, EU legislation, and environmental impacts

Over the last two decades, the insect production industry has been mainly targeting the pet food market, according to the most recent market factsheet developed by The International Platform of Insects for Food and Feed (IPIFF), which is the European organisation that represents the interests of the insect production sector towards EU policymakers to promote the use of insects as food and feed (IPIFF, 2021). However, since the EU authorization in 2017 to include insect-derived PAP into aquafeeds (EU, 2017) the aquafeed industry became the main target for insect producers and has pushed forward insect production (IPIFF, 2021). By 2030, the share of IM used in aquaculture is expected to surpass the pet food market (40% vs 30%, respectively), which will be followed by poultry and pigs (20% and <10%, respectively) (Figure 1.3).

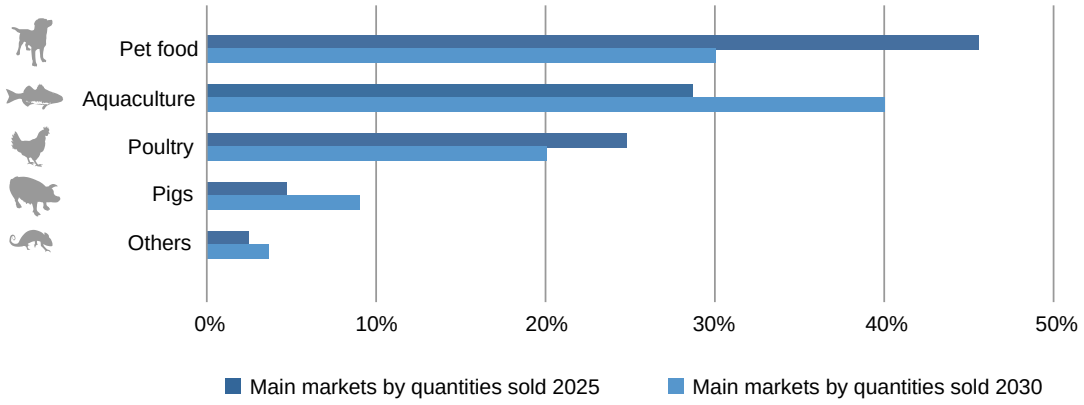

Figure 1.3. Expected main markets of insect-derived processed animal proteins (PAP) by 2025 and 2030. Adapted from IPIFF, 2021.

Over the last three years, 10,000 tonnes of insect protein were annually produced worldwide and placed in the market at a price ranging from 3,500 to 5,500 euros (EUR) per tonne (Rabobank, 2021); the EU annual production of insects is estimated in 6,000 tonnes, corresponding to an average of 2,000-3,000 tonnes of insect-derived PAP (IPIFF, 2019). In contrast, the most recent data from the European Market Observatory for Fisheries and Aquaculture Products (EUMOFA), indicate that in 2020, the EU produced

approximately 420,000 tonnes of FM, and this value was still not sufficient to meet the protein needs of animal feed producers (≈ 450,000 tonnes) (EUMOFA, 2021). Furthermore, according to the EU trade balance (export *minus* import), approximately 230,000 tonnes were imported from different non-EU countries (primarily from Peru, Morocco, and Norway), approximately at 1,220 EUR/tonne, but 184,000 tonnes were exported, approximately at 1,406 EUR/tonne (EUMOFA, 2021). This highlights the still huge price difference between FM and insect-derived PAP. On the other hand, in 2020 the EU production of the most widely used PP source in aquafeeds (i.e., SBM) only reached approximately 13,000 tonnes, and other 16,000 tonnes were imported mainly from Argentina and Brazil at around 330 EUR/tonne (Kuepper and Stravens, 2022). It is also important to point out that since the 2000's the price of both FM and SMB has risen, and the trend is to continue increasing due to the increasing demand and limited availability (FAO, 2022). Besides, with Russia's invasion of Ukraine, the price of these commodities was already pushed up to 1,600 EUR/tonne for FM and 490 EUR/tonne for SMB, highlighting the need to find new protein sources. At the moment, the insect industry still can't compete with FM or SBM production sector, neither in terms of volume nor value and need to scale-up. Accordingly, over the last two years, about 1 billion EUR have been invested in the insect production industry in the EU, and it is expected that the production of insect-derived PAP to be used in animal feed could reach 500,000 tonnes by 2030 (IPIFF, 2021; Rabobank, 2021). According to Rabobank (2021), the price of insect protein has stabilised at around 3,500-5,500 EUR/tonne. However, a droplet of 1,000 EUR/tonne is expected once the industry completes the initial scale-up phase, followed by another 1,000 EUR/tonne in the maturity-phase, reaching the price of 1,500-2,500 EUR/tonne by 2030. This represents a major turning point for the insect industry after which much less time will be required to double or even quadruple production volume and exceed 1 million tonnes (Rabobank, 2021). However, for the industry to reach its full potential some constraints related to legal details must be overcome.

Before insect-based food and feed products started attracting a significant level of interest in the European market, small-scale production and trade of edible insects had not been considered sufficiently important to be subject to legislative regulation. However, a report released by FAO in collaboration with the Laboratory of Entomology at Wageningen University (Wageningen, The Netherlands) in 2013, titled "Edible insects: future prospects for food and feed security" (van Huis et al., 2013), triggered the interest of European policymakers and stakeholders in such protein source. In 2015, the European Commission asked the European Food Safety Authority (EFSA) to assess the biological, chemical, allergenicity, and environmental hazards arising from the production

and consumption of insects as food and feed. In this connection, on 8 October 2015, EFSA released the first scientific opinion on this topic, stating that as long as insects are fed with permitted raw materials, the potential occurrence of hazards is expected to be similar to that of other non-processed protein sources (EFSA, 2015). In 2017 the use of IM from seven insect species in aquafeed, namely, black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*), was approved by the EU (European Commission, 2017). Like other farmed animals, insects can only be fed with authorized substrates like “feed grade materials” (e.g., plant-based materials, vegetable and fruit residues, wheat bran, grass, and brewery by-products, among others). However, insect producers have shown interest in using other media for insect rearing, such as low value organic wastes. Indeed, the short list of allowed substrates for mass rearing is a bottleneck for the development of the insect industry. Thus, to promote changes in legislation, more research is needed to assess the risks deriving from chemicals, mycotoxins, heavy metals, pesticides, and other residues in low quality substrates intended for insect rearing (Zuk-Golaszewska et al., 2022) before they can be authorised.

Insects have unique characteristics that enable them to produce protein in an efficient and environmentally friendly way as mentioned in section 1.1. (Halloran et al., 2016). Nonetheless, to obtain a true assessment of the environmental sustainability of insects as an alternative protein source for aquafeeds, more information is needed about their production systems through the life cycle assessment (LCA) approach, which is recognized as the most complete method of environmental impact assessment of a commercial product (Halloran et al., 2016). Few studies are still available in the literature comparing the environmental impact of industrial insect production with other alternative protein sources for aquafeeds through the LCA approach. But available data indicate that *H. illucens* and *T. molitor* production on a pilot scale requires less land and water than SBM and/or microalgae biomass production. On the other hand, the production of IM from H. illucens *H. illucens* and *T. molitor* results in higher CO_2 emissions, higher acidification, higher eutrophication, and energy demand; the highest environmental impacts observed during insect production were associated with their rearing substrate and energy used for growth and processing. Thus, it was suggested that efforts should be made to identify alternative sources of feed for insects with lower environmental impact, such as low-value agricultural by-products. Finally, it has been suggested the use of renewable energy as the key to reducing the impact of insects’ production on global warming when compared

with nature-based production systems not so strongly dependent on energy (Thévenot et al., 2018; Smetana et al., 2019; Maiolo et al., 2020; Modahl and Brekke, 2022).

1.2.3. Insects in aquafeeds for European sea bass and other important fish species produced in European and Mediterranean aquaculture

European sea bass is one of the most important fish species in European aquaculture, particularly in the Mediterranean. The production of this marine fish species has increased year by year and reached a new record in 2019, when accounted for 11% and 29% of total fish species produced in European and Mediterranean aquaculture, respectively, with 86,149 tonnes and 491 million EUR in Europe, and 219 million tonnes and more than 2 billion EUR in Mediterranean countries (FAO, 2020; European Commission and Directorate-General for Maritime Affairs and Fisheries, 2021). Over the last two decades, the aquaculture of marine fish species in Turkey increased by more than 615% and is nowadays the biggest producer of European sea bass among the 21 Mediterranean countries. In 2020, Turkey produced almost 150 million tonnes of sea bass valued at more than 545 million EUR (3.67 EUR/Kg) and the trend is to continue increasing (Eurostat, 2022). Greece and Spain are the second and third major producers of European sea bass among Mediterranean countries, respectively. In 2019, the European sea bass production in Greece decreased by 12% in comparison to 2018, reaching 41 million tonnes, which were sold at an average price of 4.86 EUR/kg, representing a price drop of 5%, yielding 200 million EUR. On the other hand, Spanish production of European sea bass grew 12% from 2018 to 2019, reaching its 10-year highest production level with 25 million tonnes and almost 155 million EUR (6.12 EUR/Kg). Although France is a relatively small sea bass producer, it is worth noting that its production increased by more than 40% between 2018 and 2019, reaching 2 million tonnes and almost 21 million EUR (8.43 EUR/Kg) in 2019 (European Commission and Directorate-General for Maritime Affairs and Fisheries, 2021; Eurostat, 2022). Since European sea bass is a carnivorous fish species with a high protein requirement (45-55%, depending on its development stage (NRC, 2011)), it is of paramount importance to identify new protein sources able to minimize the use of FM and support the expansion of its sustainable production.

Several studies have evaluated the feasibility of IM protein use as FM and/or PP substitute in diets for European sea bass and other important fish species produced in Europe, such as Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*). The main results obtained in these studies concerning growth performance, nutrient utilization, whole-body composition, intermediary

metabolism, and nutritional and organoleptic quality are summarized in Table 1.3. Most studies selected *H. illucens* or *T. molitor*, both full fat (HI and TM, respectively) and defatted (*d*HI and *d*TM, respectively), probably due to their greater market availability. Overall, growth performance results were very encouraging, since in most studies final body weight (FBW) and FCR remained unaltered when 10-75% FM was replaced by IM (corresponding to an 8-50% IM dietary inclusion) for 45-180 days. Furthermore, total FM replacement by partially *d*HI does not seem to impair the growth performance and feed efficiency of Atlantic salmon (Belghit et al., 2019b) and rainbow trout (Biasato et al., 2022), whereas the use of partially *d*TM even improved growth performance and feed efficiency of rainbow trout after 90 days of feeding (Rema et al., 2019). As far as we are aware, in gilthead sea bream and European sea bass total FM and/or PP replacement by IM was never assessed before. Despite the promising results of growth performance and feed efficiency, in Atlantic salmon, rainbow trout, and gilthead sea bream, the dietary inclusion of IM was associated with an impairment of nutrients digestibility and utilization (Belforti et al., 2015; Belghit et al., 2018b; Belghit et al., 2019b; Chemello et al., 2020; Fabrikov et al., 2021b; Weththasinghe et al., 2021; Bordignon et al., 2022; Melenchón et al., 2022), which in the long-term may impair fish growth performance and health status. But in European sea bass, according to Magalhães et al. (2017), nutrient digestibility was not affected when 45% of FM was replaced by defatted HI (19.5 defatted HI dietary inclusion).

Since fish have a pivotal role in human nutrition due to their unique content of n-3 LC-PUFA, particularly EPA and DHA, and insects have low amounts or even absence of these FA, the impact of FM substitution by IM on the nutritional quality of fish for human consumption has been extensively studied (Table 1.3) (Belforti et al., 2015; Lock et al., 2016; Iaconisi et al., 2017; Renna et al., 2017; Secci et al., 2019; Bruni et al., 2020a; Bruni et al., 2020b; Gasco et al., 2020; Mastoraki et al., 2020; Fabrikov et al., 2021a; Pulido-Rodriguez et al., 2021; Bordignon et al., 2022; Mancini et al., 2022). However, the available results in the literature are still controversial and vary greatly among fish species, insect species, and their fat content; other factors like the basal dietary formulations, the IM dietary inclusion levels, or the trial duration are also largely variable among studies. For example, the use of 19.5% of partially defatted HI to substitute 30% of FM did not alter the whole-body FA profile of European sea bass after 84 days of feeding (Mastoraki et al., 2020). Nonetheless, in the same trial, when similar inclusion levels of full fat TM and full fat *M. domestica* were used also to replace 30% of FM, the whole-body's EPA and DHA content of European sea bass decreased (Mastoraki et al., 2020). Since all diets tested by Mastoraki et al., 2020 were isolipidic, these distinct results were probably associated with the inclusion level of FO in each dietary treatment. In fact, either the

defatted HI diet or the control diet had similar inclusion levels FO (i.e., 10% as feed basis), which was higher than that included in TM and *M. domestica* diets (i.e., 6% as feed basis). Thus, 6% FO dietary inclusion was not enough to maintain a similar whole-body EPA and DHA content between European sea bass fed TM and M. domestica, and control diets.

In the case of European sea bass, the impact of dietary inclusion of IM on flesh nutritional quality for human consumption remains to be ascertained. Therefore, further studies are needed to better understand the impact of IM on the metabolic pathways controlling nutrients' metabolism and deposition in the major edible part of European sea bass (i.e., the muscle).

1.2.4. Insects as alternative protein source for aquafeeds: SWOT analysis

SWOT (Strengths, Weaknesses, Opportunities, and Threats) analyses are performed assessing: (i) internal factors to the system (i.e., strengths and weaknesses), that offer the products a competitive advantage or disadvantage; (ii) external factors to the system (i.e., opportunities and threats), capable of facilitating or hindering the development of a product. Thus, the benefits and limitations of using insects as an alternative protein source will be summarized herein by a SWOT analysis (Figure 1.4).

Figure 1.4. SWOT analysis: Internal (Strengths and Weaknesses) and External (Opportunities and Threats) factors on the use of insects as a protein source for animal feeds.

Table 1.3. Summary of the main results obtained in available studies (2000-2022) focused on the impact of insect meal (IM) as fishmeal (FM) and/or plant protein meal (PP) substitute, on growth performance, nutrient utilization, whole-body composition, intermediary metabolism, and nutritional and organoleptic quality of Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*).

Fish species	**Fish IBW (g) [trial duration]**	**Insect species and stage of development [full-fat or defatted; % CF]**	**IM inclusion level (%)**	**[FM or PP] replacement level (%)**	**Main results of IM-based diets *versus* CTRL diet**	**Reference**
D. labrax	50 [62 days]	*Hermetia illucens* prepupae [defatted; 6% CF]	6.5, 13, 19.5	[FM] 15, 30, 45	↔ HSI and VSI ↔ Protein, fat and ash **[M]**	Moutinho et al. (2021)
D. labrax	6 [84 days]	*Hermetia illucens* larvae [partially defatted; 6% CF]	19.5	[FM] 30	↔ FBW, SGR, FCR, HSI and VSI ↔ DM, protein, ash and energy **[WB]** ↓ Fat **[WB]** ↔ Protein, ash and energy retention **[WB]** ↔ SFA, OA, MUFA, LA, ALA, EPA, DHA, n-3 and n-6 PUFA, and n-3/n-6 **[WB]** ↔ EAA profile **[WB]** ↔ Glucose, cholesterol, triglycerides, phospholipids and lactate **[Pla]** ↔ Aat1 and Ast1 **[Pla, L]** Gdh **[L]**	Mastoraki et al. (2020)
		Tenebrio molitor larvae [full-fat; 24% CF]	19.5	[FM] 30	↔ FBW, SGR, HSI and VSI ↑ FCR ↔ Protein **[WB]** ↓ DM, fat and energy **[WB]** ↓ Protein, fat and energy retention **[WB]** ↔ Ash retention **[WB]** ↑ SFA, OA, MUFA, LA, ALA, n-6 PUFA and n-3/n-6 **[WB]** ↓ EPA, DHA and n-3 PUFA **[WB]** ↔ EAA profile **[WB]** ↔ Triglycerides, phospholipids and lactate **[Pla]** ↓ Glucose and cholesterol **[Pla]** ↔ Aat1 and Ast1 **[Pla, L]**	
		Musca domestica larvae [full-fat; 23% CF]	19.5	[FM] 30	↔ FBW, SGR, FCR, HSI and VSI ↔ DM, protein, fat, ash and energy **[WB]** ↓ Protein, fat, ash and energy retention **[WB]** ↔ SFA and ALA **[WB]** ↑ OA, MUFA and LA **[WB]** ↓ EPA, DHA, n-3 PUFA and n-3/n-6 **[WB]** ↔ EAA profile **[WB]** ↑ Cys and Tyr **[WB]**	

Table 1.3. (*Continued*).

Fish species	Fish IBW (g) [trial duration]	Insect species and stage of development [full-fat or defatted; % CF]	IM inclusion level (%)	[FM or PP] replacement level (%)	Main results of IM-based diets *versus* CTRL diet	Reference
					↑ Glucose **[Pla]** ↓ Cholesterol **[Pla]** ↔ Triglycerides, phospholipids and lactate **[Pla]** ↔ Aat1 and Ast1 **[Pla, L]** Gdh **[L]**	
D. labrax	11 [49 days]	*Tenebrio molitor* larvae (reared on broilers diet) [n.a.]	18	[FM] 50	↓ FBW and SGR ↔ FCR ↔ DM, protein, fat and ash **[M]**	Reyes et al. (2020)
		Hermetia illucens (reared on broilers diet) [n.a.]	11, 18	[FM] 30, 50	↓ FBW and SGR ↑ FCR (50% FM replacement) ↔ DM, protein, fat and ash **[M]**	
		Hermetia illucens (reared on fish by-products) [n.a.]	18	[FM] 50	↓ FBW and SGR ↑ FCR ↔ DM, protein, fat and ash **[M]**	
D. labrax	12 [56 days]	*Hermetia illucens* larvae [full-fat; 20% CF]	7, 10, 15	[FM] 25, 35, 50	↔ FBW, SGR, FCR, HSI and VSI ↔ Moisture, protein, fat and ash **[WB]** ↔ Glucose, total protein, albumin, globulin, Ast1 and Aat1 **[Pla]**	Abdel-Tawwab et al. (2020)
D. labrax	50 [62 das]	*Hermetia illucens* prepupae [defatted; 6% CF]	6.5, 13, 19.5	[FM] 15, 30, 45	↔ FBW, SGR and FCR ↔ $ADC_{DM, CP, CF, GE, AA (excepting Arg, His, Val)}$ ↑ $ADC_{Arg, His}$ (45% FM replacement) ↑ ADC_{Val} (15% FM replacement) ↔ Amylase and protease **[PI, DI]** ↓ Lipase **[PI, DI]** ↔ Glucose, total protein and triglycerides **[Pla]** ↓ Cholesterol (45% FM replacement) **[Pla]**	Magalhães et al. (2017)
D. labrax	5 [70 days]	*Tenebrio molitor* larvae [full-fat; 2. 4% CF]	25, 50		↓ FBW and SGR (50% FM replacement) ↔ FCR ↔ DM, protein, fat and ash **[WB]** ↔ SFA **[WB]** ↑ OA, LA, n-6 PUFA **[WB]** ↓ ALA (50% FM replacement) **[WB]** ↓ EPA, DHA, n-3 PUFA, n-3/n-6 **[WB]**	Gasco et al. (2016)

Table 1.3. (*Continued*).

Fish species	Fish IBW (g) [trial duration]	Insect species and stage of development [full-fat or defatted; % CF]	IM inclusion level (%)	[FM or PP] replacement level (%)	Main results of IM-based diets *versus* CTRL diet	Reference
O. mykiss	15 [77 days]	*Tenebrio molitor* larvae [full-fat; 27% CF]	18	[FM] 50	↔ FBW, SGR, FCR, HSI and VSI ↔ ADC_{CP} ↔ Villus height and width, stratum compactum, circular and longitudinal muscular layers width and lamina propria width **[PC, DI]** ↑ Enterocyte height **[DI]** ↔ Enterocyte height **[PC]** ↔ Hepatocyte nucleus and cytoplasm Ø and hepatocyte vacuolization ↔ Fbpase, Pk, Ast1, Aat1 and Gdh **[L]** ↔ Calcium and phosphorus **[M]** ↑ Protein and ash **[M]** ↓ Moisture **[M]**	Melenchón et al. (2022)
		Hermetia illucens larvae [full-fat; 26% CF]	18	[FM] 50	↔ FBW, HSI and VSI ↓ SGR ↑ FCR ↓ ADC_{CP} ↔ Villus height and width, enterocyte height, stratum compactum, longitudinal layer width and lamina propria width **[PC, DI]** ↓ Circular muscular layer width **[DI]** ↔ Circular muscular layer width **[PC]** ↔ Hepatocyte nucleus and cytoplasm Ø and hepatocyte vacuolization ↔ Fbpase, Pk, Ast1, Aat1 and Gdh **[L]**	
O. mykiss	113 [133 days]	*Hermetia illucens* larvae [partially defatted; 20% CF]	8, 16, 32	[FM] 25, 50, 100	↔ FBW, SGR, FCR, HSI and VSI ↔ $ADC_{DM, CP, CF, GE}$	Biasato et al. (2022)
O. mykiss	54 [91 days]	*Hermetia illucens* pupae [partially defatted; 21% CF]	8, 23, 45	[PP] 10, 30, 60	↔ FBW and HSI ↑ SGR ↓ FCR (30 and 60% PP replacement) ↓ Moisture **[WB]** ↔ Protein, ash, phosphorus and daily phosphorus gain and retention **[WB]** ↑ Fat and energy, energy retention (30 and 60% PP replacement) **[WB]** ↑ Daily nitrogen gain (10% PP replacement level) **[WB]** ↑ Nitrogen retention **[WB]** ↑ Hepatocyte vacuolization (60% PP replacement)	Cardinaletti et al. (2022)

Table 1.3. (*Continued*).

Fish species	Fish IBW (g) [trial duration]	Insect species and stage of development [full-fat or defatted; % CF]	IM inclusion level (%)	[FM or PP] replacement level (%)	Main results of IM-based diets *versus* CTRL diet	Reference
O. mykiss	156 [76 days]	*Hermetia illucens* larvae [partially defatted: 7% CF]	6, 12	[FM] 25, 50	↔ FBW and FCR ↓ SGR ↓ $ADC_{DM, CP, CF, GE}$ ↔ Villus height **[PI]** ↑ Nº goblet cells (50% FM replacement) **[PI]** ↑ a* and b* (50% FM replacement) **[M]** ↔ SFA, MUFA, EPA, DHA, n-3 PUFA, n-6 PUFA and n-3/n-6 **[M]**	Bordignon et al. (2022)
O. mykiss	55 [46 days]	*Hermetia illucens* larvae [full-fat; 34% CF]	5.5, 11	[FM] 14, 30	↑ SFA, OA and LA (30% FM replacement) **[M]** ↔ MUFA and ALA **[M]** ↓ EPA and n-3/n-6 **[M]** ↓ DHA and PUFA (30% FM replacement) **[M]** ↑ AI, TI **[M]**	Fabrikov et al. (2021a)
		Tenebrio molitor larvae [full-fat; 28% CF]	5.5, 11	[FM] 15, 30	↑ OA, MUFA, LA, ALA and TI **[M]** ↓ SFA, EPA, DHA, PUFA and n-3/n-6 **[M]** ↔ AI **[M]**	
O. mykiss	55 [46 days]	*Hermetia illucens* larvae [full-fat; 34% CF]	5.5, 11	[FM] 14, 30	↔ FBW, SGR, FCR, HSI and VSI ↔ Acidic proteases **[S]** and alkaline proteases **[PC+I]** ↓ Amylase **[PC+I]** ↔ Hadh, Pk, Cs, Ldh, Fbpase, Gyk, Ast1, Aat1 and Gdh **[L]**	Melenchón et al. (2020)
		Tenebrio molitor larvae [full-fat; 28% CF]	5.5, 11	[FM] 15, 30	↔ FBW, SGR, FCR and HSI ↓ VSI (30% FM replacement) ↔ Acidic proteases **[S]** and amylase **[PC+I]** ↑ Alkaline proteases **[PC+I]** ↔ Hadh, Pk, Cs, Ldh, Fbpase, Gyk, Ast1, Aat1 and Gdh **[L]**	
O. mykiss		*Hermetia illucens* prepupae [full-fat; 33% CF]	10.5, 21	[FM] 25, 50	↔ HSI ↔ *cd36, pparα, pparβ pparγ pparδ, elovl1* and *elovl2* [**L, PC, MI]** ↑ *fads2* (50% FM replacement) **[PC]** ↑ Fat **[L]** ↑ OA, LA, MUFA and n-6 PUFA (25% FM replacement) **[L]** ↓ EPA **[L]** ↓ b* (25% FM replacement) **[M]** ↔ SFA, DHA, n-3 PUFA **[L]**	Bruni et al. (2020b)

Table 1.3. (*Continued*).

Fish species	Fish IBW (g) [trial duration]	Insect species and stage of development [full-fat or defatted; % CF]	IM inclusion level (%)	[FM or PP] replacement level (%)	Main results of IM-based diets *versus* CTRL diet	Reference
					↑ SFA **[M]** ↔ OA, LA, ALA, MUFA, n-3 PUFA, n-6 PUFA, n-3/n-6, TI and h/H **[M]** ↑ AI (50% FM replacement) **[M]** ↔ pH, LPO, WHC, L* and a* **[M]**	
O. mykiss	78 [154 days]	*Tenebrio molitor* larvae [partially defatted; n.a.]	5, 10, 20	[FM] 25, 50, 100	↔ FBW, SGR, FCR and VSI ↑ HSI ↔ $ADC_{DM, CF, GE}$ ↓ ADC_{CP} (50 and 100% FM replacement) ↔ Aat1, Ast1, Gdh, G6pdh, Me and Fas **[L]**	Chemello et al. (2020)
O. mykiss	116 [90 days]	*Tenebrio molitor* larvae [partially defatted; n.a.]	25, 50	[FM] 35, 67	↑ Total Leu and Ala **[M]** ↑ Total Cys, Pro, Tyr and *Tau* (67% FM replacement) **[M]** ↓ Total His, Thr and Gln (67% FM replacement) **[M]** ↓ Total Asn **[M]** ↑ Free His and Gly (67% FM replacement) **[M]** ↓ Free Ile, Leu, Lys, Val and Ala (67% FM replacement) **[M]**	Iaconisi et al. (2019)
O. mykiss	53 [71 days]	*Hermetia illucens* prepupae [full-fat; 33.5% CF]	20	[FM] 30	↔ FBW, SGR, FCR ↔ Villus height, width **[PI]**	Józefiak et al. (2019)
		Tenebrio molitor larvae [full-fat; 25% CF]	20	[FM] 41	↔ FBW, SGR, FCR ↔ Villus width **[PI]** ↓ Villus height **[PI]**	
O. mykiss	137 [98 days]	*Hermetia illucens* prepupae [full-fat; 33% CF]	10.5, 21	[FM] 25, 50	↔ FBW, SGR and FCR ↔ Cholesterol, triglycerides, glucose, total protein and albumin **[Pla]** ↔ *igf-i* and *mstn1a* **[L]**	Cardinaletti et al. (2019)
O. mykiss	179 [92 days]	*Hermetia illucens* larvae [partially defatted; 18% CF]	20, 40	[FM] 25, 50	↔ pH, WHC, L*, a*, b* shear force, total lipids and LPO **[M]** ↑ SFA **[M]** ↔ LA **[M]** ↓ ALA, MUFA, EPA, DHA, n-3 PUFA, n-6 PUFA and PUFA (**M**) ↓ OA (50% FM replacement) **[M]**	Secci et al. (2019)

Table 1.3. (*Continued*).

Fish species	Fish IBW (g) [trial duration]	Insect species and stage of development [full-fat or defatted; % CF]	IM inclusion level (%)	[FM or PP] replacement level (%)	Main results of IM-based diets *versus* CTRL diet	Reference
O. mykiss	5 [90 days]	*Tenebrio molitor* larvae [defatted; 14% CF]	5, 7.5, 15, 25	[FM] 20, 30, 60, 100	↑ FBW and SGR ↓ FCR ↔ $ADC_{DM, CP, CF, GE, phosphorus}$ ↔ Moisture, protein, fat, energy, ash and phosphorus; fat retention **[WB]** ↑ Protein, phosphorus, energy retention **[WB]**	Rema et al. (2019)
O. mykiss	179 [92 days]	*Hermetia illucens* larvae [partially defatted; 18% CF]	20, 40	[FM] 25, 50	↔ Moisture, protein, fat and ash **[M]** ↑ SFA **[M]** ↔ OA, LA, ALA and n-6 PUFA **[M]** ↓ MUFA (50% FM replacement) **[M]** ↓ EPA, DHA, n-3 PUFA and PUFA **[M]** ↔ L* and a* **[M]** ↓ b* (50% FM replacement) **[M]**	Mancini et al. (2018)
O. mykiss	116 [90 days]	*Tenebrio molitor* larvae [full-fat; 24% CF]	25, 50	[FM] 35, 67	↔ Moisture, protein, total lipids and ash **[M]** ↔ pH, WHC, shear force, L*, a* and b* **[M]** ↔ SFA, AI and h/H **[M]** ↑ OA, MUFA, LA, n-6 PUFA and TI **[M]** ↓ ALA, EPA, DHA, n-3 PUFA and n-3/n-6 **[M]**	Iaconisi et al. (2018)
O. mykiss	179 [78 days]	*Hermetia illucens* larvae [partially defatted; 18% CF]	20, 40	[FM] 25, 50	↔ FBW, SGR, FCR, HSI, VSI ↔ $ADC_{DM, CP, CF, GE}$ ↔ Villus height **[PI]** ↑ DM, fat, SFA, AI **[M]** ↔ protein **[M]** ↓ Ash (25% FM replacement) **[M]** ↑ TI (50% FM replacement) **[M]** ↓ OA, MUFA, n-6 PUFA **[M]** ↓ n-3/n-6 (50% FM replacement) **[M]**	Renna et al. (2017)
O. mykiss	116 [90 days]	*Tenebrio molitor* larvae [full-fat; 24% CF]	25, 50	[FM] 35, 67	↔ FBW and VSI ↑ SGR (50% FM replacement) ↓ FCR and HSI ↔ $ADC_{DM, CF}$ ↓ ADC_{CP} (50% FM replacement) ↓ SFA, ALA, EPA, DHA, n-3 PUFA and n-3/n-6, AI **[M]**	Belforti et al. (2015)

Table 1.3. (*Continued*).

Fish species	Fish IBW (g) [trial duration]	Insect species and stage of development [full-fat or defatted; % CF]	IM inclusion level (%)	[FM or PP] replacement level (%)	Main results of IM-based diets *versus* CTRL diet	Reference
					↔ MUFA **[M]** ↑ n-6 PUFA and TI **[M]**	
O. mykiss	339 [63 days]	*Hermetia illucens* prepupae [full-fat; 33% CF]	15, 30	[FM] 25, 50	↓ FBW and FCR (50% FM replacement) ↓ Moisture, fat and energy **[WB]** ↔ Protein **[WB]** ↔ Total lipids **[M]** ↓ AO, LA, ALA, EPA, DHA **[M]**	St-Hilaire et al. (2007)
		Musca domestica pupae [full-fat; 16% CF]	9	[FM] 25	↓ FBW ↔ FCR ↔ Moisture, protein, fat and energy **[WB]** ↔ Total lipids, OA, ALA, EPA and DHA **[M]** ↓ LA **[M]**	
S. salar	34 [49 days]	*Hermetia illucens* larvae [full-fat; 32% CF]	8, 16, 32	[FM + PP] 6, 12.5, 25	↓ FBW and SGR (25% FM + PP replacement level) ↔ FCR ↔ $ADC_{CP, GE, AA (expecting Tyr)}$ ↓ $ADC_{CF, Tyr}$ (12.5 and 25% FM+ PP replacement levels) ↔ Protein and phosphorus retention ↓ Lipids and energy retention (25% FM+ PP replacement levels) ↔ Phosphorus and nitrogen fecal losses	Weththasinghe et al. (2021)
S. salar	1398 [114 days]	*Hermetia illucens* larvae [partially defatted; 18% CF]	5, 10, 15	[FM] 33, 66, 100	↔ pH, WHC, L*, a*, b*, hardness, cohesiveness, resilience and adhesiveness **[M]** ↔ Moisture, ash, protein and total lipids **[M]** ↓ Polar OA and MUFA (100% FM replacement level) **[M]** ↓ Polar LA, ALA and n-6 PUFA (66 and 100% FM replacement levels) **[M]** ↔ Polar SFA, EPA, DHA and n-3 PUFA **[M]** ↓ Neutral OA, LA, ALA, MUFA and n-6 PUFA **[M]** ↑ Neutral SFA **[M]** ↑ Neutral EPA, DHA and n-3 PUFA (66 and 100% FM replacement levels) **[M]**	Bruni et al. (2020a)

Table 1.3. (*Continued*).

Fish species	Fish IBW (g) [trial duration]	Insect species and stage of development [full-fat or defatted; % CF]	IM inclusion level (%)	[FM or PP] replacement level (%)	Main results of IM-based diets *versus* CTRL diet	Reference
S. salar	49 [56 days]	*Hermetia illucens* larvae [partially defatted; 22% CF]	60	[FM + PP] 83	↔ Ast1, total protein and triglycerides **[Pla]** ↑ Cholesterol **[Pla]** ↓ Aat1 **[Pla]** ↔ ADC_{FA} ↑ Total FA, SFA, LA and n-6 PUFA **[WB]** ↓ OA, MUFA, ALA, EPA, DHA, n-3 PUFA and n-3/n-6 **[WB]** ↓ Total FA, SFA, OA, MUFA, ALA and EPA **[L]** ↔ LA, DHA, n-3 PUFA, n-6 PUFA and n-3/n-6 **[L]** ↓ Triglycerides **[L]** ↑ Neutral/polar lipids **[L]** ↔ *lxra, ppar-α, ppar-γ, srebp1, fas, cpt1a, acox* and *apob100* **[L]**	Belghit et al. (2018a)
S. salar	1398 [114 days]	*Hermetia illucens* larvae [partially defatted; 18% CF]	5, 10, 15	[FM] 33, 66, 100	↔ FBW, SGR, FCR, HSI and VSI ↔ $ADC_{CP, CF, AA, FA}$ ↔ Aat1, Ast1, glucose, FFA, triglycerides, cholesterol and total protein **[Pla]** ↔ Trypsin, bile acids and leucine aminopeptidase **[PI, MI, DI]** ↔ AA profile **[WB]** ↓ Total FA (66 and 100% FM replacement) **[WB]** ↓ OA, LA and n-6 PUFA **[WB]** ↓ ALA and MUFA (66 and 100% FM replacement) **[WB]** ↑ EPA, n-3 PUFA and n-3/n-6 (66 and 100% FM replacement) **[WB]** ↑ SFA and DHA **[WB]** ↔ Total lipids and lipid classes **[L]**	Belghit et al. (2019b)
S. salar	49 [56 days]	*Hermetia illucens* larvae [partially defatted; 22% CF]	60	[FM + PP] 83	↔ FBW, SGR and FCR ↑ HSI and VSI ↓ $ADC_{CP, CF, ash, AA}$ ↔ Trypsin and bile acids **[PI, MI, DI]** ↑ Leucine aminopeptidase **[DI]** ↓ Leucine aminopeptidase **[PI, MI]** ↔ DM, protein, fat and ash **[WB]**	Belghit et al. (2018b)

Table 1.3. (*Continued*).

Fish species	Fish IBW (g) [trial duration]	Insect species and stage of development [full-fat or defatted; % CF]	IM inclusion level (%)	[FM or PP] replacement level (%)	Main results of IM-based diets *versus* CTRL diet	Reference
S. salar	247 [105 days]	*Hermetia illucens* larvae [25.5% CF]	5, 10, 25	[FM] 25, 50, 100	↓ FBW (100% FM replacement) ↑ FCR (50 and 100% FM replacement) ↑ HSI and VSI (100% FM replacement) ↔ $ADC_{AA, FA}$ ↓ n-3/n-6 **[WB]**	Lock et al. (2016)
		Hermetia illucens larvae [17% CF]	5, 25	[FM] 25, 100	↓ FBW ↓ FCR (25% FM replacement) ↑ FCR (100% FM replacement) ↑ HSI, VSI (25 and 100% FM replacement) ↔ $ADC_{AA, FA}$ ↔ FA profile **[WB]**	
S. aurata	144 [180 days]	*Hermetia illucens* larvae [defatted; n.a]	8, 11, 16	[FM] 25, 35, 50	↔ FBW ↔ Moisture, protein, total lipids, ash, OA and ALA, TI, h/H **[M]** ↑ SFA and AI (50% FM replacement) **[M]** ↓ MUFA (50% FM replacement) **[M]** ↑ LA, n-6 PUFA (35 and 50% FM replacement) **[M]** ↓ EPA, n-3 PUFA and PUFA (25% FM replacement) **[M]** ↓ DHA, n-3/n-6	Oteri et al. (2022)
S. aurata	181 [144 days]	*Hermetia illucens* larvae [partially defatted; 21% CF]	9, 18, 28	[FM] 25, 50, 75	↔ L*, a* and b* **[M]** ↔ Total lipids, MUFA and n-3/n-6 **[M]** ↑ SFA and OA (50 and 75% FM replacement) **[M]** ↓ EPA, DHA and n-3 PUFA (50 and 75% FM replacement) **[M]** ↑ n-6 PUFA **[M]**	Pulido et al. (2022)
S. aurata	22 [45 days]	*Hermetia illucens* larvae [full-fat; 34% CF]	6, 11	[FM] 14, 30	↑ SFA, LA, AI and TI **[M]** ↔ OA, MUFA, ALA, DHA and PUFA **[M]** ↓ EPA (30% FM replacement) **[M]** ↓ n-3/n-6 **[M]**	Fabrikov et al. (2021a)
		Tenebrio molitor larvae [full-fat; 28% CF]	5, 11	[FM] 15, 30	↓ SFA, n-3/n-6 and AI **[M]** ↑ LA **[M]** ↔ MUFA, DHA and PUFA **[M]** ↓ ALA and EPA (30% FM replacement) **[M]** ↑ OA and TI (30% FM replacement) **[M]**	

Table 1.3. (*Continued*).

Fish species	Fish IBW (g) [trial duration]	Insect species and stage of development [full-fat or defatted; % CF]	IM inclusion level (%)	[FM or PP] replacement level (%)	Main results of IM-based diets *versus* CTRL diet	Reference
S. aurata	7 [32 days]	*Hermetia illucens* larvae [full-fat; 26% CF; reared on vegetable substrate]	11, 18	[FM] 30, 50	↓ FBW, FCR, HSI and VSI ↓ ADC_{CP} ↑ Moisture (30% FM replacement) **[M]** ↑ Ash (50% FM replacement) **[M]** ↔ Fat and protein **[M]** ↔ Pk, Fbpase, G6pdh, Aat1, Ast1 and Gdh **[L]** ↑ Alkaline protease, amylase (50% FM replacement) **[I]** ↓ Acid protease **[S]**	Fabrikov et al. (2021b)
		Hermetia illucens larvae [full-fat; 28% CF; reared on fish by-products]	18	[FM] 50	↓ FBW, FCR, HSI and VSI ↔ ADC_{CP} ↑ Moisture **[M]** ↔ Fat and protein **[M]** ↔ Pk, Fbpase, G6pdh, Aat1, Ast1 and Gdh **[L]** ↑ Alkaline protease **[I]** ↔ Amylase **[I]** ↓ Acid protease **[S]**	
		Tenebrio molitor larvae [full-fat; 27% CF]	18	[FM] 50	↓ FBW, FCR, HSI and VSI ↔ ADC_{CP} ↔ Moisture, protein, fat and ash **[M]** ↔ Pk, Fbpase, G6pdh, Aat1, Ast1 and Gdh **[L]** ↑ Alkaline protease **[I]** ↔ Amylase **[I]** ↓ Acid protease **[S]**	
S. aurata	49 [147 days]	*Hermetia illucens* pupae [partially defatted; 21% CF]	8, 16, 32	[PP] 12, 24, 47	↔ FBW, SGR, FCR and HSI ↔ pH, shear force, L*, a* and b* **[M]** ↑ SFA **[M]** ↓ ALA **[M]** ↔ OA, LA, MUFA, EPA, DHA, n-3 PUFA, n-6 PUFA, n-3/n-6 and LPO **[M]**	Pulido-Rodriguez et al. (2021)

Table 1.3. (*Continued*).

Fish species	Fish IBW (g) [trial duration]	Insect species and stage of development [full-fat or defatted; % CF]	IM inclusion level (%)	[FM or PP] replacement level (%)	Main results of IM-based diets *versus* CTRL diet	Reference
S. aurata	116 [90 days]	*Tenebrio molitor* larvae [full-fat; n.a]	5, 11	[FM] 33, 67	↑ Total Lys and Ala (67% FM replacement) **[M]** ↓ Total His and Phe **[M]** ↑ Free Arg, His and Gly **[M]** ↑ Free Leu, Phe, Val, Pro and EAA/NEAA (67% FM replacement) **[M]** ↓ Free *Tau* **[M]**	Iaconisi et al. (2019)
S. aurata	105 [163 days]	*Tenebrio molitor* larvae [full-fat; 24% CF]	25, 50	[FM] 33, 74	↔ FBW, SGR and FCR ↑ HSI and VSI ↓ $CTTAD_{DM, CP, CF}$	Piccolo et al. (2017)

The abbreviations stand for: a*, redness; AA, amino acids; *acox,* peroxisomal acyl-coenzyme A oxidase; ADC, apparent digestibility coefficient; AI, atherogenicity index; Ala, alanine; ALA, α-linolenic acid (C18:3n-3); Aat1, alanine aminotransferase; *apob100,* apolipoprotein B-100; Arg, arginine; Ast1, aspartate aminotransferase; Asn, asparagine; b*, redness; *cd36,* platelet glycoprotein 4; CF, crude fat; CP, crude protein; *cpt1a,* Carnitine palmitoyltransferase 1a; Cs, cysteine synthase; CTRL, control; CTTAD, coefficient of total tract apparent digestibility; Cys, cysteine; DHA, docosahexaenoic acid (C22:6n-3); DM, dry matter; EAA, essential amino acids; *elovl1,* elongation of very long chain fatty acid 1; *elovl2,* elongation of very long chain fatty acid 2; EPA, eicosapentaenoic acid (C20:5n-3); FA, fatty acids; *fads2,* fatty acid desaturase 2; Fas, fatty acid synthetase; Fbpase, Fructose 1,6-bisphosphatase; FBW, final body weight; FCR; feed conversion ratio; FFA, free fatty acids; FM, fishmeal; G6pdh, glucose-6-phosphate dehydrogenase; Gdh, Glutamate dehydrogenase; GE, gross energy; Gln, glutamine; Gly, lycine; GyK, glycerol kinase; h/H, hypo/hypercholesterolemic ratio; His, histidine; *hadh*, 3-hydroxyacyl-CoA dehydrogenase; HSI, hepatosomatic index; IBW, initial body weight; *igf-i,* insulin like growth factor I; Ile, isoleucine; IM, insect meal; L*, lightness; LA, linoleic acid (C18:2n-6); Ldh, lactate dehydrogenase; Leu, leucine; LPO, lipid peroxidation; *lxrα,* liver X receptor*α;* Lys, lysine; Me, malic enzyme; *mstn1α,* myostatin 1-*α;* MUFA, monounsaturated fatty acids; NEAA, non-essential amino acids; OA, oleic acid (C18:1n-9); Phe, phenylalanine; Pk, Pyruvate-kinase; PP, plant protein meal; *pparα,* peroxisome proliferator activated receptor-*α; pparβ,* peroxisome proliferator activated receptor-*β; pparγ,* peroxisome proliferator activated receptor-*γ; pparδ,* peroxisome proliferator activator receptor-*δ;* Pro, proline; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SGR, specific growth rate; *srebp1*, sterol regulatory element-binding protein 1; Tau, taurine; Thr, Threonine; TI, thrombogenicity index; Tyr, tyrosine; Val, valine; VSI, viscerosomatic index; WHC, water holding capacity.
The abbreviations in bold indicate the corresponding tissue of analysed parameters and stand for: DI, distal intestine; I, intestine; L, liver; MI, mid intestine; M, muscle; Pla, plasma; PC, pyloric caeca; PI, proximal intestine; S, stomach; WB, whole-body.
The symbols stand for: ↑ significantly higher than control diet; ↓ significantly lower than control diet; ↔ similar to control diet.

1.3. Nutritional quality of ingredients: from regulation of feed intake to fish flesh quality

Several authors have primarily evaluated the nutritional quality of new ingredients for aquafeeds through their impact on fish growth performance. Indeed, several strategies can be used to evaluate the nutritional quality of ingredients. However, and according to Glencross (2020): “the choice of strategies used can have a strong impact on the interpretation of that information”. Since the quality of an ingredient sequentially influences the growth performance of fish, it must be analysed hierarchically to define the nature of the responses (Figure 1.5) (Glencross, 2020).

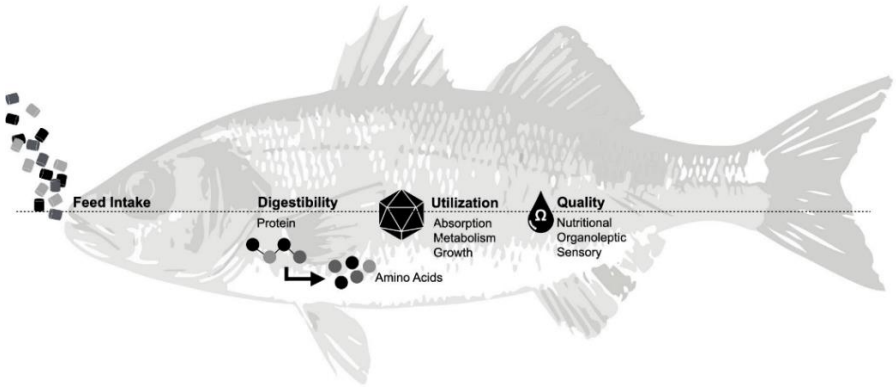

Figure 1.5. Schematic representation of hierarchical analyses of ingredients’ quality.

1.3.1. Central regulation of feed intake

First, to assess the impact of the dietary inclusion of an ingredient on fish growth, obviously, the animal must ingest the feed. Therefore, understanding the impact of an ingredient (e.g., IM) on the regulation of food/feed intake is crucial when assessing the quality of that ingredient. Food intake in mammals is regulated through positive and negative loops acting at three different temporary scales: (1) short-term regulatory level: when food intake is influenced by the size of a single meal; (2) mid-term regulatory level: when food intake is regulated over several days; (3) long-term regulatory level: when food intake is regulated over weeks, months or even years. The positive loop (the beginning of a meal) is triggered by the relationship among prior experience with nutrient availability, the sensory quality of food, physiological status of the animal, and environmental factors. On the other hand, the negative loop, which leads to the end of a meal, is related to metabolic and gastrointestinal signals displaying changes prior to and after nutrient absorption (Langhans, 1999; Schwartz et al., 2000). Similar loops of regulatory mechanisms are expected to be present in the regulation of feed intake in fish (Soengas et al., 2018).

The regulation of food/feed intake is framed commonly into both homeostatic (food/feed intake necessary to maintain body weight and metabolic function) and hedonic feeding (food/feed intake driven by sensory perception and/or pleasure). While the homeostatic circuits (Morton et al., 2014) receive energetic information from adipose tissue and/or satiety signals from the gastrointestinal system, the non-homeostatic circuits (Rossi and Stuber, 2018) receive sensorial and environmental information and convey data related to learning and previous experiences, hedonic, stress, and many other factors. Both homeostatic and non-homeostatic circuits have been partially characterized in fish with similarities and differences from those known in mammals (Delgado et al., 2017; Soengas et al., 2018). All this information determining food intake and feeding behaviour is integrated into the central nervous system, with the hypothalamus being the main centre involved in homeostatic regulation, whereas the corticolimbic system and nucleus accumbens are mainly involved in hedonic regulation (Figure 1.6). Nevertheless, recent findings suggest that both areas work in parallel (Berthoud et al., 2017; Rossi and Stuber, 2018). Two populations of neurons in these areas have specific receptors for peripheral hormones and mechanisms able to sense changes in nutrient levels. The first population co-expresses the orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP), which increase food intake. On the other hand, the second population co-expresses the anorexigenic pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), which decrease food intake (Blouet and Schwartz, 2010). Moreover, these two populations of neurons inhibit each other resulting in signalling to other higher-order neurons (Conde-Sieira and Soengas, 2017; Delgado et al., 2017; Soengas et al., 2018). Comparable integrative mechanisms have been partially characterized in fish in recent years (Soengas et al., 2018; Soengas, 2021).

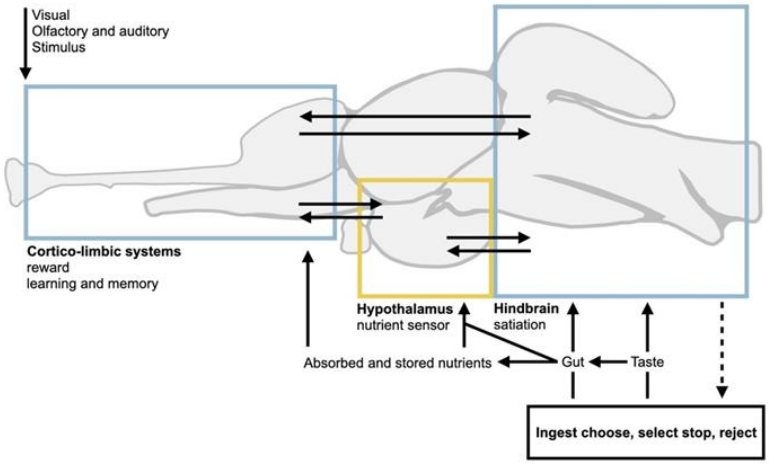

Figure 1.6. Schematic diagram showing interconnected major brain areas constituting the core processor for the control of ingestive behaviour and its relation to peripheral organs involved in energy homeostasis. Adapted from Berthoud et al., 2017.

1.3.2. Nutrients utilization

After intake, food/feed only has value if the animal can digest and absorb its nutrients for further metabolism and growth. The term digestibility is used for nutrients that undergo the digestive process prior to absorption, whereas bioavailable nutrients are those that are absorbed and can be used by the animal. Nutrients that are not absorbed are excreted as faeces (NRC, 2011). Thus, the quality of an IM as a feed ingredient depends not only on its nutritional characteristics but also on its nutrient's bioavailability.

In fish, the dietary proteins are hydrolysed to tri- or di-peptides and free AA (FAA) by digestive enzymes (i.e., proteases and peptidases) secreted into the gastrointestinal tract and absorbed predominantly in the anterior (or proximal) intestine. In the posterior or distal intestine, small proteins and peptides with nutritional and immunological importance can also be absorbed by endocytosis, but at a reduced rate. The tri- and di-peptides are transported from the intestinal lumen into the cytosol of enterocytes through the apical epithelial cells of brush border membrane (BBM), by the H^+-dependent symporters peptide transporter 1 and 2 (PepT1 and PepT2, respectively). On the other hand, FAA are transported either by Na^+-independent facilitated transport or by the Na^+-dependent active transport. In the cytosol of enterocytes, tri- and di-peptides are hydrolysed and the resulting AA, together with those previously absorbed, are released into the circulatory system throughout the enterocyte's basolateral transporters. The dietary AA present in the blood, together with those obtained either from *de novo* synthesis from other metabolic intermediates or from the breakdown of tissue proteins, originate the pool of FAA. Most of the AA present in this pool are directed to the liver through the hepatic portal vein, where they are catabolized (Ray and Ringø, 2014); others follow to the muscle where they are mainly used to synthesize enzymes or structural proteins, contributing to protein accretion, or serve as substrates for energy production (Valente et al., 2013).

The first step of AA catabolism consists of the separation of the amino group from the carbon skeleton (α-keto acid), by direct deamination or transamination pathway. The direct deamination only occurs for some AA (e.g., aspargine (Asn), Glu, glutamine (Gln), His, and Serine (Ser)) through the action of specific enzymes, whereas most AA follow the transaminase pathway. The transamination pathway is a reversible reaction catalysed by specific transaminases (also known as aminotransferases) that transfer the amino group to an α-keto acid (produced by deamination) to form a new AA. These AA give rise to pyruvate and/or intermediates of the tricarboxylic acid (TCA) cycle (also known as the Krebs or citric acid cycle) to be converted into energy-storage products, namely glucose/glycogen and FA, through gluconeogenic/glycogenic and lipogenic pathways,

respectively (Figure 1.7 and Figure 1.8) (Cowey and Walton, 1989; Médale and Guillaume, 1999). Leucine (Leu) and Lys are exclusively ketogenic, meaning that they are converted into acetyl-coenzyme A (CoA) or acetoacetyl-CoA and can be used as ketone body precursors. Isoleucine (Ile), phenylalanine (Phe), threonine (Thr), Trp, and tyrosine (Tyr) give rise to both glucose and ketone body precursors, thus they are both glucogenic and ketogenic (NRC, 2011). According to Engelking (2015), glutamate dehydrogenase (Gdh), alanine aminotransferase (Aat1), and aspartate aminotransferase (Ast1) are considered the most relevant enzymes of AA metabolism. The Gdh is a mitochondrial enzyme that reversibly converts Glu to α-ketoglutarate and NH_4^+; Ast1 and Aat1 are responsible for the transamination of Ala and Asp to Glu, respectively. Thus, the study of the activity of these enzymes in the liver of fish fed new dietary ingredients provides valuable information about the impact of such new ingredients on AA metabolism.

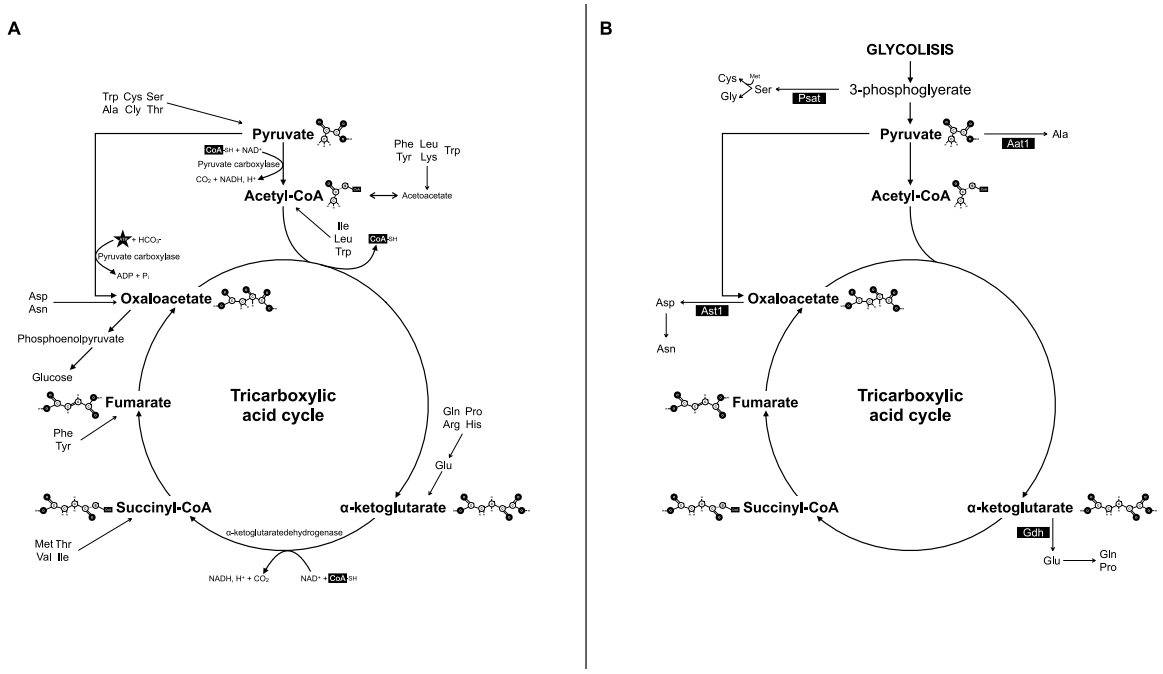

Figure 1.7. Tricarboxylic acid (TCA) cycle intermediates formed following amino acids catabolism (A) and biosynthesis (B) of essential amino acids from TCA cycle intermediates. Aat1, alanine aminotransferase; ADP, adenosine diphosphate; Ala, alanine; Arg, arginine; Ast1, aspartate aminotransferase; Asn, asparagine; Asp, aspartate; CO_2, carbon dioxide; CoA-SH, coenzyme A with sulphydryl functional group; Cys, cysteine; Gdh, glutamate dehydrogenase; Gln, glutamine; Glu, glutamate; Gly, glycine; H^+, hydrogen proton; His, histidine; Iso, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; NADH, nicotinamide-adenine dinucleotide reduced form; Phe, phenylalanine; Pro, proline; Psat, phosphoserine aminotransferase; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine. Adapted from Engelking, 2015.

In fish, as in other vertebrates, the longevity of body proteins is dependent on the continuous renewal and repair of protein structures, through the sequential cycling of its synthesis (i.e., anabolism) and degradation (i.e., catabolism), known as “protein turnover” (Konrad and Guderley, 2003). This process of protein turnover is tissues specific, e.g., in fish liver and gastrointestinal tract, more than 90% of proteins are catabolized and returned to the FAA pool, whereas in muscle only 30-50% of proteins synthesized are turned over and the remaining 50-70% are retained. But overall, 40-50% of fish proteins are catabolized and return to the pool of FAA to be reused for the synthesis of new proteins (Cowey and Luquet, 1983). Fish growth is fundamentally regulated by the rates of protein synthesis and protein degradation in muscle fibres. Protein synthesis is promoted through the activation of the phosphoinositide 3-kinases/protein kinase B/target of rapamycin (PI3K/AKT/TOR) and growth hormone/insulin-like growth factor (GH/IGF) pathways. Protein degradation is mediated by (1) calpains; (2) caspases; (3) lysosomes; and (4) the ubiquitin-proteasome system. The coordinated protein synthesis and degradation induces protein deposition and consequently fish growth. Fish growth is highly plastic and implies adaptive responsiveness in the dynamics of myotomal muscle (favouring hyperplasia vs hypertrophy of existing fibres) to changing environmental factors such as temperature and food supply, and has considerable intra- and interspecific variation (Valente et al., 2013).

European sea bass, like other animals, does not have a true protein requirement but requires a well-balanced mixture of essential and non-essential AA (NEAA). The amino acids that fish cannot synthesize at all or that are incapable of synthesizing in adequate quantities, and must be supplied by the diet, are referred as EAA. In contrast, the NEAA are efficiently synthesized *de novo* by the organism from precursors, such as an EAA and an α-keto acid as abovementioned, and therefore do not need to be supplied by the diet. Among the amino acids that are synthesized from other EAA, some are considered conditionally essential amino acids (CEAA), if under some circumstances their metabolic demand may rise above the biosynthetic capacity of the organism (NRC, 2011). The ten EAA for European sea bass, as well as CEAA and NEAA are represented in Table 1.4. It is worth noting that despite *Taurine* (*Tau*) being often referred as a CEAA, this is not truthful. *Tau* is an organic acid derived from cysteine, but it does not have a carboxyl group (NRC, 2011).

Table 1.4. Nutritionally essential (EAA), conditionally essential (CEAA), and non-essential amino acids (NEAA) for European sea bass.

EAA	CEAA*	NEAA
Arginine	Cysteine	Alanine
Histidine	Glutamine	Asparagine
Isoleucine	Hydroxyproline	Aspartate
Leucine	Proline	Glutamate
Lysine	Tyrosine	Glycine
Methionine	*Taurine*	Serine
Phenylalanine		
Threonine		
Tryptophane		
Valine		

*Depending on diet formulation, physiological and environmental factors. Adapted from NRC (2011).

Quantification of EAA requirements is generally established by dose-response curves with weight gain used as a response criterion, i.e., the minimum dietary requirement of an EAA is identified when the lowest level of such EAA guarantees the maximum growth of fish. However, quantitative data for amino acid requirements of European sea bass juveniles estimated based on dose-response is only available for Arg, Lys, Met, Thr, Trp, and *Tau* (Table 1.5). For other amino acids, indirect estimations were made based on the ideal protein concept, which states that even though the requirement of individual amino acids may vary at different stages of development, the ratio between EAA and NEAA (A/E ratio) remains constant (Kaushik et al., 1998).

Table 1.5. Amino acids requirements (% dietary protein) of European sea bass.

Amino acid	Requirement	Method	Reference
Arginine	3.9	Dose-response (broken-line model)	Tibaldi et al. (1994)
Histidine	1.6	Ideal protein (whole-body composition)	Kaushik (1998)
Isoleucine	2.6	Ideal protein (whole-body composition)	Kaushik (1998)
Leucine	4.3	Ideal protein (whole-body composition)	Kaushik (1998)
Lysine	4.8	Dose-response (broken-line model)	Tibaldi and Lanari (1991)
Methionine	2.7	Dose-response (broken-line model)	Tulli et al. (2010)
Phenylalanine + Tyrosine	2.6	Ideal protein (whole-body composition)	Kaushik (1998)
Threonine	2.7	Dose-response (broken-line model)	Tibaldi and Tulli (1999)
Tryptophane	0.6	Ideal protein (whole-body composition)	Kaushik (1998)
Valine	2.9	Ideal protein (whole-body composition)	Kaushik (1998)
Taurine	0.5	Dose-response (saturation kinetic model)	Martins et al. (2018)

Lipids, along with proteins, are the major organic constituents of fish (Tocher, 2003). The dietary lipids are hydrolysed along the gastrointestinal tract by digestive enzymes and absorbed predominantly in the anterior intestine. In fish, the digestion of lipids in the anterior intestine involves three key constituents: bile acid salts, pancreatic lipases (triacylglycerol hydrolases), and carboxyl ester lipases. Bile acid salts are metabolized from cholesterol in the liver by up to 16 enzymes, with cholesterol 7α-hydroxylase (Cyp7a1) being the first rate-limiting enzyme on their "classic biosynthesis pathway". Then, they are stored in the gallbladder and further released in the intestinal lumen when reduced due to the increased circulating levels of cholecystokinin. Once in the intestinal lumen, bile acid salts act as surfactant by emulsifying lipids into micelles, enhancing the digestion of lipids by lipases (Romano et al., 2020). Pancreatic lipases predominantly hydrolyse triacylglycerols (TAG), whereas carboxyl ester lipases can hydrolyse different lipid classes (i.e., tri-, di- and monoacylglycerols, phosphoacylglycerols, wax esters, ceramides, among others). Furthermore, the presence of phospholipase A_2, which hydrolyse phosphoacylglycerols, has also been reported in fish, but its role still is under debate (Tocher, 2003). The products resulting from lipids hydrolysis (i.e., mostly free fatty acids (FFA) and to a lesser extent di- and monoacylglycerols, glycerol, and cholesterol, among others) are mainly transported from the intestinal lumen into the cytosol of enterocytes by passive diffusion, and some FFA, monoacylglycerols, and cholesterol are transported through protein transporters (Corraze, 2001). In the endoplasmic reticulum of enterocytes, FFA and monoacylglycerols are re-esterified into TAG and phosphoglycerides by glycerol-3-phosphate and monoacylglycerol pathways (Oxley et al., 2005; Oxley et al., 2007). On the hand, the cholesterol is easily transported by passive diffusion from enterocytes but can also be esterified into cholesteryl ester by the action of Acyl-CoA cholesterol acyltransferase. Afterward, the re-esterified TAG and phospholipids and the esterified cholesteryl esters are predominantly integrated into TAG-rich chylomicrons and in a smaller degree in very-low-density lipoproteins (VLDL), depending on the degree of unsaturation of dietary lipids. High amounts of dietary PUFA lead to the production of TAG-rich chylomicrons, whereas high amounts of dietary SFAs result in smaller VLDL. In fish, despite most of the intestinal chylomicrons and VLDL being secreted by exocytosis to the lymphatic system before reaching the circulatory system and being delivered to the liver, they can also be transported directly to the liver via the portal vein. In addition to the VLDL of intestinal origin, teleost fish also have VLDL particles of hepatic origin and the main difference between them is that the first are mainly formed by dietary lipids, whereas the seconds are predominantly formed by lipids synthetized endogenously (i.e., lipids synthetized *de novo*) (Corraze, 2001; Tocher, 2003). Once in the circulatory system, both VLDL and

chylomicrons suffer the action of lipoprotein lipase and lecithin-cholesterol-acyl-transferase, resulting in low-, intermediate- and high-density lipoproteins (LDL, IDL, and HDL, respectively). The HDL are the principal class of lipoproteins present in the plasma of fish, which suggests a key role in the transport of FA to peripheral tissues, where they can be used for energy production or storage (Turchini et al., 2009).

In marine fish species, such as European sea bass, the liver and viscera are the main tissues for lipid storage, whereas in salmonids muscle is the main target. Despite muscle being the major site of FA catabolism, since it comprises about 60% of the fish body, the liver is the organ with the highest β-oxidation capacity (Glencross, 2009). The β-oxidation of FA occurs in both mitochondria and peroxisomes through identical sequential chemical reactions (i.e., dehydrogenation, hydration, dehydrogenation again, and thiolytic cleavage) by which FA are sequentially oxidized into acetyl-CoA. Acetyl-CoA can be further used for other metabolic purposes or energy production via the TCA cycle, through the production of ATP (Figure 1.8) (Tocher, 2003). The net production of adenosine triphosphate (ATP) is 16 ATP molecules per oxidation of each acetyl-CoA unit, which turns this metabolic process the most energy productive of all cellular processes (Glencross, 2009). Although the β-oxidation mechanisms in mitochondria and peroxisomes are chemically similar, there are five major differences between them: (1) the chemical reactions are catalysed via a completely different set of enzymes; (2) mitochondrial enzymes catalysing the first step of β-oxidation are flavin adenine dinucleotide- (FAD) dependent dehydrogenases, whereas the corresponding peroxisomal enzymes are FAD-dependent acyl-CoA oxidases donating their electrons directly to molecular oxygen (O_2); (3) FA are transported across the peroxisomal membrane as acyl-CoAs, or as FFA, whereas before entering mitochondria fatty acyl-CoA are conjugated with carnitine by the action of carnitine palmitoyltransferase 1 (Cpt1) forming fatty acylcarnitine. Fatty acylcarnitine is then translocated into mitochondria and a reverse reaction mediated by carnitine palmitoyltransferase 2 (Cpt2) occurs with the release of fatty acyl-CoA; (4) mitochondria can completely oxidize FAs to CO_2 and H_2O, whereas peroxisomes can only partially oxidize FA to acetyl-CoA, propionyl-CoA, and different medium-chain acyl-CoA; (5) carnitine does not play a role in the uptake of FA into peroxisomes but is required for the transport of the end products of peroxisomal β-oxidation to mitochondria for full oxidation (Tocher, 2003; Glencross, 2009; Wanders et al., 2015).

Since there is no described mechanism for the long-term storage of protein and carbohydrates in animals, when they are included at high levels in diets, they can be converted into lipids before storage in adipocytes through *de novo* synthesis of FA, also

known as lipogenesis (Figure 1.8) (Corraze, 2001; Tocher, 2003; Glencross, 2009). Lipogenesis in fish, contrary to mammals, occurs predominantly in the liver, instead of adipose tissue. Lipogenesis is initiated by the conversion of citrate into acetyl-CoA, and oxaloacetate through the action of ATP citrate lyase (Acly). Afterward, occurs the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase and the final step of lipogenesis is characterized by the action of FA synthetase (Fas) under malonyl-CoA, giving rise to myristic acid (C14:0), PA (C16:0) or SA (C18:0) (Figure 1.8)(Corraze, 2001; Tocher, 2003; Glencross, 2009). In this process, a considerable amount of nicotinamide-adenine dinucleotide phosphate reduced form (NADPH) is required for reducing power, and it is supplied by the action of dehydrogenases of the pentose phosphate pathway (PPP), namely glucose-6-phosphate dehydrogenase (G6pdh), malic enzyme (Me) and nicotinamide-adenine dinucleotide phosphate oxidized form ($NADP^+$) dependent isocitrate dehydrogenase ($NADP^+$-Idh). The activity of these NADPH-forming enzymes is thought to vary between species according to their nutritional and hormonal conditions. Nonetheless, in the case of European sea bass, NADPH reducing equivalents are mainly provided by G6pdh, regardless of their nutritional and hormonal state (Figure 1.8) (Dias et al., 1998; Castro et al., 2015).

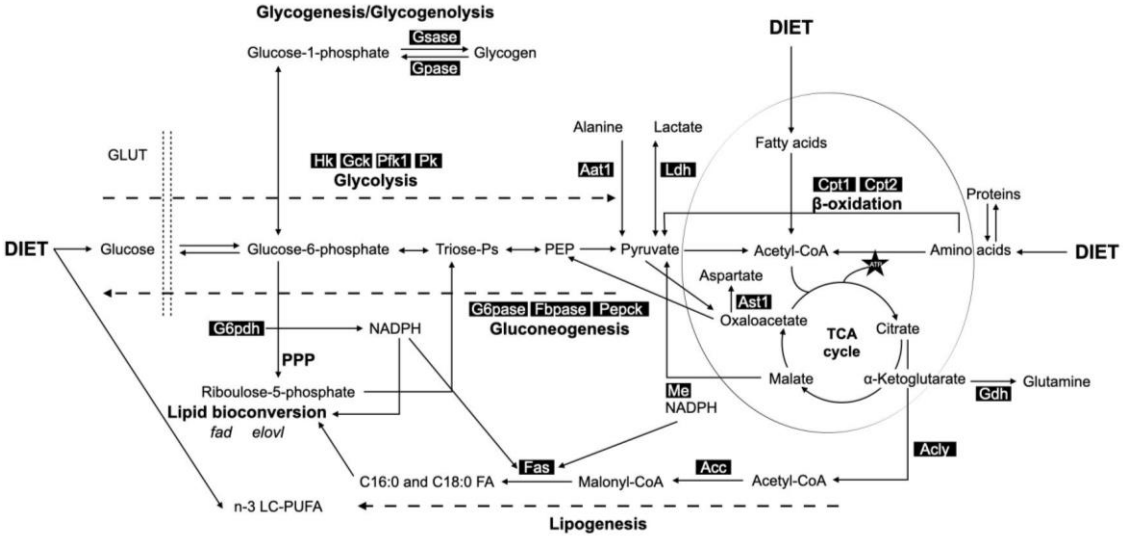

Figure 1.8. Schematic representation of the major metabolic pathways involved in intermediary metabolism. The target metabolic enzyme markers of the glycolytic, gluconeogenic, glycogenesis, glycogenolysis, fatty acid, and amino acid synthesis, pentose phosphate (PPP), oxidation, and bioconversion pathways are represented: Aat1, alanine aminotransferase; Acly, adenosine triphosphate citrate lyase; Acc, acetyl CoA carboxylase; Ast1, aspartate aminotransferase; Cpt1 and Cpt2, carnitine palmitoyl transferase-1 and -2, respectively; Elovl, elongases; Fad, fatty acyl desaturases; Fas, fatty acid synthase; Fbpase, fructose-1,6-bisphosphatase; GcK, glucokinase; Gdh, glutamate dehydrogenase; G6pase, glucose-6-phosphatase; Gpase, glycogen phosphorylase; G6pdh, glucose-6-phosphate dehydrogenase; Gsase, glycogen synthase; Ldh, lactate dehydrogenase; Me, malic enzyme; Pepck, phosphoenolpyruvate carboxykinase; Pfk1, 6-phosphofructo 1-kinase; Pk, pyruvate-kinase.

Fish, like other vertebrates, can convert the short-chain SFA obtained through lipogenesis into longer and unsaturated FA, such as palmitoleic acid (C16:1n-7) and oleic acid (C18:1n-9) by the action of fatty acyl desaturases (Fad). Nonetheless, they cannot biosynthesize PUFA *de novo*, and therefore they are essential dietary nutrients. However, some fish species can convert LA (C18:2n-6) and ALA (C18:3n-3) into LC-PUFA by successively desaturating and elongation reactions catalysed by Fad and proteins for elongation of very long-chain FA (Elovl), respectively, as presented in Figure 1.9. In general, freshwater fish species have a higher ability for conversion of C_{18} PUFA to LC-PUFA than marine fish species, mainly due to the limited mRNA abundance of *Fad5*, *Fad6,* and *Elovl2* genes in marine fish species (Tocher, 2010; Monroig et al., 2018). Available data regarding quantitative n-3 LC-PUFA requirements for European sea bass still are limited, but the NRC (2011) recommends that aquafeeds for European sea bass juveniles must contain 1% (on a DM basis) of n-3 LC-PUFA, with at least 0.5% of DHA. Nonetheless, these values are based on the results obtained by Coutteau et al. (1996), where only two different levels of LC-PUFA were studied, namely 1 and 2.5% (on a DM basis). According to the study of Skalli and Robin (2004), where six dietary levels of LC-PUFA were tested (0.2-1.9% DM), it was possible to conclude that European sea bass juveniles (14-27 g) require at least 0.7% (on a DM basis) of n-3 LC-PUFA and a DHA:EPA ratio of 1.5:1. However, Huston (2018) demonstrated that the optimum dietary level of EPA+DHA for European sea bass juveniles with 24-80 g is 1.3-1.5% (as feed basis), whereas fish with 80-200 g require 1.1-1.2% of EPA+DHA (as feed basis). The ideal DHA:EPA ratio for European sea bass was not assessed by Huston (2018).

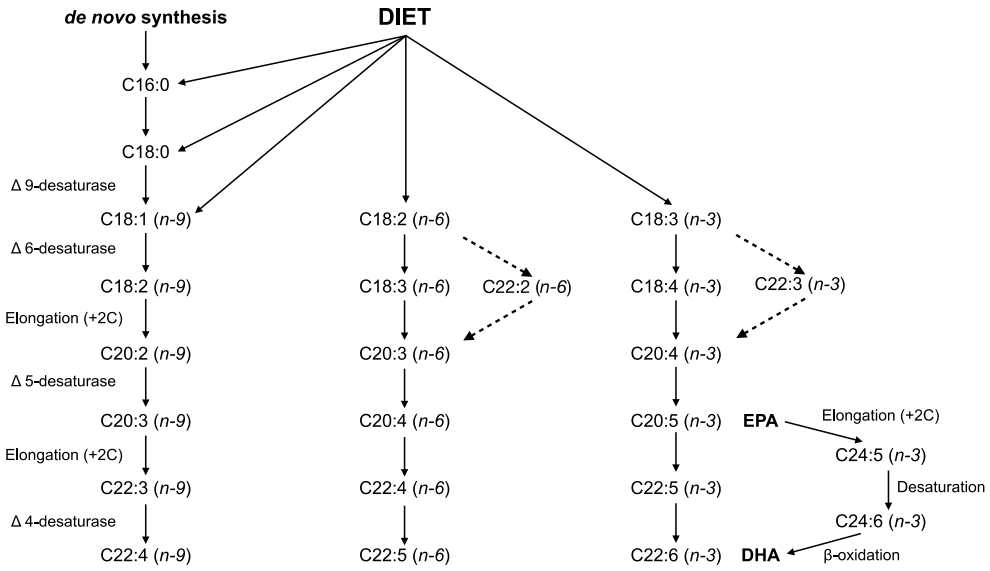

Figure 1.9. Bioconversion of fatty acids: elongation and desaturation of n-9, n-6 and n-3 PUFA. Adapted from Monroig et al., 2018.

Despite all fish species having enzymatic mechanisms for the hydrolysis and absorption of simple and complex carbohydrates, irrespective of their feeding habits and digestive tract anatomy, the herbivorous and omnivorous fish species use these nutrients more efficiently than carnivorous fish species. The great capacity of herbivorous and omnivorous fish species to use carbohydrates is not only a consequence of their singular intestinal morphology and microbiota but also their higher activity of pancreatic α-amylase and BBM disaccharidases (Krogdahl et al., 2005). The pancreatic α-amylase hydrolyses α-1,4-glycosidic bonds of glycogen and starch into low molecular-weight oligosaccharides, which are further hydrolysed by disaccharidases into monosaccharides (mainly glucose). On the other hand, either herbivorous, omnivorous, or carnivorous fish have limited capacity to hydrolyse β-glycoside linkages due to the limited or even absence of specific enzymes, such as chitinases and cellulases. Despite some activity of these two enzymes (chitinase and cellulase) having been detected in some fish species, whether such enzymes are produced by fish, obtained through feed, or produced by gut microbiota is still a matter of debate (Stone, 2003).

Glucose and other monosaccharides are absorbed and released by cells (i.e., enterocytes, hepatocytes, adipocytes, etc.) predominantly through Na^+-non-dependent glucose transport proteins (GLUT) and Na^+-dependent glucose transporters (SGLTs). After feeding, glucose is completely catabolized by glycolysis, the TCA cycle, and the respiratory chain for ATP production or through the PPP leading to the production of NADP. When in high levels, glucose may be converted into glycogen (glycogenesis), through the action of glycogen synthase (Gsase), or into lipids (lipogenesis). On the other hand, under food deprivation conditions, glucose requirements can be satisfied by glycogenolysis, through the action of glycogen phosphorylase (Gpase), or by *de novo* glucose synthesis, also known as gluconeogenesis (Figure 1.10) (Polakof et al., 2012).

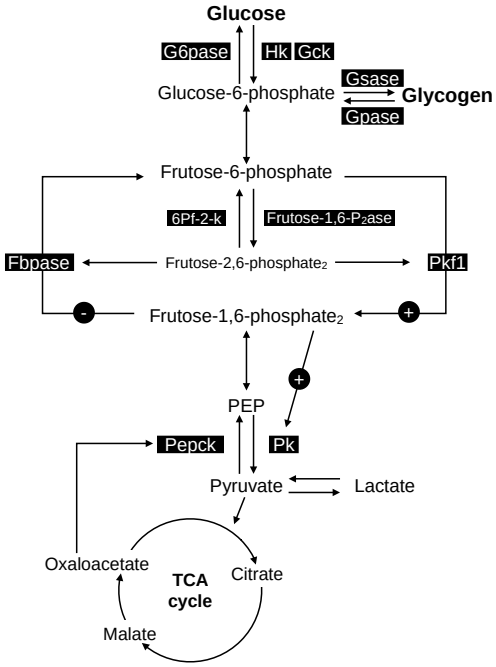

Figure 1.10. Glycolysis and gluconeogenesis pathways. The key enzymes of the glycolytic pathway are: Gck, glucokinase; Pfk1, 6-phosphofructokinase; Pk, pyruvate-kinase. The key gluconeogenic enzymes are: Pepck, phosphoenolpyruvate carboxykinase; Fbpase, fructose-1,6-bisphosphatase; G6pase, glucose-6-phosphatase. The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF-2-K/Fru-2,6-P2ase) is also represented. PEP means phosphoenolpyruvate. Adapted from Enes et al., 2009.

Glycolysis is a 10-step pathway, where one molecule of glucose yields two molecules of pyruvate, two molecules of ATP, two molecules of NADH, and two molecules of H_2O. Under anaerobic conditions, which prevail in the white muscle, pyruvate is converted into lactate via lactate dehydrogenase (Ldh), whereas under aerobic conditions pyruvate is converted into acetyl-CoA by the mitochondrial pyruvate dehydrogenase complex. Acetyl-CoA can be completely oxidized to produce ATP, through the TCA cycle or used as a substrate for lipogenesis. Glycolysis and gluconeogenesis share several enzymes that catalyse reversible reactions. However, three reactions are considered irreversible and different enzymes are used. The first step of glycolysis (i.e., conversion of glucose into glucose-6-phosphate) is catalysed by glucokinase (Gck), only present in the liver, or hexokinases (Hk). The other crucial steps in glycolysis are the phosphorylation of fructose 6-phosphate into fructose-1,6-bisphosphate by phosphofructokinase-1 (Pfk1) and the conversion of phosphoenolpyruvate into pyruvate by pyruvate-kinase (Pk). These three enzymes catalyse the limiting and unidirectional steps of glycolysis. On the other hand, phosphoenolpyruvate carboxykinase (Pepck), fructose-1,6-bisphosphatase (Fbpase), and glucose-6-phosphatase (G6pase) are the rate-limiting enzymes of gluconeogenesis. Pepck catalyses the conversion of oxaloacetate into phosphoenolpyruvate; Fbpase, hydrolyse fructose-1,6-bisphosphate into fructose-6-

phosphate; and is responsible for dephosphorylation of glucose-6-phosphate into glucose (Krogdahl et al., 2005; Enes et al., 2009; Polakof et al., 2012).

In conclusion, understanding the impact of new ingredients on fish metabolism is crucial for the formulation of strategic diets capable of providing all essential nutrients to the fish and contributing to precision nutrition (Zhang et al., 2020).

1.3.3. Flesh quality for human consumption

The quality of fish is a complex concept to define, but it is globally accepted that it is related to the attributes that determine the value of fish products and consumers' expectations in relation to these attributes. In the field of Food Science, nutritional value, organoleptic or sensory attributes and freshness are the most important characteristics for evaluating fish quality. These characteristics are strongly dependent on fish chemical composition, which is in its turn depend on endogenous (i.e., species, size, etc.) and exogenous factors (i.e., diet, temperature, salinity, etc.) (Matos et al., 2016). The nutritional quality of fish for human consumption is particularly attributed to its unique n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) content, predominantly EPA and DHA, due to their recognised health associated effects (EFSA, 2010). In fact, dietary recommendations for EPA and DHA based on cardiovascular risk considerations for European adults are between 250 and 500 mg day-1 according to the European food safety authority (EFSA, 2012). The fish organoleptic properties are generally associated with visual appearance, odour, taste, and texture. These properties are sensory or instrumentally evaluated and used to determine freshness, which is a meta-attribute that reflects physical, chemical, biochemical, and microbiological changes that occurred since slaughter. The fish visual appearance is characterized by external characteristics (i.e., shape, eye and gills coloration, and the presence of species-specific marks) or fillet colour and has a pivotal role in product freshness determination and consequently with its marketability (Matos et al., 2016). Odour and taste sensory information result from chemoreception mediated through gustative or olfactory receptors, respectively. Odour is also directly associated with fish freshness and marketability. The odour of fresh marine fish is simple and generally composed of alcohols, aldehydes, and ketones derived from PUFA mainly by lipoxygenases activity (Grigorakis, 2007). Texture has a critical role in determination of the fish ultimate quality and acceptability by consumers and depends on physical properties (i.e., fish species, size, rearing conditions, etc.), chemical composition (e.g., water, protein, fat, and collagen content) and other factors (i.e., storage time, slaughter method, processing methods, etc.). Hardness or firmness, adhesiveness, springiness, cohesiveness, chewiness, and resilience have been commonly used to

characterize the textural profile of a food product, being the hardness the most relevant trait associated with the freshness and acceptability of fish (Cheng et al., 2014). Several studies have demonstrated a direct relationship between hardness and muscle cellularity (i.e., the number, diameter, and type of fibres, their distribution, and density of fibres) (Periago et al., 2005; Ayala et al., 2010; Johnston et al., 2011). Muscle tissue with a higher number of small-sized fibres presents higher fibre density and more intramuscular collagen, resulting in harder texture (Periago et al., 2005).

1.4. Objectives of the thesis

Since little is still known about the feasibility of using IM as an FM substitute in aquafeed for European sea bass, this thesis hypothesized that if an IM was included in a well-balanced diet, FM could be replaced in diets for this species without affecting feed intake, digestibility, and metabolism, resulting in high fish growth and flesh nutritional value. Thus, the present thesis aimed to test the previously mentioned hypothesis through a multidisciplinary approach including conventional and cutting-edge methodologies. Specific objectives included:

1. Identification of the IM with the highest nutrient bioavailability for European sea bass through a deep characterization of the nutritional value of IMs available in the market, either full fatted or defatted, and *in vivo* determination of their bioavailability.

2. Identification of the maximum dietary FM possible to replace by the selected IM through evaluation of feed intake regulation, digestion capacity, intermediary metabolism, and flesh nutritional value.

3. Assessment of the impact of feeding IM on market-sized seabass flesh nutritional, organoleptic, and sensory quality for human consumption.

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Chapter 2.

Nutritional value of different insect larvae meals as protein sources for European sea bass (*Dicentrarchus labrax*) juveniles

by

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Abstract

Apparent digestibility coefficients (ADCs) of five commercially available insect larvae meals, *H. illucens* (HI and HI*d*), *T. molitor* (TM and TM*d*) and a locust meal (LM), obtained from different producers, were assessed in European sea bass (*Dicentrarchus labrax*) juveniles. Each experimental diet was obtained by replacing 20% of a commercial-based diet used as reference (DREF), with 1% of chromic oxide added, by a test ingredient. Each diet was fed to quadruplicate groups of fish (initial weight: 33 ± 1 g) and, after an acclimatization period, daily collection of faeces was performed for 12 days. Dry matter ADC was highest in TM (85%) and lowest in LM (40%). Protein ADC was high in TM*d* (93%); intermediate in HI*d* and TM (87 and 89%, respectively); and moderate in LM and HI (74 and 76%, respectively). Overall, the ingredient with highest digestible total essential amino acids (EAAs) content was TM*d*, followed by TM which was higher than HI*d*; HI had a moderate amount of digestible EAAs, and LM had the lowest values. Fat ADC was highest for HI*d*, TM*d* and TM (93%–95%). Energy and phosphorus ADC were highest in TM*d* (84% and 91%, respectively) and lowest in LM (53% and 27%, respectively). In conclusion, the high protein level, high protein digestibility and high amount of digestible EAAs of TM and TM*d* (> 89%) suggests that *Tenebrio molitor* larvae meals are the most promising protein sources tested for European sea bass, with potential to replace fishmeal without impairing nutrient digestibility.

Keywords: *Hermetia illucens*; insect meal; insect digestibility; sustainable protein sources; *Tenebrio molitor.*

2.1. Introduction

European sea bass (*Dicentrarchus labrax*) is one of the most important fish species in Mediterranean aquaculture, having reached its highest production level in the European Union in 2016, with 82.000 tonnes (EUMOFA, 2018). It is a carnivorous fish with a high protein requirement ranging from 45 to 55%, depending on the stage of development (NRC, 2011). Diets for European sea bass still rely on marine ingredients such as fishmeal (FM) due to its well-balanced essential amino acid (EAA) profile, excellent palatability and high protein digestibility (Jasour et al., 2018). However, the fast grow of aquaculture (FAO, 2018) is challenging the industrial and scientific community to reduce pressure on fisheries and find eco-friendlier alternatives to fish-based ingredients (Campos et al., 2017; Torrecillas et al., 2017; Glencross et al., 2019; Reis et al., 2019; Valente et al., 2019). The inclusion of insect protein in aquafeeds was authorized by the European Union (EU) in 2017 (EC Regulation 893/2017; European Commission, 2017), rising the interest in such protein sources. Insects have several advantages when compared with other conventional feed resources, as they grow fast, reproduce easily, have low feed conversion ratios, have no need for arable land and may require little water and energy, depending on their life stage and substrate used to feed and breed them (Henry et al., 2015; Bosch et al., 2019). Furthermore, some insects may be raised on bio-wastes contributing to a circular economy. The nutritional composition of insects differs according to species, life stage, rearing conditions and substrates. The generality of the insect species has high protein content (up to 70% on dry matter (DM) basis) and well-balanced EAA profile; they can also be rich sources of fat (10-50% DM), vitamins, and minerals such as potassium, calcium, iron, magnesium, and selenium (Makkar et al., 2014; Payne et al., 2016; Nogales-Mérida et al., 2018; Koutsos et al., 2019). The quality of insect meals (IMs) as feed ingredient depends not only on their nutritional characteristics, but also on their nutrient's bioavailability. Some species have such high fat contents that often require technological processing methods for defatting by applying solvents, such as petroleum ether, or mechanical pressure. These processes can further increase IM protein content through generation of different combinations of free amino acids (AAs) and decrease the excess of undesired fat (Choi et al., 2017). *Hermetia illucens* and *Tenebrio molitor* larvae meal (HI and TM, respectively) are currently the most frequently IMs used for animal feed, because they are easy to breed and feed, and have the capacity to recycle nutrients from manure and organic wastes into biomass (Varelas, 2019). For these reasons, standard mass-rearing techniques for industrial production of these species are already available (van Huis and Oonincx, 2017). HM protein content can vary from 31 to 59% DM, with an amino acid profile particularly rich in lysine; its fat

content depends on the type of diet supplied and can range from 28 to 49% DM (Makkar et al., 2014). TM protein content ranges between 44 and 70% DM and is rich in isoleucine, leucine and lysine, whilst its fat content varies between 23 and 47% DM (Ravzanaadii et al., 2012). Beyond their great nutritional characteristics and sustainable nature, both HI and TM are amongst the few insect species recently authorized by EU for feeding aquaculture animals (EC Regulation 893/2017; European Commission, 2017). Locusts are one of the insect species most consumed by humans worldwide, but have also been used either as complementary protein source for poultry or as fish bait (van Huis et al., 2013). They have a widely variable protein (29-70% DM) and fat content (4 to 22% DM) (Makkar et al., 2014; Paul et al., 2016). However, locust meal utilisation as FM substitute in aquaculture diets has still been poorly studied, and no information is available on its use in diets for marine fish species (Alegbeleye et al., 2012; Grace, 2015).

In freshwater species and salmonids, several studies have already evaluated the inclusion of insects as alternative protein sources to FM (Bondari and Sheppard, 1981; Ng et al., 2001; St-Hilaire et al., 2007; Belforti et al., 2016; Renna et al., 2017; Mancini et al., 2018; Rema et al., 2019; Bruni et al., 2020). In marine fish species, the suitability of full-fat TM, as partial substitute of FM, was evaluated in diets for European sea bass (Gasco et al., 2016; Henry et al., 2018), gilthead sea bream (*Sparus aurata*) (Piccolo et al., 2017) and black spot sea bream (*Pagellus bogaraveo*) (Iaconisi et al., 2017). Magalhães et al. (2017) tested the partial substitution of FM by defatted *Hermetia illucens* pre-pupae meal in diets for sea bass and de Haro et al. (2016) tested the partial substitution of FM by *Lucilia sericata* in diets for gilthead seabream. However, all these studies were mainly focused on the dietary impact of IM on fish growth performance and feed utilisation, paying little attention to the nutrient bioavailability of such new feedstuffs. The bioavailability of macronutrients and EAAs in commercially available insect meals *per se* is of major importance to formulate balanced diets, but still is largely unknown for most fish species. Recently, Fontes et al. (2019) have evaluated the digestibility of *Nauphoeta cinerea*, *Zophobas morio*, *Gromphadorhina portentosa*, *Gryllus assimilis*, and *Tenebrio molitor* in Nile tilapia (*Oreochromis niloticus*), and Mo et al. (2019) evaluated the digestibility of maggot meal in Mandarine fish (*Siniperca chuatsi*).

This study aimed to assess the *in vivo* apparent digestibility coefficient (ADC), in European sea bass, of five different commercially available insect larvae meals *H. illucens* (HI and HI*d*), *T. molitor* (TM and TM*d*) and a locust meal (LM) obtained from different producers.

2.2. Materials and methods

The study was directed by accredited scientists in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGAV-Portugal, following FELASA category C recommendations) and conducted according to the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals for scientific purposes.

2.2.1. Ingredients and experimental diets

Five different commercially available insect larvae meals *H. illucens* (HI and HI*d*), *T. molitor* (TM and TM*d*) and a locust meal (LM) were used as test ingredients (Table 2.1). Due to the large variety of ingredients and/or products in the market, two *H. illucens* and two *T. molitor* meals, from different producers, were selected based on their technical files, in particular the fact of being either defatted (HI*d* and TM*d*) or full fat meals (HI and TM). Locust meal could only be obtained in its full fat form. Proximate composition and amino acid profile of each test ingredient is presented in Table 2.1. An anchovy fishmeal (FM) was also included in the table for better comparison of the ingredients' nutritional value (NRC, 2011). A fish meal-based diet with 48% crude protein and 20% crude fat (Table 2.2) was formulated and extruded by SPAROS Lda. (Portugal) and used as reference diet (DREF). To this reference diet, 1% chromic oxide (Cr_2O_3; Merck KGaA, Germany) was added as inert marker for the evaluation of apparent digestibility coefficients (ADC). Five other experimental diets (D-) were obtained by mixing 80% of DREF and 20% of each test ingredient. The insect inclusion level of 20% was selected due to the high fat content of some insects (e.g. HI and LM) and to avoid technological difficulties during extrusion of diets with such extreme fat contents. Proximate composition and amino acid profile of the experimental diets are presented in Table 2.2.

Table 2.1. Proximate composition (% of dry matter (DM) or kJ g^{-1}), amino acid profile (mg g^{-1} of DM) of anchovy fishmeal (5-01-985; NRC, 2011) and the test ingredients.

	FM	HI	HId	TM	TMd	LM
Proximate composition						
Dry matter	*92.0*	91.7	92.3	93.1	94.0	94.1
Ash	*13.2*	7.8	6.0	4.1	4.3	5.6
Crude protein (N × 4.76)	-	32.2	38.9	39.9	49.3	17.3
Crude protein (N × 5.60)	-	37.9	45.8	47.0	58.0	24.0
Crude protein (N × 6.25)	*60.2*	46.1	55.4	56.3	68.9	24.2
True protein (∑ AA)	*na* [c]	45.5	51.7	54.4	66.4	23.2
Crude fat	*7.0*	20.2	20.4	29.4	11.9	8.0
Gross energy (kJ g^{-1})	*na* [c]	21.9	24.7	26.5	23.6	20.5
Phosphorus	*na* [c]	0.8	0.9	0.9	0.7	0.5
Crude fibre	*0.9*	9.2	9.0	5.9	7.2	35.3
ADF [a]	*na* [c]	9.7	9.1	7.0	7.4	44.9
Chitin	*na* [c]	6.5	6.1	4.6	4.8	43.1
NFE [b]	*18.7*	16.6	9.2	4.3	7.8	26.9
Essential amino acids						
Arginine	*33.9*	30.8	36.9	39.5	51.3	14.1
Histidine	*14.4*	14.5	16.2	17.7	23.4	6.3
Lysine	*47.0*	26.7	36.9	29.6	39.1	15.4
Threonine	*25.9*	19.2	21.6	24.2	26.9	9.3
Isoleucine	*28.2*	25.8	24.4	28.2	34.6	12.7
Leucine	*46.0*	34.9	36.5	41.6	53.8	18.5
Valine	*32.3*	33.0	35.2	36.5	46.4	17.0
Methionine	*17.9*	8.8	13.7	7.4	9.6	3.3
Phenylalanine	*24.5*	23.5	22.3	25.1	31.6	11.5
∑ EAA (mg g^{-1} DM)	*270.1*	217.2	243.8	249.8	316.9	108.0
Non-essential amino acids						
Cystine	*5.6*	1.6	2.3	3.6	1.5	vest [d]
Tyrosine	*19.8*	35.6	44.2	42.1	55.1	17.0
Aspartic acid + Asparagine	*na* [c]	42.6	47.0	45.4	54.5	24.4
Glutamic acid + Glutamine	*na* [c]	47.2	56.8	65.3	79.8	25.6
Alanine	*na* [c]	33.1	39.9	39.5	45.7	18.4
Glycine	*na* [c]	33.0	28.6	37.4	43.8	15.9
Proline	*na* [c]	27.0	33.2	36.8	40.6	13.9
Serine	*na* [c]	17.3	20.2	22.4	26.5	8.4
Taurine	*na* [c]	vest [d]	1.2	1.6	vest [d]	vest [d]
∑ Non-EAA (mg g^{-1} DM)	*na* [c]	237.4	273.4	294.2	347.5	123.6

The abbreviations for the test ingredients stand for: FM – anchovy fishmeal; HI - *Hermetia illucens* larvae meal; HId - defatted *Hermetia illucens* larvae meal; TM - *Tenebrio molitor* larvae meal; TMd - defatted *Tenebrio molitor* larvae meal; LM - locust larvae meal; [a] ADF, acid detergent fibre; [b] NFE, nitrogen-free extract (%) = 100 - (% ash + % crude protein + % crude fat + % crude fibre); [c] na, non-available data; [d] vest, vestigial amount of amino acid (< 1 g kg^{-1} DM).

Table 2.2. Ingredients (%), proximate composition (% DM or kJ g^{-1}) and amino acid profile (mg g^{-1} of DM).

	DREF	DHI	DHId	DTM	DTMd	DLM
Ingredients						
Fishmeal Super Prime [a]	45	36	36	36	36	36
Soy protein concentrate [b]	8	6	6	6	6	6
Soybean meal 48 [c]	10	8	8	8	8	8
Rapeseed meal [d]	5	4	4	4	4	4
Wheat meal [e]	16	12.8	12.8	12.8	12.8	12.8
Salmon oil [f]	13	10.4	10.4	10.4	10.4	10.4
Vitamin and mineral premix [g]	1	0.8	0.8	0.8	0.8	0.8
Binder [h]	1	0.8	0.8	0.8	0.8	0.8
Chromic oxide	1	0.8	0.8	0.8	0.8	0.8
HI [i]	-	20	-	-	-	-
HId [j]	-	-	20	-	-	-
TM [i]	-	-	-	20	-	-
TMd [k]	-	-	-	-	20	-
LM [l]	-	-	-	-	-	20
Proximate composition						
Dry matter	95.0	93.9	93.9	92.9	91.9	94.3
Ash	12.5	11.3	13.4	11.2	10.8	11.2
Crude protein (N × 6.25)	47.5	47.9	49.0	50.3	53.9	44.4
Crude fat	20.1	19.4	19.2	19.6	17.1	17.3
Gross energy (kJ g^{-1})	21.7	21.5	22.0	22.2	21.7	21.2
Phosphorus	1.3	1.3	1.3	1.2	1.2	1.0
Crude fibre	1.1	2.7	2.9	2.3	2.8	6.0
ADF [m]	1.6	4.0	2.8	2.6	3.0	7.8
NFE [n]	18.8	18.7	15.4	16.6	15.3	21.0
Essential amino acids						
Arginine	39.4	39.2	39.3	39.8	42.4	39.2
Histidine	11.2	12.5	12.6	12.7	12.6	10.9
Lysine	32.2	31.4	32.0	32.7	38.3	36.3
Threonine	18.4	18.7	19.4	20.0	20.1	19.2
Isoleucine	23.6	24.0	24.1	26.4	27.3	21.2
Leucine	36.1	36.3	36.3	39.4	42.1	33.3
Valine	24.3	26.3	26.0	28.8	30.2	24.0
Methionine	10.0	9.2	10.0	11.4	11.3	9.9
Phenylalanine	22.3	24.2	23.2	22.6	23.4	18.2
∑ EAA (mg g^{-1} DM)	217.6	221.8	222.9	233.8	247.7	212.4
Non-essential amino acids						
Cystine	2.0	2.0	2.0	2.2	2.2	2.9
Tyrosine	16.4	22.1	20.7	22.1	25.0	16.6
Aspartic acid + Asparagine	42.2	42.3	43.2	47.4	51.7	40.2
Glutamic acid + Glutamine	71.6	66.9	68.9	75.8	82.6	70.8
Alanine	27.6	29.6	28.9	32.0	33.7	25.3
Glycine	35.5	36.5	36.6	36.7	36.1	27.5
Proline	23.9	25.1	24.6	27.6	27.9	23.1
Serine	17.5	17.3	19.1	20.4	19.3	19.2
Taurine	2.3	1.7	2.0	1.9	1.8	3.4
∑ Non-EAA (mg g^{-1} DM)	239.2	243.5	246.0	266.1	280.2	228.9

The abbreviations for the experimental diets stand for: DREF - reference diet; DHI - *Hermetia illucens* larvae meal diet; DHId - defatted *Hermetia illucens* larvae meal diet; DTM - *Tenebrio molitor* larvae meal diet; DTMd - defatted *Tenebrio molitor* larvae meal diet; DLM – locust larvae meal diet; [a] Fishmeal super prime: 71% crude protein (CP), 11% crude fat (CF), Exalmar S.A.A., Peru; [b] Soy protein concentrate: 65% CP, 0.7% CF, ADM, Netherlands; [c] Soybean meal 48: 48% CP, 2% CF, Cargill S.A., Portugal; [d] Rapseed meal: 36% CP, 3% CF, Premix Lda., Portugal; [e] Wheat meal: 10% CP, 1% CF, Casa Lanchinha Lda., Portugal; [f] Salmon oil: Sopropêche, France; [g] Vitamin and mineral premix: WISIUM, ADM Portugal S.A., Portugal; [h] Kielseguhr (natural zeolite), Ligrana GmbH, Germany; [i] HI and TM: EntoGreen, Portugal; [j] HId: Protix, Netherlands; [k] TMd: Entomo Farm, France; [l] LM: ACRS, France; [m] ADF, acid detergent fibre; [n] NFE, nitrogen-free extract (%) = 100 - (% ash + % crude protein + % crude fat + % crude fibre).

2.2.2. Digestibility trial

The digestibility trial was performed in the Fish Culture Experimental Unit of CIIMAR (Porto, Portugal) with European sea bass juveniles provided by Acuinuga – Acuicultura y Nutrición de Galicia, S.L. (Coruña, Spain). To adapt to the experimental conditions, fish were kept in quarantine for 5 weeks prior to the experiment and hand fed with a commercial diet (AQUASOJA – 50% crude protein, 20% crude fat). After acclimatization, homogeneous groups of 28 fish (body weight 33 ± 1 g) were distributed by fibreglass tanks of 55 L with individual faeces sedimentation columns (Guelph system), specially designed according to Cho and Slinger (1979). Fish were then acclimatized to the new conditions for 15 days (water temperature of 22 ± 1 °C, salinity of 35 ± 0.5 g L^{-1}, flow rate at 4 L min^{-1} and 12 h light/12 h dark photoperiod regime) and hand fed each experimental diet before the faeces collection began. During the period in which faeces were collected, fish continued to be hand fed until apparent satiation once a day. Approximately 30 minutes after feeding, every tank was carefully cleaned to assure that no remains of uneaten feed were left in the bottom of the tank or in the sedimentation column. Faeces were collected from the sedimentation column every morning, before feeding, and then centrifuged at 3 000 *g* to eliminate water excess before freezing at -20 °C until analysis. Daily collection of the faeces was performed for each experimental diet for 12 days. Since the recirculating water system utilised was only constituted by 12 tanks, this procedure was repeated with a new lot of fish with the same size-range. All experimental diets were hence tested in quadruplicate, and in each time series duplicates of each diet were tested; in the second round it was ensured that each experimental diet was not allocated twice to the same tank.

2.2.3. Chemical analysis of ingredients, experimental diets and faeces

Test ingredients, experimental diets and faeces collected from each tank were ground (faeces were freeze-dried and sifted) and homogenised before analysis. Proximate composition analysis was performed according to the AOAC (2006) methods. All samples were analysed for dry matter (105 °C for 24 h); ash by combustion in a muffle furnace at 500 °C for 5 h (Nabertherm L9/11/B170, Bremen, Germany); crude protein (N × 4.76, N × 5.60 or N × 6.25) using a Leco nitrogen analyser (Model FP 528; Leco Corporation, St. Joseph, USA); crude fat by petroleum ether extraction using a Soxtec extractor (Model ST 2055 Soxtec™; FOSS, Hillerod, Denmark) and phosphorus content by digestion at 230 °C in a Kjeldatherm block digestion unit followed by digestion at 75 °C in a water bath and absorbance determination at 820 nm (adapted from AFNOR V 04-

406). Gross energy was determined in an adiabatic bomb calorimeter (Model Werke C2000, IKA, Staufen, Germany) whilst chromic oxide content in diets and faeces was determined according to Bolin et al. (1952). Crude fibre content of ingredients was analysed according to the intermediate filtration method (ISO 6865:2000) and acid detergent fibre (ADF) according to ISO 13906:2008. Chitin was estimated using the indirect method of Finke (2007) modified by Marono et al. (2015): ADF (%) – ADIP (%), where ADIP is the amount of protein linked to ADF.

Samples from test ingredients, experimental diets and faeces were hydrolysed (6 m HCl at 116 °C over 72 h in nitrogen-flushed glass vials) before total amino acid analyses. All samples were then pre-column derivatized with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). Analyses were performed by ultra-high-performance liquid chromatography (UPLC) in a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard. Tryptophan was not determined, as it is partially destroyed by acid hydrolysis. The resultant peaks were analysed with EMPOWER software (Waters). True protein of tested ingredients was calculated trough the sum of individual AAs.

2.2.4. Calculations

The apparent digestibility coefficients (ADCs) of the experimental diets were calculated according to Maynard et al. (1979): ADC (%) = 100 × (1 − (dietary Cr_2O_3 level/faeces Cr_2O_3 level) × (faeces nutrient or energy level/dietary nutrient or energy level). ADC of dry matter was calculated as follows: ADC (%) = 100 × (1 − (dietary Cr_2O_3 level/faeces Cr_2O_3 level). The ADCs of nutrients and energy of the test ingredients were estimated according to NRC (2011): ADC_{ing} (%) = ADC_{test} + [(ADC_{test} − ADC_{ref}) × ((0.8 × D_{ref})/(0.2 × D_{ing}))]; where ADC_{test} = ADC (%) of the experimental diet, ADC_{ref} = ADC (%) of the reference diet, D_{ref} = g kg^{-1} nutrient (or kcal kg^{-1} gross energy) of the reference diet (DM basis); D_{ing} = g kg^{-1} nutrient (or kcal kg^{-1} gross energy) of the test ingredient (DM basis). The digestible amino acids (DAAs) content of each insect meal was calculated as follows: DAA (mg g^{-1} of DM) = ADC (%) of the amino acid in the test ingredient × AA_{ing}; where AA_{ing} = mg g^{-1} amino acid of the test ingredient (DM basis).

2.2.5. Statistical analysis

Data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov and Levene's tests, respectively, and log-transformed whenever required before being submitted to a one-way ANOVA with the statistical program IBM SPSS

STATISTICS, 25.0 package, IBM corporation, New York, USA (2011). When this test showed significance, individual means were compared using HSD Tukey Test. Differences were considered significant when $p < 0.05$.

2.3. Results

The proximate composition of the test ingredients is presented in Table 2.1. The crude protein of IMs was calculated through the sum of AAs (true protein), and by using three nitrogen-to-protein conversion factors (Kp): Kp of 4.76, Kp of 5.60 and Kp of 6.25. Crude protein content of the selected ingredients ranged from 17 to 49% DM when using Kp of 4.76, from 24 to 58% DM when using Kp of 5.60 and from 24 to 69% DM when using Kp of 6.25, whilst varied from 23 to 66% when calculated through the sum of AAs. Crude fat content of IMs varied between 8 and 29% DM and gross energy from 21 to 27 kJ g^{-1} DM. Crude fibre varied from 7 to 35% DM among test ingredients, acid detergent fibre from 7 to 45% DM and chitin from 5 to 43% DM. Total EAAs content of the test ingredients varied between 108 and 317 mg g^{-1} DM and total non-EAAs from 124 to 348 mg g^{-1} DM. TM*d* was the test ingredient with the highest protein, and therefore, amino acid content (both essential and non-essential), although methionine level was highest in HI*d*. LM was the test ingredient with the lowest content of amino acids (both essential and non-essential). The replacement of 20% of the reference diet by each of the test ingredients resulted in experimental diets with variable chemical composition: 44 to 54% DM of crude protein, 17 to 20% DM of crude fat, 21 to 22 kJ g^{-1} gross energy DM, 1 to 6% DM of crude fibre, 212 to 248 mg g^{-1} DM EAAs and 229 to 280 mg g $^{-1}$ DM non-EAAs, reflecting the variability in the composition of each ingredient (Table 2.2).

Fish promptly accepted all the experimental diets and no mortality occurred during the trial. The ADCs of macro-nutrients, energy and individual amino acids of the experimental diets are presented in Table 2.3. Fish fed DHI, DHI*d* and DLM registered significantly lower macro-nutrients and energy ADC values than those fed DREF, except for phosphorus, which was significantly better digested in DHI*d* than in DREF. Protein ADC was highest in fish fed DREF, DTM and D TM*d*, followed by DHI*d* that did not differ significantly from DTM and DLM. The lowest protein ADC value was displayed by fish fed DHI. The crude fat ADC was highest in fish fed DREF and DTM*d* followed by DTM which was similar to DTM*d*, DHI and DHI*d*. The lowest value of crude fat ADC was observed in fish fed DLM. All individual amino acids (either essential and non-essential) showed high ADC values (>88 %) with the exception of cystine and glycine.

The ADCs of macro-nutrients, energy and individual amino acids of the test ingredients are presented in Table 2.4. The ADC for dry matter varied significantly between the test ingredients (40%-85%) and TM had the highest value followed by TM*d*, HI*d*, HI and LM. The highest protein digestibility value (93%) was obtained in TM*d* and the lowest values were obtained in LM and HI (74% and 76%, respectively). HI*d* and TM displayed moderate values for protein ADC (87% and 89%, respectively). Fat digestibility was significantly higher in TM (95%), TM*d* (94%) and HI*d* (93%) than in LM (67%) and HI*d* did not differ significantly from HI (91%). TM*d* had the highest values of gross energy and phosphorus ADC (84% and 91%, respectively), while LM had the lowest values (53% and 27%, respectively). The ADCs of individual amino acids (either EAAs or non-EAAs) of the test ingredients diets were generally moderate to high. Only ADC of cystine in HI and ADC of glycine in LM displayed values below to 50%. The protein nutritional value of each test ingredient, in terms of digestible amino acids content (mg g^{-1} of DM) is presented in Table 2.5 and Figure 2.1. Overall, TM*d* had the highest values of digestible amino acids (either essential or non-essential) followed by TM, HI*d*, HI and LM. However, HI*d* displayed the highest content of digestible methionine.

Table 2.3. Apparent digestibility coefficients (%) of macro-nutrients, energy and individual amino acids of the experimental diets.

	DREF	DHI	DHId	DTM	DTMd	DLM
Dry matter	76.9 ± 0.9 ^{b}	71.3 ± 0.1 ^{c}	72.3 ± 0.1 ^{c}	78.5 ± 0.1 ^{a}	76.0 ± 0.2 ^{b}	69.6 ± 0.4 ^{d}
Crude protein	92.6 ± 0.7 ^{a}	89.3 ± 0.1 ^{d}	91.3 ± 0.1 ^{bc}	91.8 ± 0.2 ^{ab}	92.6 ± 0.2 ^{a}	90.5 ± 0.2 ^{c}
Crude fat	98.2 ± 0.3 ^{a}	96.8 ± 0.2 ^{c}	97.1 ± 0.3 ^{c}	97.2 ± 0.2 ^{bc}	97.7 ± 0.2 ^{ab}	95.3 ± 0.1 ^{d}
Gross energy	86.5 ± 0.6 ^{a}	82.7 ± 0.4 ^{c}	81.7 ± 0.2 ^{c}	84.8 ± 0.3 ^{b}	86.1 ± 0.6 ^{a}	80.1 ± 0.4 ^{d}
Phosphorus	60.4 ± 1.5 ^{b}	57.2 ± 0.3 ^{c}	62.3 ± 0.4 ^{a}	63.6 ± 0.2 ^{a}	63.9 ± 0.2 ^{a}	57.4 ± 0.2 ^{c}
Essential amino acids						
Arginine	96.5 ± 0.1 ^{a}	95.6 ± 0.04 ^{c}	96.1 ± 0.2 ^{ab}	96.1 ± 0.1 ^{b}	94.9 ± 0.1 ^{d}	95.5 ± 0.1 ^{c}
Histidine	95.2 ± 0.2 ^{c}	96.8 ± 0.1 ^{a}	93.4 ± 0.2 ^{d}	96.1 ± 0.2 ^{b}	95.0 ± 0.1 ^{c}	93.9 ± 0.1 ^{d}
Lysine	96.2 ± 0.03 ^{a}	95.6 ± 0.1 ^{b}	96.2 ± 0.1 ^{a}	95.8 ± 0.1 ^{b}	95.5 ± 0.1 ^{b}	95.5 ± 0.1 ^{b}
Threonine	93.4 ± 0.3	92.2 ± 0.1	92.3 ± 0.2	92.9 ± 0.5	90.8 ± 0.2	92.3 ± 0.3
Isoleucine	92.6 ± 0.1 ^{b}	92.6 ± 0.2 ^{b}	93.1 ± 0.04 ^{b}	94.0 ± 0.6 ^{a}	92.9 ± 0.1 ^{b}	91.0 ± 0.3 ^{c}
Leucine	92.8 ± 0.3 ^{c}	92.5 ± 0.2 ^{c}	93.1 ± 0.1 ^{bc}	94.3 ± 0.3 ^{a}	93.8 ± 0.1 ^{ab}	91.8 ± 0.2 ^{d}
Valine	91.6 ± 0.3 ^{c}	92.0 ± 0.6 ^{bc}	91.2 ± 0.1 ^{c}	93.4 ± 0.1 ^{a}	92.8 ± 0.2 ^{ab}	90.5 ± 0.2 ^{d}
Methionine	94.6 ± 0.01 ^{bc}	95.7 ± 0.018 ^{a}	94.9 ± 0.1 ^{b}	94.4 ± 0.2 ^{cd}	95.7 ± 0.03 ^{a}	94.2 ± 0.2 ^{d}
Phenylalanine	91.7 ± 0.1 ^{c}	91.9 ± 0.1 ^{bc}	92.7 ± 0.1 ^{ab}	93.1 ± 0.3 ^{a}	92.0 ± 0.2 ^{bc}	88.9 ± 0.4 ^{d}
∑ EAA	94.2 ± 0.4 ^{a}	93.7 ± 0.1 ^{ab}	93.9 ± 0.1^{a}	94.5 ± 0.1 ^{a}	93.8 ± 0.1 ^{a}	92.9 ± 0.2 ^{b}
Non-essential amino acids						
Cystine	87.6 ± 0.6 ^{d}	81.5 ± 0.2 ^{f}	84.1 ± 0.2 ^{e}	95.4 ± 0.02 ^{a}	91.8 ± 0.3 ^{c}	93.2 ± 0.2 ^{b}
Tyrosine	91.6 ± 0.5 ^{b}	95.0 ± 0.1 ^{a}	92.2 ± 0.2 ^{b}	94.1 ± 0.1 ^{a}	94.8 ± 0.1 ^{a}	89.2 ± 0.3 ^{c}
Aspartic acid + Asparagine	93.6 ± 0.2 ^{a}	91.6 ± 0.2 ^{c}	93.8 ± 0.1 ^{a}	93.3 ± 0.2 ^{ab}	92.9 ± 0.2 ^{b}	91.5 ± 0.2 ^{c}
Glutamic acid + Glutamine	95.4 ± 0.2 ^{a}	93.9 ± 0.1 ^{b}	95.2 ± 0.1 ^{a}	95.5 ± 0.2 ^{a}	94.4 ± 0.1 ^{b}	94.4 ± 0.2 ^{b}
Alanine	93.8 ± 0.1 ^{b}	93.6 ± 0.1 ^{bc}	93.0 ± 0.1 ^{d}	94.6 ± 0 ^{a}	93.3 ± 0.1 ^{cd}	92.3 ± 0.2 ^{e}
Glycine	71.7 ± 2.02 ^{ab}	75.5 ± 0.4 ^{ab}	71.8 ± 0.3 ^{a}	72.7 ± 1.7 ^{ab}	70.6 ± 0.3 ^{b}	68.5 ± 0.6 ^{b}
Proline	94.4 ± 0.3 ^{a}	94.3 ± 0.1 ^{a}	93.0 ± 0.1 ^{b}	94.7 ± 0.2 ^{a}	93.0 ± 0.1 ^{b}	93.4 ± 0.2 ^{b}
Serine	91.9 ± 0.3 ^{abc}	91.0 ± 0.2 ^{cd}	92.5 ± 0.04 ^{a}	92.2 ± 0.3 ^{ab}	90.9 ± 0.2 ^{d}	91.3 ± 0.3 ^{bcd}
∑ Non-EAA	94.0 ± 0.1 ^{a}	93.2 ± 0.1 ^{b}	93.4 ± 0.1 ^{b}	94.4 ± 0.01 ^{a}	93.5 ± 0.1	92.5 ± 0.2 ^{c}

Values are presented as mean ± standard deviation (n = 6 for macro-nutrients and energy of DREF; n = 4 for macro-nutrients and energy of DHI, DHId, DTM, DTMd and DLM; n = 3 for indivudual amino acids of DREF; n = 2 for individual amino acids of DHI, DHId, DTM, DTMd and DLM). Values in the same row without a common superscript letter differ significantly ($p < 0.05$). Absence of superscript indicates no significant difference between treatments.

Table 2.4. Apparent digestibility coefficients (%) of macro-nutrients, energy and individual amino acids of the test ingredients.

	HI	HId	TM	TMd	LM
Dry matter	48.3 ± 0.6 ^{d}	53.7 ± 0.7 ^{c}	85.2 ± 0.6 ^{a}	72.4 ± 0.8 ^{b}	40.1 ± 1.8 ^{e}
Crude protein	75.8 ± 0.8 ^{c}	87.2 ± 0.5 ^{b}	89.2 ± 0.99 ^{b}	92.8 ± 0.7 ^{a}	74.1 ± 1.5 ^{c}
Crude fat	91.4 ± 1.2 ^{b}	92.7 ± 1.7 ^{ab}	94.5 ± 0.7 ^{a}	94.4 ± 1.3 ^{a}	66.5 ± 1.4 ^{c}
Gross energy	67.2 ± 1.99 ^{c}	64.8 ± 1.1 ^{c}	79.2 ± 1.4 ^{b}	84.4 ± 2.7 ^{a}	53.0 ± 1.9 ^{d}
Phosphorus	35.8 ± 1.6 ^{d}	73.4 ± 2.6 ^{c}	83.3 ± 1.6 ^{b}	90.7 ± 2 ^{a}	26.8 ± 2.7 ^{e}
Essential amino acids					
Arginine	90.9 ± 0.3 ^{b}	94.5 ± 0.8 ^{a}	94.4 ± 0.5 ^{a}	90.1 ± 0.3 ^{b}	84.5 ± 1.2 ^{c}
Histidine	97.6 ± 0.4 ^{a}	88.5 ± 0.7 ^{d}	95.3 ± 0.7 ^{b}	92.5 ± 0.2 ^{c}	81.4 ± 0.6 ^{e}
Lysine	92.6 ± 0.4 ^{b}	96.1 ± 0.3 ^{a}	93.7 ± 0.7 ^{ab}	93.1 ± 0.3 ^{b}	89.7 ± 1.1 ^{c}
Threonine	79.1 ± 0.5	81.3 ± 0.7	84.7 ± 2.2	77.7 ± 0.7	66.6 ± 2.3
Isoleucine	92.6 ± 0.8 ^{b}	95.0 ± 0.2 ^{ab}	98.7 ± 2.5 ^{a}	93.7 ± 0.5 ^{ab}	78.8 ± 2.2 ^{c}
Leucine	92.2 ± 0.9 ^{c}	94.2 ± 0.5 ^{bc}	100 ± 1.2 ^{a}	97.0 ± 0.5 ^{ab}	85.2 ± 1.5 ^{d}
Valine	90.3 ± 0.4 ^{b}	90.2 ± 0.6 ^{b}	95.3 ± 0.5 ^{a}	93.3 ± 0.5 ^{ab}	78.1 ± 1.6 ^{c}
Methionine	100.7 ± 0.1 ^{a}	95.8 ± 0.3 ^{b}	93.2 ± 1.3 ^{bc}	100.5 ± 0.2 ^{a}	88.8 ± 2.1 ^{c}
Phenylalanine	92.6 ± 0.7 ^{a}	96.7 ± 0.5 ^{a}	98.0 ± 1.2 ^{a}	92.9 ± 0.9 ^{a}	66.9 ± 3.6 ^{b}
∑ EAA	92.1 ± 0.6 ^{b}	92.8 ± 0.5 ^{ab}	95.8 ± 0.3 ^{a}	92.8 ± 0.4 ^{ab}	82.9 ± 1.7 ^{b}
Non-essential amino acids					
Cystine	49.4 ± 1.1 c	71.3 ± 0.7 ^{b}	112.9 ± 0.1 ^{a}	114.6 ± 1.9 ^{a}	nd *
Tyrosine	101.3 ± 0.2 ^{a}	93.1 ± 0.5 ^{c}	98.1 ± 0.2 ^{b}	98.7 ± 0.2 ^{b}	79.5 ± 1.3 ^{d}
Aspartic acid + Asparagine	83.5 ± 0.8 ^{b}	94.7 ± 0.2 ^{a}	92.3 ± 1.01 ^{a}	90.5 ± 0.7 ^{a}	76.2 ± 1.8 ^{c}
Glutamic acid + Glutamine	84.3 ± 1.04 ^{c}	94.1 ± 0.6 ^{ab}	96.0 ± 1.1 ^{a}	91.0 ± 0.4 ^{b}	82.9 ± 1.98 ^{c}
Alanine	92.7 ± 0.4 ^{b}	90.6 ± 0.4 ^{b}	96.9 ± 0.1 ^{a}	92.2 ± 0.4 ^{b}	83.4 ± 1.6 ^{c}
Glycine	91.8 ± 2.4 ^{a}	72.2 ± 1.8 ^{b}	76.7 ± 8.3 ^{ab}	67.0 ± 1.4 ^{b}	40.0 ± 6.1 ^{c}
Proline	94.3 ± 0.4 ^{a}	89.1 ± 0.5 ^{bc}	95.5 ± 0.9 ^{a}	89.9 ± 0.3 ^{b}	86.7 ± 1.3 ^{c}
Serine	87.4 ± 0.9 ^{c}	94.3 ± 0.2 ^{a}	93.1 ± 1.1 ^{ab}	88.1 ± 0.7 ^{bc}	85.9 ± 2.7 ^{c}
∑ Non-EAA	90.3 ± 0.6 ^{b}	91.6 ± 0.4 ^{b}	95.6 ± 0.1 ^{a}	92.2 ± 0.4 ^{b}	82.0 ± 1.6 ^{c}

Values are presented as mean ± standard deviation (n = 4 for macro-nutrients and energy; n = 2 for individual amino acids). Values in the same row without a common superscript letter differ significantly ($p < 0.05$). Absence of superscript indicates no significant difference between treatments; * nd, not determined. When the amount of amino acid in the test ingredient was vestigial, the ADC could not be determined.

Table 2.5. Digestible amino acids content (mg g^{-1} of DM) of the test ingredients.

	HI	HId	TM	TMd	LM
Essential amino acids					
Arginine	28.0 ± 0.1 ^{d}	34.9 ± 0.3 ^{c}	37.3 ± 0.2 ^{b}	46.2 ± 0.2 ^{a}	11.9 ± 0.2 ^{e}
Histidine	14.2 ± 0.1 ^{c}	14.3 ± 0.1 ^{c}	16.8 ± 0.1 ^{b}	21.7 ± 0.1 ^{a}	5.1 ± 0.03 ^{d}
Lysine	24.7 ± 0.1 ^{d}	35.5 ± 0.1 ^{b}	27.8 ± 0.2 ^{c}	36.4 ± 0.1 ^{a}	13.8 ± 0.2 ^{e}
Threonine	15.1 ± 0.1	17.6 ± 0.2	20.5 ± 0.5	20.9 ± 0.2	6.2 ± 0.2
Isoleucine	23.9 ± 0.2 ^{c}	23.8 ± 0.05 ^{c}	28.1 ± 0.7 ^{b}	32.5 ± 0.2 ^{a}	10.0 ± 0.3 ^{d}
Leucine	32.2 ± 0.3 ^{d}	35.2 ± 0.2 ^{c}	41.6 ± 0.5 ^{b}	52.2 ± 0.2 ^{a}	15.7 ± 0.3 ^{e}
Valine	29.8 ± 0.1 ^{d}	31.8 ± 0.2 ^{c}	34.8 ± 0.2 ^{b}	43.3 ± 0.2 ^{a}	13.3 ± 0.3 ^{e}
Methionine	9.4 ± 0.01 ^{c}	13.2 ± 0.04 ^{a}	6.9 ± 0.1 ^{d}	9.7 ± 0.02 ^{b}	2.9 ± 0.1 ^{e}
Phenylalanine	22.3 ± 0.2 ^{c}	21.7 ± 0.1 ^{c}	24.6 ± 0.3 ^{b}	29.4 ± 0.3 ^{a}	7.7 ± 0.4 ^{d}
∑ EAA	199.6 ± 1.2 ^{d}	229.0 ± 1.3 ^{c}	241.3 ± 0.8 ^{b}	295.6 ± 1.3 ^{a}	91.1 ± 1.9 ^{e}
Non-essential amino acids					
Cystine	2.1 ± 0.01 ^{d}	3.1 ± 0.01 ^{b}	4.8 ± 0.001 ^{a}	2.7 ± 0.02 ^{c}	nd *
Tyrosine	37.3 ± 0.1 ^{c}	41.2 ± 0.2 ^{b}	41.3 ± 0.1 ^{b}	54.4 ± 0.1 ^{a}	13.5 ± 0.2 ^{d}
Aspartic acid + Asparagine	35.6 ± 0.1 ^{d}	44.5 ± 0.1 ^{b}	41.9 ± 0.5 ^{c}	49.3 ± 0.4 ^{a}	18.6 ± 0.4 ^{e}
Glutamic acid + Glutamine	39.8 ± 0.5 ^{d}	53.4 ± 0.3 ^{c}	62.7 ± 0.7 ^{b}	72.6 ± 0.4 ^{a}	21.3 ± 0.5 ^{e}
Alanine	30.7 ± 0.1 ^{d}	36.1 ± 0.2 ^{c}	38.3 ± 0.03 ^{b}	42.1 ± 0.2 ^{a}	15.3 ± 0.3 ^{e}
Glycine	30.3 ± 0.8 ^{a}	20.6 ± 0.5 ^{b}	28.7 ± 3.1 ^{a}	29.4 ± 0.6 ^{a}	6.4 ± 0.97 ^{c}
Proline	25.5 ± 0.1 ^{d}	29.6 ± 0.2 ^{c}	35.2 ± 0.3 ^{b}	36.5 ± 0.1 ^{a}	12.1 ± 0.2 ^{e}
Serine	15.1 ± 0.2 ^{d}	19.0 ± 0.03 ^{c}	20.9 ± 0.2 ^{b}	23.4 ± 0.2 ^{a}	7.2 ± 0.2 ^{e}
∑ Non-EAA	215.6 ± 1.3 ^{d}	250.5 ± 1.1 ^{c}	281.3 ± 0.04 ^{b}	320.5 ± 1.5 ^{a}	101.4 ± 2.02 ^{e}

Values are presented as mean ± standard deviation (n = 2). Values in the same row without a common superscript letter differ significantly ($p < 0.05$). Absence of superscript indicates no significant difference between treatments; * nd, not determined. When the amount of amino acid in the ingredient was vestigial, their digestible amount could not be determined.

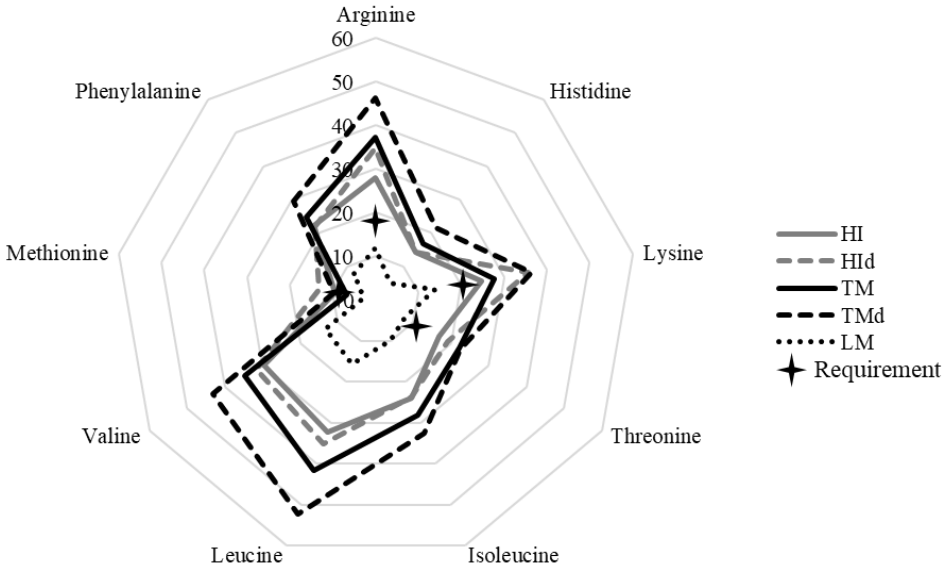

Figure 2.1. Digestible essential amino acids content (mg g^{-1} of DM) of the test ingredients and European sea bass requirements (mg g^{-1} of diet) (Tibaldi and Lanari, 1991; Tibaldi et al., 1994; Tibaldi and Tulli, 1999; Tulli et al., 2010).

2.4. Discussion

Characterization and determination of the bioavailable nutrients in feed ingredients is crucial to appropriately formulate aquafeeds that guarantee species nutritional requirements. The nutrients' availability in an ingredient is generally assessed through the *in vivo* determination of ADC values, which indicate the proportion of ingested nutrients that are not excreted in faeces and can be utilized by the organism (Glencross et al., 2007). The classic approach implicates the replacement of a certain amount of a reference diet by one ingredient of interest, which often results in diets with distinct nutritional values. This is not an optimal approach but is the most widely used and accepted in nutritional studies with fish (NRC, 2011). Insects are the most diverse group of animals worldwide and have a highly variable composition. Beyond the taxonomic group, life stage and methods of breeding and feeding strongly influence the composition of IM, often requiring different technological processes prior its inclusion in aquafeeds due to extremely high fat contents. In this study, we selected five distinct insect larvae meals from different producers, based on their technical files, in particular the fact of being either full fat (HI and TM) or defatted (HI*d* and TM*d*). According to Mæhre et al. (2018), the protein content of an ingredient should be calculated directly through the sum of its individual amino acids (true protein). But this method is often inaccessible, due to the high

costs associated with total AAs quantification, being the protein content of feedstuffs mostly based on nitrogen determination with subsequent conversion using a nitrogen-to-protein Kp of 6.25 (crude protein). Considering that insects contain non-protein nitrogen, Belghit et al. (2019a) have recently suggested that a Kp between 4.2 and 5 might be more suitable than the usual factor of 6.25 to avoid overestimation of protein content. Moreover, Janssen et al. (2017) calculated a specific Kp of 4.76 for *T. molitor* and *H. illucens* whole larvae but suggested a factor of 5.60 after protein extraction of such larvae. Considering such Kps, in the present study, crude protein of HI and TM if calculated with a Kp of 4.76 resulted in values 13 and 15% below the true protein, respectively (obtained through the sum of AAs). Likewise, the crude protein of HI*d* and TM*d* calculated with Kp of 5.60 also resulted in values 6 and 8% below the true protein, whilst the use of Kp of 6.25 has just resulted in an overestimation of 1-4% for HI, TM, HI*d* and TM*d*. In the case of LM, the use of Kp of 4.76 resulted in an underestimation of 6%, whilst the use of either Kp of 5.60 or 6.25 resulted in an overestimation of 1%. These results highlight the importance of determining true protein values in insect meals and suggest caution when choosing the most adequate nitrogen-to-protein factor to avoid either over or underestimation of total protein values.

HI*d* and TM*d* had higher protein content than HI and TM, suggesting that the defatting process used by each producer might have segregated fat, enhancing protein content, as already reported by Teh et al. (2013) and Choi et al. (2017). The similar fat contents between HI and HI*d* can be partly explained by their distinct origin, under probable distinct rearing conditions and processing technologies. This emphasizes the impact of rearing conditions and diet during insects' production on their nutritional characteristics as previously denoted by Osimani et al. (2016). Among all tested ingredients, the LM had the lowest protein and fat content and the highest crude fibre and chitin levels, which may compromise its dietary inclusion as protein source for carnivorous fish species. Although it is difficult to compare the chemical composition of ingredients obtained from different companies, the results of this study were in line with data previously reported in literature and recently reviewed by Nogales-Mérida et al. (2018). The EAA profile of HI, HI*d*, TM and TM*d* slightly differ from that reported for anchovy fishmeal (5-01-985; NRC, 2011), a recognised excellent protein source for carnivorous fish, included herewith for comparison. But all tested IMs met the ideal EAAs requirements of European sea bass according to NRC (2011). Although the sum of EAAs in HI, HI*d* and TM was lower than that observed in the reference anchovy fishmeal, HI, HI*d* and TM had higher amounts of histidine (15-18 vs 14 mg g^{-1} DM) and valine (33-37 vs 32 mg g^{-1} DM) compared to anchovy FM. HI*d* and TM also displayed higher quantities of arginine (37 and

40 vs 34 mg g^{-1} DM, respectively) compared to FM. Contrarily, in TM*d* only lysine (39 vs 47 mg g^{-1} DM) and methionine (10 vs 18 mg g^{-1} DM) were observed in lower quantities than FM. The poorness of LM in terms of EAAs was clearly demonstrated, suggesting it would not be a good alternative to replace FM. The improved EAA profile observed in HI*d* and TM*d* (defatted meals) compared to HI and TM (full-fat ingredients) emphasizes the importance of applying a technological process as a strategy to improve the nutritional value of ingredients.

The dry matter ADC provides an overall indication of an ingredient's digestibility, reflecting the digestible fraction of both organic and inorganic matter, which are highly variable according to its insoluble carbohydrates and mineral composition, respectively (NRC, 2011). In TM and TM*d* the DM digestibility was moderate to high (72-85%) in contrast to HI, HI*d* and LM (<54%). The results obtained in the present study for DM digestibility of TM are in line with those obtained by Fontes et al. (2019) in Nile tilapia. HI and HI*d* had the highest ash content, which may have contributed to the reduced DM digestibility of these ingredients compared to TM and TM*d*. Previous studies have reported a much higher ash content in *H. illucens* compared to *T. Molitor,* mainly due to its higher Ca content (7.6 *vs* 0.3% DM, respectively) (Makkar et al., 2014). Fish, principally carnivorous species such as European sea bass, have a well-known capacity to poorly digest complex carbohydrates (Stone, 2003; Enes et al., 2011). Moreover, the higher chitin content in HI and HI*d*, compared to either TM or TM*d*, may also have contributed to the reduced DM digestibility observed in these ingredients. Chitin has been associated with decreased nutrients' digestibility in turbot, Atlantic cod, Atlantic salmon and Atlantic halibut (Kroeckel et al., 2012; Karlsen et al., 2017; Fontes et al., 2019). Moreover, LM displayed the highest ADF and crude fibre contents, that together with its high ash content explain the lowest DM digestibility observed of this insect meal.

The protein quality of dietary ingredients is the foremost important factor affecting fish performance, reflecting its capacity to utilize specific ingredients (Mo et al., 2019). Thus, the determination of its bioavailability is of paramount importance. In all tested ingredients, protein ADC was moderate to high. The lowest protein ADCs values were obtained in LM and HI (74 and 76%, respectively), that were the ingredients with the lowest protein contents. This is in accordance with Campos et al. (2018) that observed that protein digestibility in European sea bass was lowest in low protein ingredients. The protein ADC value of TM*d* (93%) was even higher than values reported for a low quality FM (90-91%) and was very close to those reported for a high quality FM (94-96%) (Gomes da Silva and Oliva-Teles, 1998; Lanari and D'Agaro, 2005; Davies et al., 2009). When protein digestibility of insect meals is compared with that of other land animal

ingredients, it can be concluded that European sea bass digests TM*d* equally well, or even better than poultry by-product meal (86-93%; Davies et al., 2009; Campos et al., 2018). Moreover, the results obtained in this study show that protein digestibility of TM*d* is also higher than that of feather meals, blood meal and meat meal (77-91%; Gomes da Silva and Oliva-Teles, 1998; Davies et al., 2009; Campos et al., 2018), clearly demonstrating the high potential of this feedstuff for *D. labrax* diets. The remaining tested ingredients had lower protein ADC values than those reported for FM, regardless of its quality. Besides, both LM and HI also had considerably lower protein ADC values (74-76%) than those reported for soybean meal, one of the main fishmeal substitutes used in aquafeeds (91%; Lanari and D'Agaro, 2016). The protein digestibility of HI*d* was previously studied in both turbot (*Psetta maxima*) (Kroeckel et al., 2012) and rainbow trout (*Oncorhynchus mykiss*) (Dumas et al., 2018), with both authors reporting lower protein ADC values than those obtained in the present study (63-85 vs 87%). Fontes et al. (2019) also reported lower protein digestibility values of TM in Nile tilapia than those herein obtained (85 vs 89%). Such difference is probably related to variable nutritional value or processing methodologies used to obtain the HI*d* evaluated in each case. Previous studies have evaluated the digestibility of nutritionally balanced diets including different levels of insect species. Such results cannot be directly compared to those presently reported for experimental diets and must hence be analysed with caution. In European sea bass, the dietary inclusion of 25% TM*d*, to replace FM, resulted in protein ADC values similar to those presently observed for DTM*d* (Gasco et al., 2016). Magalhães et al. (2017), also reported that the dietary inclusion of 20% HI*d* resulted in protein ADC values similar to those observed for DHI*d* in sea bass. Likewise, in other fish species, the dietary inclusion of TM or HI*d* up to 25% resulted in protein ADC values within the range of values (91-92%) presently observed for the experimental diets (Belforti et al. 2014; Renna et al., 2017). But in rainbow trout, the protein ADC values of diets with 25% TM*d* (Rema et a., 2019) were slightly higher than those obtained for DTM*d* in the present study (94 vs 93%, respectively). Lower protein digestibility values (<89%) were reported in gilthead sea bream (*Sparus aurata*) and Atlantic salmon (*Salmo salar*), Belghit et al. (2019b) either fed 25% TM (Piccolo et al., 2017) or 15% HI*d*, to replace FM.

Fish's ability to digest dietary proteins depends on its capacity to hydrolyse them into tripeptides, dipeptides and free amino acids, the only compounds released into the portal vein as protein digest products (NRC, 2011). Shortage in any EAA leads to poor utilization of dietary protein and consequently decreases feed efficiency and reduces fish performance. The ADC of EAAs observed in TM (96%) was in line with values reported for a high quality fishmeal or for a fish by-product meal in European sea bass (96-97%), but

HI, HI*d* and TM*d* protein ADC values were slightly lower (92-93%) (Davies et al., 2009; Campos et al., 2018). The lowest essential amino acids ADC value was obtained in LM (83%), that was also the ingredient with the lowest protein content. Lysine and methionine are the most limiting EAAs in diets for European sea bass. Therefore, accurate estimation of these two amino acids bioavailability is of highest importance to formulate equilibrated aquafeeds for this species. The ADC values of lysine varied between 90 and 96%, being lower than values attributed to FM and fish by-product meal in European sea bass (98 and 99%, respectively). However, methionine ADC values displayed by both HI and TM*d* (≈100%) were equivalent to those observed in FM, and even higher than those obtained in fish processing by-product meal (100 and 90%, respectively) (Davies et al., 2009; Campos et al., 2018). Moreover, lysine ADC of TM and HI*d* (94% and 96%, respectively) was higher than in poultry by-product (93%) or hydrolysed feather meals (< 90%) (Campos et al., 2018). ADC values close to, or even above 100% can be found in literature for several ingredients (Allan et al., 2000; Mo et al., 2019), and can be explained by a low nutrient content in the ingredient (that comprises 15-30% of the experimental diet), leading to its higher uptake from the reference diet constituents (70-85% of the experimental diet) to meet the species requirement. In fact, methionine content of HI and TM*d* halved that reported for fishmeal. The EAAs bioavailability of HI*d* was previously assessed by Dumas et al. (2018) in *O. mykiss,* and only threonine displayed higher ADC values than those obtained in the present study (87 vs 81%, respectively). The remaining EAAs of HI*d*, including lysine and methionine, seem to be better digested in sea bass. Moreover, compared to broilers, European sea bass also seems to digest better the EAAs contained in HI, HI*d* and TM (De Marco et al., 2015; Schiavone et al., 2017). Lysine and methionine ADC values presently reported for DHI*d* are also higher than those observed in sea bass fed diets including 20% HI*d*, to replace FM (Magalhães et al., 2017).

The EAAs requirements of sea bass are clearly defined for arginine, lysine, threonine and methionine. With the exception of LM, the digestible arginine, lysine and threonine of all remaining tested IMs surpassed sea bass requirements, with TM*d* being the IM with the highest content of these three digestible EAAs. However, in the particular case of methionine, HI*d* was the feedstuff with values closer to sea bass requirements (8-9 mg g^{-1} of diet) when compared to TM*d* (13 vs 10 mg g^{-1} DM, respectively) (Tulli et al., 2010). Overall, the highest amount of digestible EAAs in TM and TM*d* makes these ingredients particularly valuable for European sea bass diets, since besides being good sources of protein, they also have a high bioavailability of EAAs able to meet this species requirements (NRC, 2011). Contrarily, despite the moderate to high values of digestible

EAAs in LM, its limited protein content (24%) is of concern and its further utilization in sea bass diets must be carefully evaluated.

Fat digestibility was high (> 91%) for all test ingredients except for LM (67%). The fatty acid profile and the fat source structure have great influence in its digestibility. It is well known that the digestibility of individual fatty acids is extremely variable according to the degree of saturation, chain length, and melting point, being impaired when the fatty acid profile is rich in saturated fatty acids (Hua and Bureau, 2009). The fatty acid profile of HI, HI*d*, TM and TM*d* was shown to be highly variable depending on rearing conditions, diet supplied during the insect's production and stage of development (Nogales-Mérida et al., 2018) making comparisons very difficult. Although the fatty acid profile of the tested ingredients was not evaluated in the present study, fat ADC values obtained for HI, HI*d*, TM and TM*d* (90-95%) are close to or even exceed fishmeal ADC values (93%) reported by Davies et al. (2009) for European sea bass. TM displayed higher fat digestibility values than those obtained for TM in Nile tilapia (Fontes et al., 2019). Fat digestibility values of HI*d* were higher than values reported by Kroeckel et al. (2012) and Dumas et al. (2018) in diets for turbot and rainbow trout. Likewise, HI, HI*d*, TM and TM*d* displayed higher values than those previously observed for hydrolysed feather meal (85-88%), whilst fat ADC of LM (67%) resulted in lower values (Campos et al., 2018). It must, however, be highlighted that the present trial was performed at controlled temperature (22 ± 1 °C); as insects are generally rich in saturated fatty acids (Nogales-Mérida et al., 2018), impaired fat digestibility might be induced at lower temperatures and hence merits further consideration. In *D. labrax*, the dietary inclusion of TM*d* or HI*d* up to 25%, to replace FM, resulted in fat ADC values higher than those presently observed for the experimental diets (Gasco et al., 2016; Magalhães et al., 2017). Similarly, in *O. mykiss*, Renna et al. (2017) observed that the dietary inclusion of 20% HI*d* resulted in slightly higher values for fat ADC when compared to those observed for DHI*d* in sea bass (99 vs 97%, respectively). However, also in *O. mykiss*, Rema et al. (2019) reported that the dietary inclusion of 25% TM*d* resulted in fat ADC values slightly lower than those presently obtained for DTM*d* (97 vs 98%, respectively). Moreover, in other fish species, the dietary inclusion of TM or HI*d* up to 25% also resulted in fat ADC values lower than those observed for the experimental diets in sea bass (Piccolo et al., 2017; Belghit et al., 2019b).

Energy digestibility of an ingredient results from the fractional sum of its protein, fat and carbohydrates ADC (NRC, 2011). TM and TM*d* had the highest energy ADC values (79% and 84%, respectively), but still lower than those reported for TM (82%) in Nile tilapia and for fishmeal (88%) in European sea bass (Davies et al., 2009; Fontes et al., 2019). However, these values were well above those previously reported for soybean

meal (69%) in sea bass (Gomes da Silva and Oliva-Teles, 1998) and within the same range of values found in other land animal protein sources (Campos et al., 2018). HI and HI*d* had moderate energy ADC values (67% and 65%, respectively), almost in line with above mentioned values for soybean meal in European sea bass. The low protein and fat content of LM, and high content of crude fibre and NFE, explains its low energy ADC (McGoogan and Reigh, 1996; Gomes da Silva and Oliva-Teles, 1998) and limits the inclusion of this insect in diets for carnivorous fish. In *P. maxima*, the energy ADC values of HI*d* were lower than those observed for HI*d* in sea bass (Kroeckel et al., 2012). In *D. labrax*, the dietary inclusion of 20% HI*d*, to replace FM, resulted in energy ADC values very close to those observed in the present study for DHI*d* (81 vs 82%, respectively) (Magalhães et al., 2017). Contrarily, in *O. mykiss*, the dietary inclusion of 20% HI*d* resulted in lower energy ADC values to those observed for DHI*d* in sea bass (65 vs 82%, respectively) (Renna et al., 2017). Also, in *O. mykiss*, the energy ADC values of dietary inclusion of 25% TM*d* were slightly lower than those obtained for DTM*d* in the present study (84 vs 86%, respectively) (Rema et a., 2019).

Phosphorus is one of the most important minerals for fish growth and bone mineralization. However, phosphorus imbalance in the diet affects its digestibility, which is an indicator of its contribution to water pollution (Abbasi et al., 2019). Unlike plants, the phosphorus in insects is not phytate phosphorus and seems to be readily available to fish (Dashefsky et al., 1976). The IMs better digested by sea bass were those obtained from TM and TM*d*, and their phosphorus ADC values (83 and 91%, respectively) were higher than those observed for a high quality FM in *D. labrax* (80-82%) (Gomes da Silva and Oliva-Teles, 1998; Davies et al., 2009). Moreover, the phosphorus ADC values of HI*d* (73%) were slightly higher than values reported for a low quality FM (49-69%) in sea bass, and much higher than those observed for soybean meal (≈ 21%) (Pimentel-Rodrigues and Oliva-Teles, 2007). When phosphorus digestibility of insects is compared with that of other land animal ingredients, it can be concluded that European sea bass digests TM, TM*d* and HI*d* better than poultry by-product meal and feather meals (Gomes da Silva and Oliva-Teles, 1998; Campos et al., 2018). Likewise, the phosphorus ADC values of TM, TM*d* and HI*d* were higher than soybean and wheat germ by-product meals, revealing that the use of these ingredients is more environmentally friendly than fishmeal, and other animal or plant ingredients (Gomes da Silva and Oliva-Teles, 1998; Campos et al., 2018). HI and LM had the lowest values of phosphorus digestibility, contributing to a lower environmental load. In *O. mykiss*, the dietary inclusion of 25% TM*d* resulted in higher ADC values of phosphorus than those observed in the present study for DTM*d* (70 vs 64%, respectively). The higher phosphorus ADC in HI*d* and TM*d*, compared to HI and TM, may

be attributed to the defatting process that might have increased phosphorus bioavailability, or to distinct feeding schemes, since the ingredients came from different producers.

2.5. Conclusions

The high protein level and high digestibility of TM and TM*d* (> 89%) makes these ingredients particularly valuable for European sea bass diets, to replace FM, as it was clearly demonstrated that besides being rich protein sources, they also have the highest digestible total essential amino acids able to meet this species requirements. Nonetheless, the use of insect meals in European sea bass diets requires further evaluation in order to determine its maximal inclusion level able to assure good growth performance and nutrient utilisation without impairing flesh nutritional quality and fish welfare.

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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Chapter 3.

Central regulation of food intake is not affected by inclusion of defatted *Tenebrio molitor* larvae meal in diets for European sea bass (*Dicentrarchus labrax*)

by

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Abstract

The homeostatic regulation of food intake in fish has been thoroughly studied in the last years with dietary nutrient composition is one of the factors involved. Despite several studies addressed the impact of insect meal-based diets in food intake of fish, there is no knowledge about their impact in mechanisms of food intake regulation. This study aimed to explore underlying mechanisms through evaluation of dietary fishmeal (FM) replacement by defatted (*d*-) *Tenebrio molitor* (*d*TM) larvae meal in European sea bass. Fish (55 ± 2 g) fed a FM-based diet (CTRL) were compared with those fed two experimental diets containing increasing levels of *d*TM to replace 40 and 80% of FM (TM40 and TM80, respectively). After ten weeks of feeding, fish food intake was calculated; samples of plasma and different brain areas (hypothalamus and telencephalon) were collected at different post-prandial times (2, 6 and 24 hours after feeding) to evaluate circulating metabolites and mRNA abundance in hypothalamus and telencephalon of neuropeptides involved in food intake regulation. No differences occurred in food intake and weight gain among diets. Plasma cholesterol levels decreased 24 hours after feeding in fish fed TM40; fish fed *d*TM diets had higher cholesterol levels, 2 and 6 hours after feeding, than those fed CTRL diet. Increased non-esterified fatty acids (NEFA) levels occurred in plasma of fish fed TM80, regardless of the sampling time. At central level, no changes occurred in the mRNA abundance of neuropeptide Y (*npy*), agouti-related protein 2 (*agrp2*), pro-opio melanocortin a (*pomca*) or cocaine- and amphetamine-related transcript 2 (*cartpt2*). The obtained results suggest that dietary FM replacement by *d*TM up to 80% in European sea bass does not affect food intake or its central homeostatic regulation, supporting the use of *d*TM as FM replacement in diets for European sea bass.

Keywords: aquafeeds; hypothalamus; insect meal; neuropeptides; telencephalon.

3.1. Introduction

Aquaculture is the sector of animal production with the fastest growth. Fish oil (FO) and fish meal (FM) remain the major sources of proteins and lipids in aquafeeds. However, the decrease of global availability and high price of these commodities have challenged the feed industry to identify eco-friendly alternatives (FAO, 2020). In this context, the European Union (EU) authorized in 2017 the use of protein from seven insect species in aquafeeds (EC Regulation 893/2017; European Commission, 2017). Among them, *Hermetia illucens* (HI) and *Tenebrio molitor* (TM) are the most studied species as FM substitute in diets for both freshwater and marine fish species (Nogales-Mérida et al., 2018). In a recent study in European sea bass (*Dicentrarchus labrax*), Basto et al. (2020) evaluated the *in vivo* apparent digestibility coefficient of HI and TM (both full-fat and defatted). They concluded that defatted TM (*d*TM) is the most promising alternative to FM not only due to its high digestible protein content, but also due to its high amount of digestible essential amino acids (EAA) content (>89%). Authors also demonstrated that it is possible to substitute dietary FM by *d*TM up to 80% in European sea bass, without detrimental effects on nutrient digestibility, growth performance and associated genetic pathways, whilst assuring fillet nutritional value for human consumption (Basto et al., 2021).

Most feeding trials reported in literature testing insect meal- (IM) based diets in various fish species evaluated food intake, but results are quite variable, and mainly depend on the fish species, the IM fat content, and the percentage of FM replacement. In European sea bass, the replacement of 30% FM by full-fat TM or defatted HI did not alter food intake (Mastoraki et al., 2020), whereas a 71% replacement level by full-fat TM reduced food intake (Gasco et al., 2016). However, the impact of IM inclusion in the mechanisms of food intake regulation remain to be determined.

The regulation of food intake in fish occurs through interaction between homeostatic and hedonic signals. The homeostatic signals result from the necessity of maintaining body energy, while hedonic signalling results from pleasure and/or sensory perception. Both signals are integrated in brain regions, mainly the hypothalamus and telencephalon (Delgado et al., 2017; Soengas et al., 2018; Soengas, 2021). Some particular neurons in these areas possess specific receptors for peripheral hormones (leptin, insulin, ghrelin, etc.) and also mechanisms able to sense changes in nutrient levels, particularly glucose, amino acids (AA) and fatty acids (FA) (Soengas, 2014; Sieira, Soengas, 2017; Soengas et al., 2018). Information from hormones and nutrient sensors lead to changes in cellular signals and transcription factors (Soengas, 2021). The

integration of these signals (Soengas, 2021) results in changes in the mRNA abundance of neuropeptides namely the orexigens agouti-related peptide (AgRP) and neuropeptide Y (NPY), and the anorexigens cocaine- and amphetamine-related transcript (CART) and pro-opio melanocortin (POMC). These changes in neuropeptide expression result in increased or decreased food intake (Delgado et al., 2017; Soengas et al., 2018).

Since there is no knowledge about the impact of IM-based diets in the central regulation of food intake in fish, this study will explore its underlying mechanisms through evaluation of dietary FM replacement by *d*TM, at different postprandial times, in hypothalamus and telencephalon of European sea bass

3.2. Materials and methods

The animal study followed European (2010/63/EU) animal directives on the handling of experimental animals, and were approved by CIIMAR ethics committee.

3.2.1. Experimental diets

Following nutritional requirements for European sea bass (NRC, 2011), a FM-based diet with 48% crude protein on a dry matter (DM) basis was formulated and used as control (CTRL). Two other isonitrogenous and isoenergetic diets were formulated to replace 40 and 80% of FM by *d*TM (TM40 and TM80, respectively). The TM larvae used in this study were produced by Entomo Farm (France). The larvae were exclusively fed with certified organic foodstuff and processed mechanically yelding *d*TM – 65% crude protein and 12% lipids. All diets were extruded by SPAROS Lda. (Portugal). Ingredients and chemical composition of experimental diets is presented on Table 3.1. Amino acid and fatty acid profiles of diets are summarized on Table 3.2. Experimental diets are the same in this and our previous study (Basto et al., 2021).

Table 3.1. Ingredients (%) and chemical composition (% DM or kJ g^{-1} DM) of the experimental diets

	CTRL	TM40	TM80
Ingredients			
Fishmeal[1]	45	27	9
Defatted *Tenebrio molitor* larvae meal[2]	-	18	36
Soy protein concentrate[3]	8	8	8
Soybean meal[4]	10	10	10
Rapeseed meal[5]	5	5	5
Wheat meal[6]	17	17	16
Sardine oil[7]	13	13	13
Vitamin and mineral premix[8]	1	1	1
Binder[9]	1	1	1
Mono-calcium phosphate	-	-	1
DL-Methionine[10]	-	0.2	0.4
Chemical composition			
Dry matter	93.5	94.3	93.2
Protein	47.8	47.7	47.0
Lipids	18.7	19.2	20.4
Energy (kJ g^{-1} DM)	22.0	22.6	23.4
Phosphorus	1.4	1.1	1.0
Ash	11.7	9.0	7.4

The abbreviations for the test experimental diets stand for: CTRL, control diet; TM40 and TM80, diet with 40 and 80% of fishmeal replacement by defatted *Tenebrio molitor*, respectively. [1] Peruvian fishmeal super prime: 71.0% protein, 11.0% lipids, Exalmar S.A.A, Peru. [2] Defatted *Tenebrio molitor* larvae meal: 65% protein, 12% lipids, Entomo Farm, France. [3] Soy protein concentrate: 65% protein, 0.7% lipids, ADM Animal Nutrition™, The Netherlands. [4] Soybean meal 48: 47.7% protein, 2.2% lipids, Cargill, Spain. [5] Rapeseed meal: 36% protein, 2.7% lipids, Premix Lda., Portugal. [6] Wheat meal: 10.2% protein, 1.2% lipids, Casa Lanchinha Lda., Portugal. [7] Sardine oil: Sopropêche, France. [8] Vitamin and mineral premix: WISIUM, ADM Portugal S.A., Portugal. [9] Kielseguhr (natural zeolite): LIGRANA GmbH, Germany. [10] DL-Metionine 99%: Evonik Degussa GmbH, Germany.

Table 3.2. Amino acid and fatty acid profiles (% DM) of the experimental diets

	CTRL	TM40	TM80
Amino acids			
Arginine	3.8	3.7	3.8
Histidine	1.1	1.4	1.4
Lysine	3.3	2.8	2.6
Threonine	1.7	2.0	1.8
Isoleucine	2.1	2.3	2.3
Leucine	3.8	3.5	3.6
Valine	2.4	2.6	2.9
Methionine	1.5	1.4	1.3
Phenylalanine	2.5	2.3	2.2
Cystine	0.3	0.3	0.3
Tyrosine	2.4	2.6	2.9
Aspartic acid + Asparagine	4.1	4.0	3.9
Glutamic acid + Glutamine	7.8	6.4	6.5
Alanine	2.5	2.8	2.9
Glycine	3.2	3.3	3.2
Proline	3.3	3.0	2.9
Serine	1.9	2.0	1.9
Taurine	0.2	0.2	0.06
Fatty acids			
C14:0	1.1	1.1	1.1
C16:0	3.1	3.3	3.4
C18:0	0.7	0.7	0.7
Σ SFA[1]	5.3	5.5	5.6
C16:1n-7	1.2	1.1	1.1
C18:1n-9	1.6	2.2	2.8
C18:1n-7	0.5	0.4	0.4
C20:1n-9	0.4	0.3	0.2
C22:1n-11	0.06	0.04	0.03
Σ MUFA[2]	4.4	4.7	4.8
C18:2n-6	0.5	1.2	1.9
C18:3n-3	0.2	0.2	0.2
C18:4n-3	0.3	0.3	0.3
C20:4n-6	0.1	0.1	0.1
C20:5n-3, EPA	2.0	1.8	1.8
C22:5n-3	0.1	0.1	0.1
C22:6n-3, DHA	1.4	1.3	1.1
EPA+DHA	3.4	3.1	2.9
Σ PUFA[3]	5.4	5.6	6.2
Σ n-3 LC-PUFA[4]	4.3	3.9	3.7
Σ n-6 LC-PUFA[5]	0.7	1.4	2.1
Σ n-3/Σ n-6	5.9	2.8	1.8

[1] Includes C12:0, C13:0, C15:0, C17:0, C20:0, C20:0, C22:0, C24:0. [2] Includes C14:1n-5, C16:1, C17:1n-7, C20:1n-7, C20:1n-11, C22:1n-9, C24:1n-9. [3] Includes C16:2n-4, C16:3n-4, C16:4n-1, C18:2n-4, C18:3n-4, C18:3n-6, C18:4n-1, C20:2n-6, C20:3n-3, C20:4n-3, C20:3n-6, C20:3n-3, C22:2n-6, C21:5n-3 C22:4n-6. [4] Includes C20:3n-3, C20:4n-3, C21:5n-3. [5] Includes C18:3n-6, C20:3n-6, C22:4n-6.

3.2.2. Feeding trial

European sea bass juveniles were obtained from Acuinuga S. L. (Spain). Fish were held in quarantine for 2 weeks and hand fed with a commercial diet (AQUASOJA – 50% crude protein, 20% lipids). After acclimatization, all fish were individually weighed (g) and measured (total length, cm) and nine homogeneous groups of 25 fish (body weight 55 ± 2 g; total length 18 ± 1 cm) were distributed in fibreglass tanks of 160 L within a seawater recirculation system. Fish were adapted to the new conditions for 3 days (water temperature of 24 ± 1 °C, salinity of 35‰, flow rate at 6 L min^{-1}, oxygen level > 90% ± 1 saturation, and photoperiod regime 12 h light/12h dark). Levels of total ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-), as well as pH were daily monitored to ensure levels within the recommended ranges for marine fish species ($NH_4^+ \leq 0.05$ mg L^{-1}; $NO_2^- \leq 0.5$ mg L^{-1}; $NO_3^- \leq 50$ mg L^{-1}; $7.5 \leq pH \leq 8.5$). Each diet was randomly assigned to triplicate groups of fish. Fish were fed by automatic feeders three times a day until apparent satiation, as previously detailed in Basto et al. (2021). At the end of 10 weeks feeding trial, fish were anesthetized with 2-Phenoxyethanol (200 µL L^{-1}) in the tank before being sampled at 2, 6 and 24 h after feeding. These time periods were selected based on previous studies (Conde-Sieira and Soengas, 2017). All fish were individually weighed (g) and measured (total length, cm) and blood of 6 fish per dietary treatment (2 fish per tank) was collected with heparinized syringes from the caudal vein, immediately centrifuged (10,000 *g* for 5 min at 4 °C) and the resulting plasma (supernatant) was collected and stored at -80 °C until metabolite analysis. Then, fish were sacrificed by decapitation, and hypothalamus and telencephalon were collected and immediately frozen in dry ice and stored at -80 °C until RNA extraction.

3.2.3. Metabolite levels analysis

Levels of glucose, lactate, triglyceride, total cholesterol and non-esterified FA (NEFA) were determined enzymatically using commercial kits (1001190, 1001330, 1001313 and 1001090, Spinreact, Spain, and 434-91795 NEFA-HR (2) R1 and 436-91995 NEFA-HR (2) R2, Wako Chemicals, Germany, respectively), adapting manufacturer's instructions to a microplate format.

3.2.4. mRNA abundance analysis by RT-qPCR

Total RNA was extracted from hypothalamus and telencephalon using TRIzol reagent (Life Technologies, USA) and treated with RQ1-DNAse (Promega, USA)

according to the manufacturer's recommendations. Two µg of total RNA was reverse transcribed to cDNA using Superscript II reverse transcriptase and random hexamers (Promega, USA) following the standard protocol. Relative expression of genes was determined by real-time quantitative PCR (RT qPCR) using the 7900HT Fast Real Time PCR System (Applied Biosystems-Thermofisher, USA). Analyses were performed on 1 µl cDNA using MAXIMA SYBR Green qPCR Mastermix (Life Technologies, USA), in a total PCR reaction volume of 15 µl, containing 50–500 nM of each primer. Expression of *agrp2*, *npy* and *pomca* was measured using previously described primers in the same fish species (Tsalafouta et al., 2017; Brandts et al., 2018; Cerdá-Reverter et al., 2000). For *cartpt2*, new primers were designed using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) from sequences available based on those obtained from a European sea bass genome database (http://seabass.mpipz.mpg.de/cgi-bin/hgGateway). A fragment of each sequence containing the amplicon was amplified by conventional PCR and run on a 1.2% agarose gel. The corresponding bands were cut from the gel, purified with the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, USA) and sequenced in a SeqStudio Genetic Analyser (Applied Biosystems-Thermofisher, USA) in Servicio de Determinación Estructural, Proteómica y Genómica (CACTI-Universidade de Vigo). The obtained sequences satisfactorily matched the reference GenBank sequences. Thermal cycling was initiated with incubation at 95 °C for 15 min using hot-start iTaq DNA polymerase activation; 40 steps of PCR were performed, each one consisting of heating at 95 °C for 15 s for denaturing, and at specific annealing temperature for 30 s and extension at 72 °C for 30 s. Following the final PCR cycle, melting curves were systematically monitored (55 °C temperature gradient at 0.5°C/s from 55 to 95 °C) to ensure that only one fragment was amplified. Each sample was analysed in duplicate and samples without reverse transcriptase or without cDNA were run as negative controls. Relative quantification of target gene transcripts was done using elongation factor 1α (*eef1a1*) and 18s ribosomal RNA (*18s*) as housekeeping genes, following the Pfaffl (2001) method. The sequences of the forward and reverse primers used for each transcript expression are shown in Table 3.3.

3.2.5. Statistical analysis

Data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov and Levene's tests, respectively, and log-transformed whenever required before being submitted to a two-way ANOVA, with time and diet as main factors. A post-hoc Tukey test was used to identify differences between means. In all cases, significant

differences were considered when P values < 0.05. Statistical analyses were performed using Statistica™ 13.5.0.17 (TIBCO Software Inc., USA).

3.3. Results

Food intake and weight gain of European sea bass fed dietary treatments are represented in Figure 3.1. After ten weeks of feeding, the dietary inclusion of *d*TM did not affect fish food intake (Figure 3.1A) or fish growth (Figure 3.1B). The plasma levels of glucose, lactate and triglyceride did not change with *d*TM dietary inclusion level or sampling time (Figure 3.2A, 3.2B and 3.2C, respectively). P and F values of the two-way ANOVA applied to plasma parameters are shown in Table 3.4. In fish fed TM40, plasma cholesterol levels significantly ($P < 0.05$) decreased 24 hours after feeding (Figure 3.2D). Fish fed *d*TM diets had significantly higher ($P < 0.05$) cholesterol levels in plasma at 2 and 6 hours after feeding compared with those fed CTRL diet (Figure 3.2D). The levels of NEFA significantly ($P < 0.05$) increased in plasma of fish fed TM80, regardless of the sampling time (Figure 3.2E). At central level, no significant changes occurred in the mRNA abundance of orexigenic (*npy* and *agrp2*) or anorexigenic (*pomca* and *cartpt2*) neuropeptides, both in hypothalamus and telencephalon (Figure 3.3). The mRNA abundance of *pomca* was not detected in telencephalon.

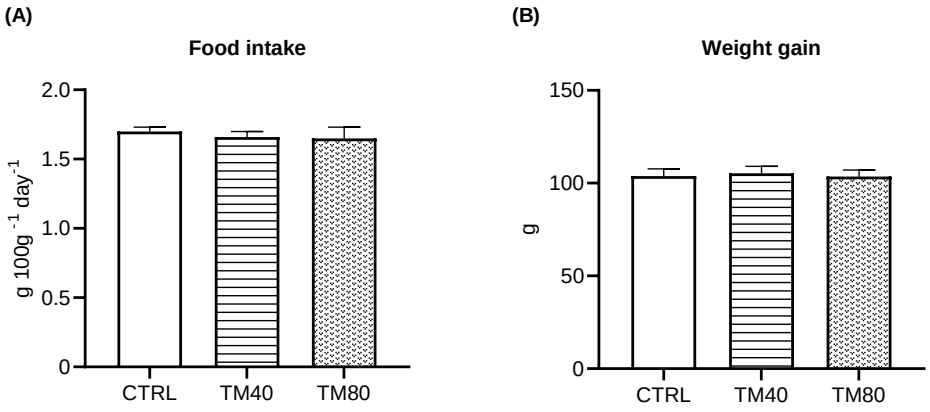

Figure 3.1. Food intake (A) and weight gain (B) of European sea bass fed for 10 weeks with control diet containing FM (CTRL) or with two experimental diets formulated to replace 40% (TM40) or 80% (TM80) of FM by *d*TM. Bars represent means + SEM of 75 fish per dietary treatment.

Table 3.3. Oligonucleotide sequences used to evaluate relative mRNA abundance of genes by RT-qPCR

Gene	Primer sequence 5'-3'	Annealing temperature (°C)	PCR Efficiency (%)	Database	Accession number
agrp2	F: GGGCAGAGGACACAAAGAAA	56	Hypothalamus: 92	GeneBank	HE660087
	R: TGTGACTTTCCTGTGGTGGA		Telencephalon: 94		
cartpt2	F: CCGAACCTGACCAGCGAGAA	62	Hypothalamus: 100	GeneBank	MZ441181
	R: GCTCCCCGACATCACACGTT		Telencephalon: 102		
eef1a1	F: CGTTGGCTTCAACATCAAGA	55	Hypothalamus: 102	GeneBank	AJ866727
	R: GAAGTTGTCTGCTCCCTTGG		Telencephalon: 104		
npy	F: ACGGAGGGATACCCGGTGAA	60	Hypothalamus: 94	GeneBank	AJ005378
	R: GCTGAGTAGTACTTGGCCAGCTC		Telencephalon: 96		
pomca	F: CCGGTCAAAGTCTTCACCTC	57	Hypothalamus: 101	GeneBank	AY691808
	R: ACCTCCTGTGCCTTCTCCTC		Telencephalon: 104		
18s	F: CCGCTTTGGTGACTCTAGATAACC	60	Hypothalamus: 105	GeneBank	AY831388.1
	R: CAGAAAGTACCATCGAAAGTTGATAGG		Telencephalon: 95		

agrp2, agouti-related protein 1; *cartpt2,* cocaine- and amphetamine-related transcript; *eef1a1,* elongation factor 1α; *npy,* neuropeptide Y; *pomca,* pro-opio melanocortin a; *18s,* 18S ribosomal RNA. * Pending validation by the National Center for Biotechnology Information.

Table 3.4. *F*- and *P*-values obtained after two-way ANOVA of parameters assessed in plasma

	Diet		Time		Diet × Time	
	F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Plasma metabolites						
Glucose	1.54	0.23	2.85	0.07	0.29	0.88
Lactate	2.72	0.08	2.87	0.07	1.82	0.15
Triglycerides	0.30	0.75	0.54	0.59	0.75	0.57
Cholesterol	21.44	< 0.001	21.73	< 0.001	4.64	< 0.001
NEFA	4.54	0.02	0.06	0.94	1.22	0.33

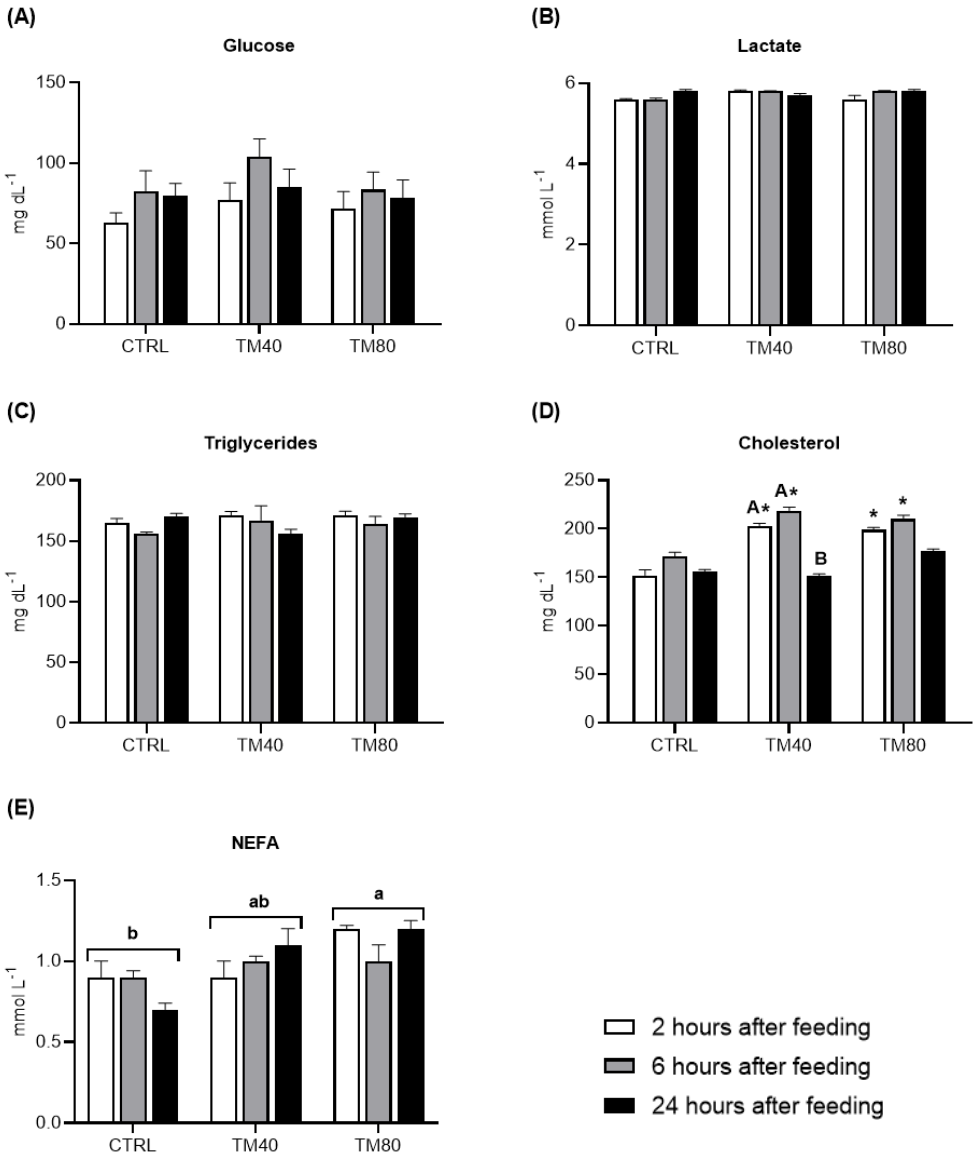

Figure 3.2. Post-feeding levels of metabolites in plasma of European sea bass previously fed for 10 weeks with control diet containing FM (CTRL) or with two experimental diets formulated to replace 40% (TM40) or 80% (TM80) of FM by *d*TM. Bars represent means + SEM of 6 fish per dietary treatment at each sampling time. Different lowercase letters indicate significant differences ($P < 0.05$) between dietary treatments, regardless sampling time. Different uppercase letters indicate significant differences ($P < 0.05$) between sampling times within the same dietary treatment; * indicate significant differences ($P < 0.05$) from CTRL at the same sampling time.

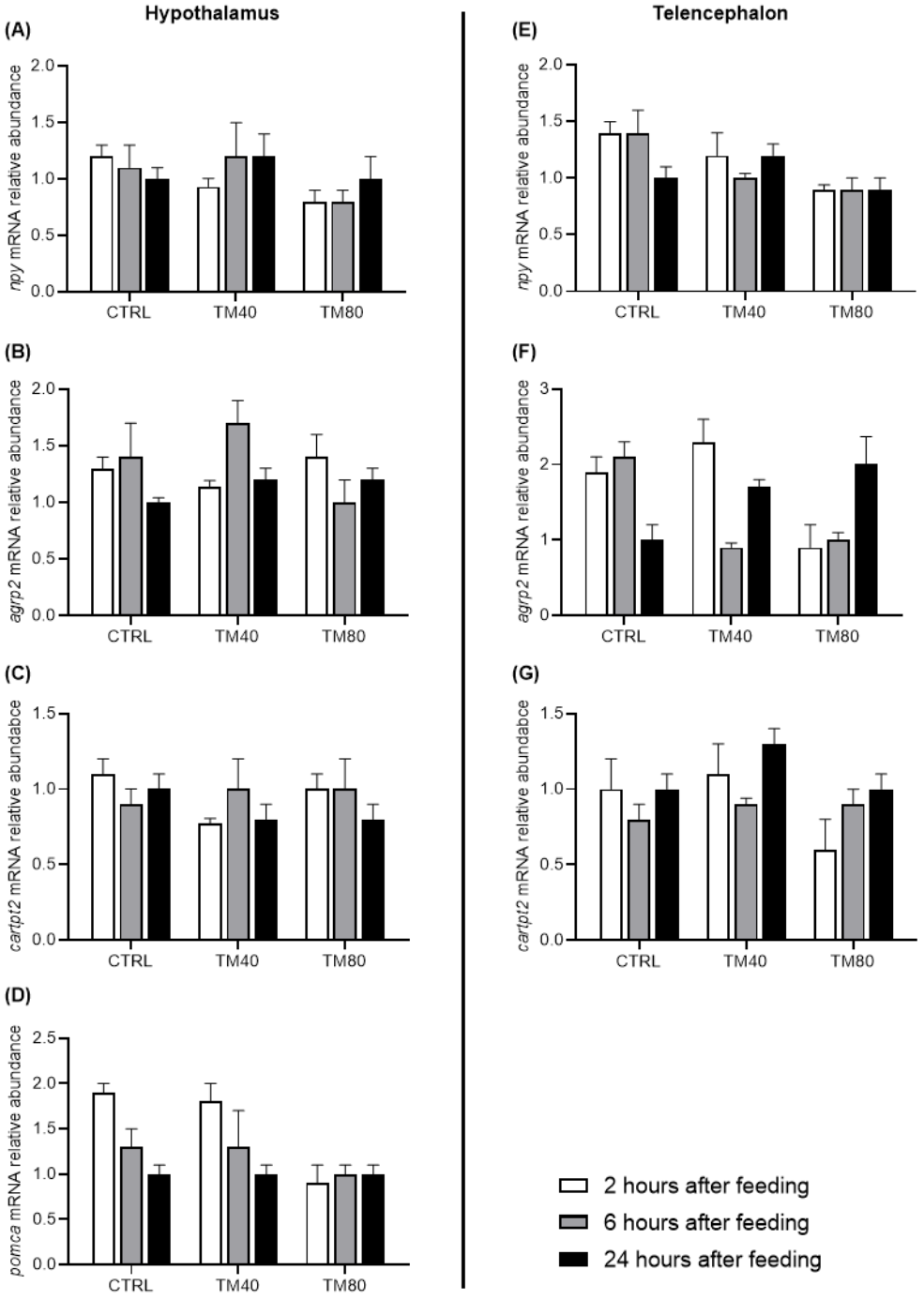

Figure 3.3. Post-feeding mRNA abundance of neuropeptides involved in regulation of food intake in hypothalamus (A-D) and telencephalon (E-G) of European sea bass previously fed for 10 weeks with control diet containing FM (CTRL) or with two experimental diets formulated to replace 40% (TM40) or 80% (TM80) of FM by *d*TM. Bars represent mean + SEM of 6 fish per dietary treatment at each sampling time. Gene expression results were previously normalized by *eef1a1* and *18s* mRNA abundance.

3.4. Discussion

All experimental diets displayed different FA profiles, mainly reflecting FM and *d*TM FA composition. The *d*TM diets were characterized by high levels of oleic (C18:1n-9) and linoleic (C18:2n-6) acids. The dietary n-6 LC-PUFA levels were increased in *d*TM diets in comparison to the CTRL diet, whereas the levels of n-3 LC-PUFA were reduced, consequently the ratio between n-3 LC-PUFA and n-6 LC-PUFA decreased with dietary inclusion of *d*TM. The dietary n-6 LC-PUFA levels were increased in *d*TM diets in comparison to the CTRL diet, whereas the levels of n-3 LC-PUFA were reduced, consequently the ratio between n-3 LC-PUFA and n-6 LC-PUFA decreased with dietary inclusion of *d*TM. It has been demonstrated that plasma cholesterol levels decrease with the increase of n-3 LC-PUFA levels, whereas the opposite occurs with the increase of n-6 LC-PUFA levels (Zhang et al., 2009), thus the above-mentioned characteristics of FA profile of dietary treatments may explain the increase of cholesterol levels in plasma at 2 and 6h after feeding in fish fed *d*TM diets. Besides, phosphorus deficiencies have been associated to hypercholesterolemia (Ballester-Lozano et al., 2015), thus the reduced content of this mineral in *d*TM in comparison with CTRL diet may also contributed to the increase of cholesterol. Some studies have been demonstrated that the chitosan present in chitin has cholesterol-lowering properties in fish (Abdel-Ghany and Salem, 2019). However, this effect was not observed in the present study probably due to the low chitin (approximately 4.8% DM) content in *d*TM. Contrarily to our results, Mastoraki et al. (2020) previously observed a decrease of glucose and cholesterol plasma levels after 30% FM replacement by full-fat TM. But the percentage of FO present in TM diet in the study of Mastoraki et al (2020) was almost half to that present in the control diet (6% vs 10%, respectively), resulting in a decrease of n-3 PUFA relative percentage in TM diet and explaining the lowering of plasma cholesterol levels. In the present study, all experimental diets were formulated with 13% FO, namely sardine oil, which is considered a premium oil due to their high content of n-3 LC-PUFA. Despite the reduced n-3 LC-PUFA levels in *d*TM diets when compared to CTRL, all experimental diets contained levels of these FA (3.8-4.3%) above the minimum recommended for marine fish species (approximately 0.7% DM; Skalli and Robin, 2004), which may have contributed to the similar growth performance of fish fed different dietary treatments. Since the dietary lipids content of an experimental diet has a direct impact on circulating NEFA levels (Díaz-Rúa et al., 2020), the increase of lipids content in TM80 may explain the increased NEFA levels in plasma of fish fed this dietary treatment. The amino acid profile of experimental diets was in general similar to that of controls except for some amino acids that describe an increase (i.e. alanine and tyrosine) or decrease (i.e. lysine and taurine).

The replacement of 40-80% of FM by *d*TM meal did not induce changes in European sea bass food intake and weight gain. Likewise, in previous study a 30% FM replacement by full-fat TM meal did not affect food intake (Mastoraki et al., 2020), but higher replacement levels (36-71%) decreased food intake (Gasco et al., 2016). However, the present results clearly suggest that European sea is able to tolerate up to 80% *d*TM without impairing food intake and growth performance. Therefore, in terms of IM inclusion, and from a food intake point of view, the use of *d*TM instead of a full fat meal allows a higher replacement of FM without compromising food intake in European sea bass. High replacement levels without compromising food intake, and even improving it, also were evident in red sea bream (*Pargus major*) and rainbow trout (*Oncorhynchus mykiss*) fed *d*TM (Ido et al., 2019; Chemello et al., 2020). However, contrarily to the present study none of these studies assessed the underlying mechanisms involved in the regulation of food intake.

In fish, previous studies have described changes in the mechanisms involved in food intake regulation due to dietary composition, especially due to glucose, AA and FA levels (Conde-Sieira, Soengas, 2017; Soengas et al., 2018). As previously discussed, all experimental diets displayed particularly different FA profiles. We have previously demonstrated in rainbow trout that an increase in the levels of the medium-chain SFA octanoate (C8:0) acid or the LC-MUFA oleic acid resulted in decreased food intake. This occurred at the same time that changes in hypothalamic mRNA abundance of neuropeptides, namely reduced values of the orexigens *npy* and *agrp2*, and increased values of the anorexigens *pomca* and *cartpt* (Librán-Perez et al., 2012; Librán-Perez et al., 2013; Librán-Perez et al., 2014). Similar responses occurred in hypothalamic mRNA abundance of neuropeptides in Senegalese sole (*Solea senegalensis*) (Conde-Sieira et al., 2015) after an increase in the levels of the SFA stearic acid (C16:0), oleic acid, and the n-3 PUFA α-linolenic acid (C18:3n-3), and EPA. No prior study assessed the impact of FA on mechanisms involved in regulation of food intake in European sea bass, though we may consider that similar mechanisms to those characterized in other fish species might be present in European sea bass. When we compare FA profile of the *d*TM diets with the CRTL diet, it is possible to observe that most FA mentioned above are similar, with the exception of oleic acid and linoleic acid that displayed higher levels in the TM80. The fact that even considering the high levels of oleic acid in that diet no significant changes occurred either in food intake or in the mRNA abundance of neuropeptides allow us to suggest that in this species the raised levels of those FA were not enough to induce changes in the mechanisms involved in food intake regulation. This different behaviour of responses attributable to a FA alone or to different FA composition also occurred in

rainbow trout (Conde-Sieira et al., 2015). We have already demonstrated that several amino acids including leucine, valine, are involved in food intake regulation in fish through different amino acid sensing mechanisms (Comesaña et al., 2018a; Comesaña et al., 2018b). Assuming these mechanisms are present in European sea bass, considering that levels of these amino acids were comparable among diets in the present study, we may suggest a minor (if any) role for them in regulation in food intake in fish fed with the experimental diets.

Besides nutrient composition of experimental diets, other factors that could have influenced food intake regulation would be time of exposure of fish during the feeding trial. In this way, is important to remark that fish fed the experimental diets for 10 weeks, after which we evaluated 2, 6 and 24h after feeding changes in neuropeptide mRNA abundance. In other fish species, changes described in food intake regulation, in response to dietary levels of FA (Librán-Perez et al., 2012; Librán-Perez et al., 2013; Librán-Perez et al., 2014) or AA (Comesaña et al., 2018a; Comesaña et al., 2018b) occurred in the first hours post-feeding after the first exposure to the nutrient. Thus, further studies are necessary to elucidate if that short-term effect can also be induced by *d*TM diets. On the other hand, the synthesis of neuropeptides in brain areas involved in food intake regulation also respond to changes in the levels of several peripheral hormones like ghrelin, leptin, insulin, glucagon-like peptide 1 or cholecystokinin (Soengas et al., 2018). No studies in fish assessed the impact of IM replacement of FM in circulating levels of any of these hormones. However, in zebrafish (*Danio rerio*), the replacement of 25-100% of FM by full-fat HI and vegetable mix resulted in decreased intestinal mRNA abundance of leptin and ghrelin gene transcripts, which occurred in parallel with a better growth suggesting enhanced food intake (Zarantoniello et al., 2020). Therefore, we cannot discard changes in hormone levels in the present study, which could interact with those of nutrient levels in the integration leading to neuropeptide synthesis, and ultimately regulating food intake (Soengas, 2021). However, the absence of changes in food intake and growth in the present study suggest a minimal (if any) impact of changes in circulating hormones in the integrative response.

3.5. Conclusions

The results obtained suggest that dietary replacement of FM by 40-80% of *d*TM for 10 weeks, despite increases circulating cholesterol levels does not affect growth, food intake, and its homeostatic regulation in European sea bass at central level. Considering that the same experimental diets did not induce detrimental effects on nutrient digestibility, growth performance, and fillet nutritional value for human consumption (Basto et al.,

2021), these results certainly will help to improve sustainability of diet formulation for this species using *d*TM meal in replacement of an important amount of FM. However, a short-term study is necessary to characterize the response of mechanisms involved in the regulation of food intake immediately after exposure to diets rich in *d*TM.

Declaration of competing interest

The authors declare that they have no competing interests.

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Chapter 4.

The use of defatted *Tenebrio molitor* larvae meal as a main protein source is supported in European sea bass (*Dicentrarchus labrax*) by data on growth performance, lipid metabolism and flesh quality

by

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Abstract

Objective: This study aims to determine the maximal inclusion level of defatted (*d*-) *Tenebrio molitor* larvae meal (TM) able to replace dietary fishmeal (FM) without compromising growth performance, general metabolism, and flesh quality traits in European sea bass, and to evaluate the major underlying physiological mechanisms.

Materials and methods: Fish (55 ± 2 g) were fed with diets containing increasing levels of *d*TM: 0, 40, 80 and 100% (CTRL, TM40, TM80, and TM100, respectively) to replace FM. After 10 weeks of feeding, the growth performance, nutrient and energy balance, intestinal integrity, plasma metabolites and the expression of genes related to growth and nutrient metabolism, in liver and muscle were determined. The fatty acids (FA) profile, textural properties and colour were also evaluated in muscle.

Results: Protein and lipids digestibility remained unaltered up to 80% *d*TM inclusion. Growth performance parameters were similar among dietary treatments. The *d*TM inclusion increased the hepatosomatic index in fish fed TM100. Muscle eicosapentaenoic acid, docosahexaenoic acid and n-3 long-chain polyunsaturated FA levels were maintained up to 80% *d*TM inclusion, but total cholesterol and non-esterified FA increased with dietary *d*TM inclusion. In liver, the expression of elongation of very long-chain FA protein 6 (*elovl6*) and FA desaturase 2 (*fads2*) did not change in fish fed TM40 and TM80, but *elovl6* decreased whilst *fads2* increased in fish fed TM100 when compared to those fed CTRL. The expression of cholesterol 7 alpha-monooxygenase (*cyp7a1*) decreased with dietary *d*TM inclusion. In muscle, the expression of myoblast determination protein-2 (*myod2*) decreased in fish fed TM80 and TM100.

Conclusion: It is feasible to substitute dietary FM by *d*TM up to 80% in European sea bass without detrimental effects on nutrient digestibility, growth performance and associated genetic pathways, whilst assuring fillet nutritional value for human consumption.

Keywords: alternative protein sources; fatty acids profile; feedstuffs; insect meal; muscle cell proliferation and differentiation; nutrient utilization.

4.1. Introduction

The use of insect protein as sustainable alternative to animal and plant protein sources has been encouraged for direct human consumption and incorporation into animal feeds (Nogales-Mérida et al., 2018; Salter, 2019). Insects have several advantages when compared with the conventional protein sources, as they grow fast and reproduce easily, have low feed conversion ratio (FCR) and small need of arable land and water (Gasco et al., 2020). Additionally, insects are rich protein sources, with a well-balanced essential amino acid (EAA) profile; being also rich sources of fat and some vitamins and minerals (Koutsos et al., 2019). Insects can also be valuable sources of healthy compounds, such as chitin and antioxidant and antimicrobial peptides. Recent studies showed that insect-based diets modulate fish microbiota and improve the immune system, which may reduce the use of antibiotics in aquaculture (Bruni et al., 2018: Henry et al., 2018; Antonopoulou et al., 2019; Rimoldi et al., 2019; Stenberg et al., 2019; Li et al., 2021). In 2017, European Union (EU) authorized the use of insect meal (IM) from seven species, including yellow mealworm (*Tenebrio molitor*), in aquafeeds (European Commission, 2017). Basto et al. (2020) have recently demonstrated that defatted (*d-*) *T. molitor* larvae meal (TM) is a highly digestible protein source able to meet European sea bass (*Dicentrarchus labrax*) EAA requirements (Basto et al., 2020). The potential of TM to partially replace fishmeal (FM) in aquafeeds has been previously assessed in various marine fish species, such as European sea bass or sparids (Gasco et al., 2016; Piccolo et al., 2017; Henry et al., 2018; Antonopoulou et al., 2019; Iaconisi et al., 2019; Ido et al., 2019), whilst total replacement was only evaluated in red sea bream (*Pargus major*) (Ido et al., 2019). Results among authors are controversial, and the maximal replacement level of FM by TM are extremely variable and never exceeding 71% in diets for European sea bass (Gasco et al., 2016).

The main nutritional limitation of including insects in aquafeeds is their low content of long-chain polyunsaturated fatty acids (LC-PUFA), such as the eicosapentaenoic (EPA; 20:5n−3) and docosahexaenoic (DHA; 22:6n−3) acids. Fish muscle is the main dietary source of these two n-3 LC-PUFA for humans, which are associated with beneficial health effects (Lands, 2014). Thus, IM may compromise not only flesh quality, but also growth performance when included at high levels in diets for marine fish species. Fish oil (FO) and FM are the main dietary sources of n-3 LC-PUFA, such as EPA and DHA. Thus, high FM replacement levels, or the concomitant replacement of FM and FO, have to be carefully addressed to not compromise the recommended dietary levels of n-3 LC-PUFA for marine fish species (approximately 0.7% on dry matter (DM) basis) (Skalli and Robin,

2004). In blackspot sea bream (*Pagellus bogaraveo*), full-fat TM did not affect fillet EPA or DHA content, when included at 21% of feed, but decreased the EPA content when included at 40% (Iaconisi et al., 2017). This suggest that dietary IM may induce changes in lipid metabolism, depending on its dietary inclusion level. Besides, IM also has a limited content of phosphorus (P), which may affect not only lipid metabolism, but also fish growth performance (Sugiura et al., 2004; Prabhu et al., 2014).

The present study aimed to explore the impact of partial and total replacement of FM by *d*TM in a comprehensive approach focusing not only on growth performance and nutrient utilization, but also on the underlying mechanisms involved in nutrient metabolism, namely lipid metabolism, in European sea bass. This is one of the most important fish species in Mediterranean aquaculture, where production surpassed 194,000 tons in 2018 (Eurostat, 2020). Muscle quality traits and fillet nutritional value for human consumption were also evaluated.

4.2. Materials and methods

4.2.1. Diets

Extruded practical diets were formulated and produced by SPAROS Lda. (Portugal) to meet all the known nutritional requirements of European sea bass (NRC, 2011). All diets contained FO (12–13% inclusion level) as the main dietary lipid source. In the control diet (CTRL), FM was added at 45% and was progressively replaced in experimental diets (40%, TM40; 80%, TM80; 100%, TM100) by *d*TM from Entomo Farm (France). The inclusion level of *d*TM increased up to 60% in TM100, and was due to the concomitant replacement of FM and plant proteins, resulting in increased protein and lipid content (Table 4.1). All experimental diets were properly supplemented with DL-methionine and mono-calcium phosphate. For determination of the apparent digestibility coefficients (ADC), 1% chromium oxide (Cr_2O_3) was added, as an inert marker, to each experimental diet. Details of dietary fatty acid (FA) and amino acid (AA) profile are shown in Table 4.2 and Supplementary Table S1 (Appendix), respectively.

4.2.2. Fish husbandry

Juvenile fish of Atlantic origin were obtained from Acuinuga S. L. (Spain). Fish, for both the digestibility and the growth trials, were held in quarantine (2000 L tanks) for 2 weeks and hand-fed with a commercial diet (AQUASOJA, Portugal – 50% crude protein and 20% crude fat on DM basis). During quarantine period, fish were held in a recirculation aquaculture system (RAS) at 24 ± 1°C, 35 ± 0.5 ‰ and 6 L min^{-1}. Total

ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and pH levels were maintained within the recommended ranges for marine species ($NH_4^+ \leq 0.05$ mg L^{-1}; $NO_2^- \leq 0.5$ mg L^{-1}; $NO_3^- \leq 50 \leq$ mg L^{-1}; $7.5 \leq$ pH ≤ 8.5). Dissolved oxygen level was kept above 90% saturation and an artificial photoperiod of 12 h' light/12 h' dark cycle was fixed.

Table 4.1. Ingredients and chemical composition of the experimental diets.

	CTRL	TM40	TM80	TM100
Ingredients, g/kg				
Fishmeal[1]	450	270	90	-
Defatted *Tenebrio molitor* larvae meal[2]	-	180	360	600
Soy protein concentrate[3]	80	80	80	-
Soybean meal[4]	100	100	100	-
Rapeseed meal[5]	50	50	50	-
Wheat meal[6]	170	168	155	240
Sardine oil[7]	130	130	130	121
Vitamin and mineral premix[8]	10	10	10	10
Binder[9]	10	10	10	10
Mono-calcium phosphate	-	-	11	13
DL-Methionine[10]	-	2	4	6
Chemical composition, g/100 g DM				
Dry matter	93.5	94.3	93.2	94.2
Protein	47.8	47.7	47.0	50.6
Lipids	18.7	19.2	20.4	22.1
Energy, kJ/g DM	22.0	22.6	23.4	24.5
Phosphorus	1.4	1.1	1.0	1.0
Ash	11.7	9.0	7.4	6.5

CTRL, control diet; DM, dry matter; TM40, TM80 and TM100, diet with 40, 80 and 100% of fishmeal replacement by defatted *Tenebrio molitor* larvae meal, respectively. [1] Peruvian fishmeal super prime: 71.0% protein, 11.0% lipids, Exalmar S.A.A, Peru. [2] Defatted *Tenebrio molitor* larvae meal: 65% protein, 12% lipids, 4.8% chitin, Entomo, France. [3] Soy protein concentrate: 65% protein, 0.7% lipids, ADM Animal Nutrition™, The Netherlands. [4] Soybean meal 48: 47.7% protein, 2.2% lipids, Cargill, Spain. [5] Rapeseed meal: 36% protein, 2.7% lipids, Premix Lda., Portugal. [6] Wheat meal: 10.2% protein, 1.2% lipids, Casa Lanchinha Lda., Portugal. [7] Sardine oil: Sopropêche, France. [8] Vitamin and mineral premix: WISIUM, ADM Portugal S.A., Portugal. [9] Kielseguhr (natural zeolite): LIGRANA GmbH, Germany. [10]DL-Metionine 99%: Evonik Degussa GmbH, Germany.

Table 4.2. Fatty acid profile of the experimental diets.

	CTRL	TM40	TM80	TM100
Fatty acids, g/100 g DM				
14:00	1.1	1.1	1.1	1.2
16:00	3.1	3.3	3.4	4.2
18:00	0.7	0.7	0.7	0.9
Σ SFA[1]	5.3	5.5	5.6	6.8
16:1n-7	1.2	1.1	1.1	1.1
18:1n-9	1.6	2.2	2.8	4.7
18:1n-7	0.5	0.4	0.4	0.5
20:1n-9	0.4	0.3	0.2	0.2
22:1n-11	0.06	0.04	0.03	0.02
Σ MUFA[2]	4.4	4.7	4.8	6.8
18:2n-6	0.5	1.2	1.9	3.6
18:3n-3	0.2	0.2	0.2	0.3
18:4n-3	0.3	0.3	0.3	0.3
20:4n-6	0.1	0.1	0.1	0.1
20:5n-3, EPA	2.0	1.8	1.8	1.8
22:5n-3	0.1	0.1	0.1	0.1
22:6n-3, DHA	1.4	1.3	1.1	1.1
EPA + DHA	3.4	3.1	2.9	2.9
Σ PUFA[3]	5.4	5.6	6.2	7.9
Σ n-3 LC-PUFA[4]	4.3	3.9	3.7	3.8
Σ n-6 LC-PUFA[5]	0.7	1.4	2.1	3.8
Σ n-3/Σ n-6	5.9	2.8	1.8	1.0

DHA, docosahexaenoic acid; DM, dry matter; EPA, eicosapentaenoic acid: LC-PUFA, long-chain PUFA; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids. [1] Includes 12:0, 13:0, 15:0, 17:0, 20:0, 20:0, 22:0, 24:0. [2] Includes 14:1n-5, 16:1, 17:1n-7, 20:1n-7, 20:1n-11, 22:1n-9, 24:1n-9. [3] Includes 16:2n-4, 16:3n-4, 16:4n-1, 18:2n-4, 18:3n-4, 18:3n-6, 18:4n-1, 20:2n-6, 20:3n-3, 20:4n-3, 20:3n-6, 20:3n-3, 22:2n-6, 21:5n-3 22:4n-6. [4] Includes 20:3n-3, 20:4n-3, 21:5n-3. [5] Includes 18:3n-6, 20:3n-6, 22:4n-6.

4.2.3. Digestibility trial

Fish (58 ± 1 g) from the initial stock were randomly distributed in 8 tanks of 50 L (18 fish per tank) equipped with feces sedimentation columns (Cho and Slinger, 1979). Fish were fed the experimental diets (with 1% chromium oxide (Cr_2O_3) until apparent satiation, once a day, during 5 days for adaptation to the diets, before the feces collection began. Approximately 30 min after feeding, every tank was carefully cleaned to assure that no remains of uneaten feed were left in the bottom of the tank or in the sedimentation column. Feces were collected from the sedimentation column every morning, before feeding, and then centrifuged at 3000 *g*, to eliminate water excess, and kept at −20°C until chemical analysis. Daily collection of the feces was performed for 10 consecutive days. In order to test each diet in quadruplicate and since the RAS was only constituted by 12 tanks equipped with feces sedimentation columns, this procedure was repeated over two consecutive rounds. In each round, each diet was tested in duplicate. In the second round, diets were allocated to different tanks and fed to new groups of 18 fish per tank from the same initial stock and with the same size range (58 ± 1 g) used in the first round.

4.2.4. Growth trial

Twelve homogeneous groups of 25 fish (55 ± 2 g) from the initial stock were randomly distributed in 160 L tanks. Each diet was distributed to triplicate groups of fish, by automatic feeders until visual satiation, three times daily, for 10 weeks. The amount of feed supplied to each tank was adjusted daily, and when some uneaten feed remained in the bottom of the tank, the total amount of feed distributed each day was reduced by 5%, until no feed losses were recorded. When no feed losses were observed, the amount of feed was maintained for 2 days, and then augmented by 5%.

4.2.5. Fish sampling

After overnight fasting, ten fish from the initial stock, and five fish from each tank at the end of growth trial were sampled, sacrificed by anaesthetic overdose (2-phenoxyethanol, 500 µL/L), and kept at −20°C until whole-body composition analysis. Also, at the end of the growth trial, five additional fish per tank were slightly anesthetized with 2-Phenoxyethanol (200 µL/L) for blood and tissue sampling. Blood was collected from the caudal vein with heparinized syringes, centrifuged at 10,000 *g* for 5 min at 4°C, and the collected plasma was stored at −80°C until metabolite analysis. Prior to sampling the remaining tissues, fish were sacrificed by a sharp blow on the head. Liver and white skeletal muscle (dorsal right side) portions (100 mg) were then collected, frozen in liquid nitrogen and stored at −80°C until RNA extraction. Sections of anterior intestine after the pyloric caeca (5 mm thick) were excised, washed, fixed in phosphate-buffered formalin 4% (pH 7) for 24 h, and posteriorly preserved in ethanol 70% until histological analysis. A cross-sectional slab with skin (5 mm thick) was taken just before the dorsal fin, and photographed (with a scale reference) for determination of the cross-sectional area (CSA). A portion from the left part of the fillet (1 × 1 cm) was frozen in isopentane, cooled by dry ice, and stored at −80°C for later histological analysis. Two other muscle samples, from the left part of the fillet, were taken, frozen in liquid nitrogen and stored at −80°C for DM, total lipid content and FA profile analyses. Additionally, the right dorsal fillet was collected with skin for instrumental colour and texture analysis.

4.2.6. Proximate composition analysis

Freeze-dried fish, diets and feces were ground and homogenized prior to proximate composition analysis. Ash, DM, crude protein (N × 6.25), lipids, P and energy were determined according to AOAC methods (AOAC, 2006) as described by Basto et al. (2020). The Cr_2O_3 content in diets and feces was determined according to Bolin et al. (1952).

4.2.7. Total lipids and fatty acids analyses

Muscle total lipids were extracted and quantified gravimetrically according to Folch et al. (1957), using dichloromethane instead of chloroform. The muscle FA methyl esters (FAME) were obtained through transesterification of lipid extracts, whilst in diets FAME were obtained by direct transesterification. The identification and quantification of FAME was performed as described by Campos et al. (2017), using the tricosaenoic acid (23:0) as internal standard.

4.2.8. Histological analysis

Samples of anterior intestine were embedded in paraffin and processed according to standard histological procedures. Intestinal cross-sections (3 µm) were stained with specific Alcian Blue/PAS staining (pH 2.5) and examined under a light microscope (Olympus BX51, GmbH, Germany) coupled with a camera (Olympus DP50, GmbH, Germany). An imaging software (Olympus cellSens Dimension Desktop) was used to determine the following parameters: intestine CSA (mm^2); width of *muscularis* externa (µm), outer longitudinal and inner circular layers (OLL and ICL, respectively); submucosa and lamina propria width (µm); *villus* length and width (µm); number of neutral and acid goblet cells (NGC and AGC, respectively) per *villus.* The OLL, ICL, and submucosa width were measured in eight points of each transverse section analyzed, and the mean was considered. The lamina propria width (measured in the middle of the *villus*), and *villus* length (measured from the top to the bottom following their natural curves) and width (measured at the base) were measured in the eight highest *villus.* Goblet cells were counted on these selected highest *villi.* All measurements are exemplified in Figure 4.1.

Frozen cross-sections of muscle (12 µm) were stained with hematoxylin-eosin and examined using the previously mentioned imaging software. The following parameters were calculated: muscle CSA (mm^2); total number of fibers per CSA; fiber number per unit of area (density; n°/mm^2); and frequency of fibers classes according to their diameter (≤20 µm and >140 µm) as reported in Lopes et al. (2017).

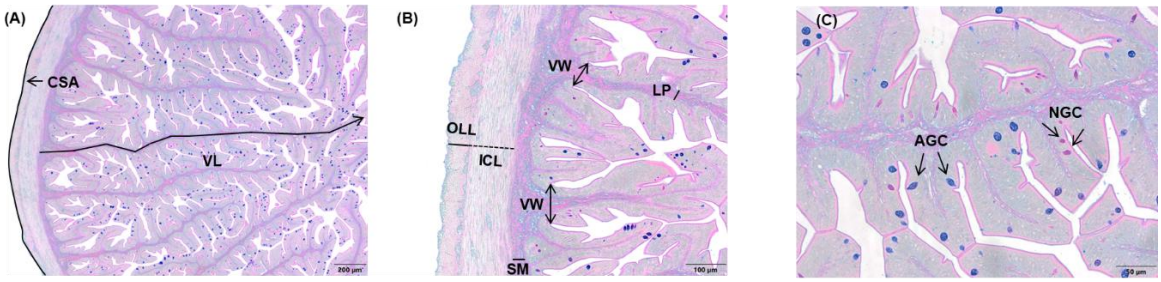

Figure 4.1. Histological sections (Alcian blue/PAS staining, pH = 2.5) of the anterior intestine of European sea bass. (A) CSA, cross-sectional area, VL, *villus* length (40×); (B) ICL, inner circular layer, LP, lamina propria, OLL, outer longitudinal layer, SM, submucosa VL, *villus* width (100×); (C) AGC, acid goblet cell, NGC, neutral goblet cell (200×).

4.2.9. Instrumental texture and colour analysis

Muscle colour measurements were done with a CR-400 chromameter (Konica Minolta Inc., Japan) with an aperture of 8 mm, with respect to CIE standard illuminant D65. The chromameter was applied onto each sample and three replicates of lightness, redness and yellowness (L^*, a^*, and b^*, respectively) values were recorded. From a^* and b^* values the hue angle [$H° = \tan^{-1}(b^*/a^*)$] and the chroma [$C^* = (a^{*2} + b^{*2})^{1/2}$] were calculated (Choubert et al., 1997). Muscle texture was analyzed using a TA.XT.plus Texture Analyser with a 5 kg load cell and a 2.0 mm diameter probe (Stable Micro systems Inc., United Kingdom). Texture profile parameters [hardness (N), adhesiveness (J), springiness (-), cohesiveness (-), chewiness (J), and resilience (-)] were obtained by double penetration (probe speed of 1 mm/s; probe penetration depth of 4 mm; wait time between penetrations of 5 s) on the thickest part of each raw fillet according to Batista et al. (2020).

4.2.10. Plasma biochemistry

Glucose, total protein, triglycerides, total cholesterol and non-esterified FA (NEFA) levels were determined enzymatically using commercial kits (1001190, 1001291, 1001313, and 1001090, Spinreact, Spain; 434-91795 NEFA-HR (2) R1 and 436-91995 NEFA-HR (2) R2, Wako Chemicals, Germany, respectively), adapting manufacturer's instructions to microplate format.

4.2.11. Gene expression

Total RNA from liver and skeletal muscle was extracted using the MagMAX-96 total RNA isolation kit (Life Technologies, United States) after tissue homogenization in TRI reagent following manufacturers' instructions. The RNA quantity and purity were

determined by Nanodrop (Thermo Fisher Scientific, United States) with absorbance ratios (A260/A280) of 1.9–2.1. Reverse transcription (RT) of 500 ng of total RNA was performed with random decamers using the High-Capacity cDNA Archive Kit (Applied Biosystems, United States) following manufacturer's instructions. The RT reactions were incubated for 10 min at 25°C and 2 h at 37°C. Negative control reactions were run without reverse transcriptase. The synthetized cDNA was used for PCR quantification with a SYBR Green Master Mix (Bio-Rad, Hercules, CA, United States), and specific primers at a final concentration of 0.9 μM (Supplementary Table S2; Appendix). Two customized PCR-array layouts were designed for the simultaneous gene expression profiling of 49 genes covering a number of markers of growth-hormone/insulin-growth-factors (GH/IGF) system (10), lipid metabolism (12), energy metabolism (11), muscle cell proliferation and differentiation (12), and protein turnover (4) (Table 4.3). The program used for qPCR reactions included an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 60 s at 60°C. All the pipetting operations were made by means of an EpMotion 5070 Liquid Handling Robot (Eppendorf, Germany) to improve data reproducibility. The efficiency of qPCRs was checked, and the specificity of reactions was verified by analysis of melting and linearity of serial dilutions of RT reactions. Fluorescence data acquired during the extension phase were normalized by the delta-delta CT method (Livak and Schmittgen, 2001), using β-*actin* as housekeeping gene due to its stability among different experimental conditions (average CT among experimental groups varied less than 0.2).

Table 4.3. PCR-array layout for hepatic and white skeletal muscle gene expression profiling.

Function	Gene	Symbol	Accession number[1]
PERFORMANCE	[1,2]Growth hormone receptor-type I	*ghr-i*	AF438177
GH/IGF system	[1,2]Growth hormone receptor-type II	*ghr-ii*	AY642116
	[1,2]Insulin-like growth factor I	*igf-i*	AY800248
	[1,2]Insulin-like growth factor II	*igf-ii*	AY839105
	[1]Insulin-like binding-protein 1b	*igfbp1a*	(LG10:13787250-13788417)
	[1]Insulin-like binding-protein 2b	*igfpb2b*	EU526670
	[2]Insulin-like binding-protein 3a	*igfbp3a*	(LG4:1920612-1938180)
	[1]Insulin-like binding-protein 4	*igfbp4*	MN045298
	[2]Insulin-like binding-protein 5b	*igfpb5b*	(LG15:3836279-3847001)
	[2]Insulin-like binding-protein 6b	*igfbp6b*	(LG22-25:348158-350835)
LIPID METABOLISM	[1]Elongation of very long chain fatty acids 1	*elovl1*	KF857295
FA elongases,	[1]Elongation of very long chain fatty acids 4	*elovl4*	KF857296
FA desaturases,	[1]Elongation of very long chain fatty acids 5	*elovl5*	FR717358
Lipases,	[1]Elongation of very long chain fatty acids 6	*elovl6*	KF857297
Transcription factors	[1,2]Stearoyl-CoA desaturase 1b	*scd1b*	FN868643
	[1]Fatty acid desaturase 2	*fads2*	EU647692
	[1]Lipoprotein lipase	*lpl*	AM411614
	[1]Hepatic lipase	*hl*	KF857289
	[1]Adipose triglyceride lipase	*atgl*	KF857294
	[1]Hormone sensitive lipase	*hsl*	KF857293
	[1]Peroxisome proliferator-activated receptor α	*pparα*	AY590300
	[1]Peroxisome proliferator-activated receptor γ	*pparγ*	AY590303
ENERGY	[1,2]Carnitine palmitoyltransferase 1a	*cpt1a*	KF857302
METABOLISM-	[1,2]Citrate synthase	*cs*	KF857304
OXPHOS,	[1]NADH dehydrogenase subunit 5	*nd5*	KF857307
Cholesterol metabolism,	[1]Succinate dehydrogenase cytochrome b560 subunit	*sdhc*	KF857305

Table 4.3. (*Continued*).

Function	Gene	Symbol	Accession number[1]
Energy sensing, Respiration uncoupling	[1]Cytochrome b	*cyb*	EF427553
	[1]Cytochrome c oxidase subunit I	*coxi*	KF857308
	[1]Cholesterol 7-alpha-monooxygenase	*cyp7a1*	KF857306
	[1,2]Sirtuin 1	*sirt1*	MH138004
	[1,2]Sirtuin 2	*sirt2*	MK983171
	[1]Mitochondrial respiratory uncoupling protein 1	*ucp1*	MH138003
	[2]Mitochondrial respiratory uncoupling protein 3	*ucp3*	(LG14:12134586-12136013)
MUSCLE CELL PROLIFERATION & DIFFERENTIATION	[2]Myoblast determination protein 1	*myod1*	(LG6:934633-937237)
	[2]Myoblast determination protein 2	*myod2*	(LG5:26406310-26408511)
	[2]Myogenic regulatory factor 4	*mrf4*	(LGx:14305213-14306264)
	[2]Myogenic factor 5	*myf5*	(LGx:14298644-14300040)
	[2]Myogenin	*myog*	(LG1A:13290583-13292182)
	[2]Myostatin	*mstn*	AY839106
	[2]Follistatin	*fst*	MK983166
	[2]Fibroblast growth factor 4	*fgf4*	(LG5:29962695-29967359)
	[2]Fibroblast growth factor 6	*fgf6*	AY831723
	[2]Muscle RING-finger protein-1	*murf1*	(UN:85299200-85300236)
	[2]Muscle atrophy F-box	*mafbx/atrogin-1*	MK983167
	[2]Myomaker	*mymk*	(LG20:21064893-21067975)
PROTEIN TURNOVER	[2]Calpain 1	*capn1*	FJ821591
	[2]Calpain 2	*capn2*	MK983168
	[2]Calpain 3	*capn3*	MK983169
	[2]Calpastatin	*cpst*	MK983170

[1]Obtained from GenBank database or the European sea bass genome project (http://seabass.mpipz.mpg.de). Accession number of sea bass genome is shown in parentheses.

4.2.12. Calculations

The ADC of the experimental diets were calculated according to Maynard et al. (1979): dry matter ADC (%) = 100 × [1 - (dietary Cr_2O_3 level/feces Cr_2O_3 level)] and nutrients or energy ADC (%) = 100 × [1 - (dietary Cr_2O_3 level/feces Cr_2O_3 level) × (feces nutrient or energy level/dietary nutrient or energy)]; Average body weight (ABW) = (final body weight + initial body weight)/2; Digestible nitrogen (N), lipids (L), P or energy (E) intake = (dry feed consumption × N, L, P (%) or E (kJ/g) in the diet × ADC N, L, P, or E)/ABW/days; N, L, P, or E gain = (final carcass N, L, P or E content - initial carcass N, L, P, or E content)/ABW/days; N, L, P, or E retention efficiency (NRE, LRE, PRE, or ERE) = (N, L, P, or E gain/digestible N, L, P or E intake) × 100; Fecal N, L, P, or E losses = crude N, L, P, or E intake × [1 - (ADC N, L, P, or E/100)]. Metabolic N, L, or P losses = digestible N, L, or P intake−N, L or P gain; Branchial and urinary E losses = non-fecal N losses × 25 kJ/N; Total N, L, P, or L losses = crude N, L, or P intake−N, L, or P gain; Metabolizable energy (ME) = digestible E intake - branchial and urinary E losses; Total heat loss = E intake – E gain; Total heat production = ME - energy gain. Condition factor (K) = [final body weight/(final body length)^3] × 100; Daily growth index (DGI) = 100 × [(final body weight)$^{1/3}$ - (initial body weight)$^{1/3}$]/days; Voluntary feed intake (VFI) = 100 × dry feed intake/average body weight/day; Feed conversion ratio (FCR) = dry feed intake/weight gain; Protein efficiency ratio (PER) = weight gain/crude protein intake; Hepatosomatic index (HSI) = 100 × liver weight/body weight; Viscerosomatic index (VSI) = 100 × weight of viscera/body weight.

4.2.13. Statistical analysis

Data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov and Levene's tests, respectively, and log-transformed whenever required before submitted to a one-way ANOVA using IBM SPSS Statistics 26.0 (IBM corporation, United States). When one-way ANOVA showed significance ($p < 0.05$), individual means were compared using Student-Newman-Keuls post-test. Fold-changes of genes were submitted to a student's t-test with significance set at $p < 0.05$. A bivariate spearmen's rank correlation coefficient (*rs*) test was applied to all variables. Significant correlations were considered at the bilateral level of 0.05.

4.3. Results

4.3.1. Digestibility and nutrient balance

Overall, all diets were well digested (DM ADC > 76%; protein ADC ≥ 89%; lipids ADC ≥ 97%; energy ADC > 86%; P ADC > 62%), but differences on nutrient digestibility were found among groups in the digestibility trial (Table 4.4). Fish fed TM100 had the lowest protein apparent digestibility, and the highest lipid and P digestibility. As a result, N intake and metabolic N losses decreased with total FM replacement, and the opposite pattern was found for N retention efficiency. Fecal N losses increased both in fish fed TM100 and in those fed TM80. Digestible P intake decreased with FM replacement, being significantly lower in fish fed TM80 and TM100 compared to fish fed CTRL. Fecal P losses decreased significantly with the concomitant increase of dietary *d*TM. Total P losses of fish fed *d*TM decreased significantly compared to fish fed CTRL, regardless of the dietary *d*TM level. Conversely, in comparison to fish fed CTRL and other experimental groups, both lipid and energy gain and retention efficiency were consistently higher in fish fed TM100, whilst lower branchial and urinary E losses, total E losses and total heat production were observed. Fecal E losses increased significantly in fish fed TM40 and TM80. The net balance (total gain, fecal and metabolic losses) of N, P, and L is summarized in Figure 4.2.

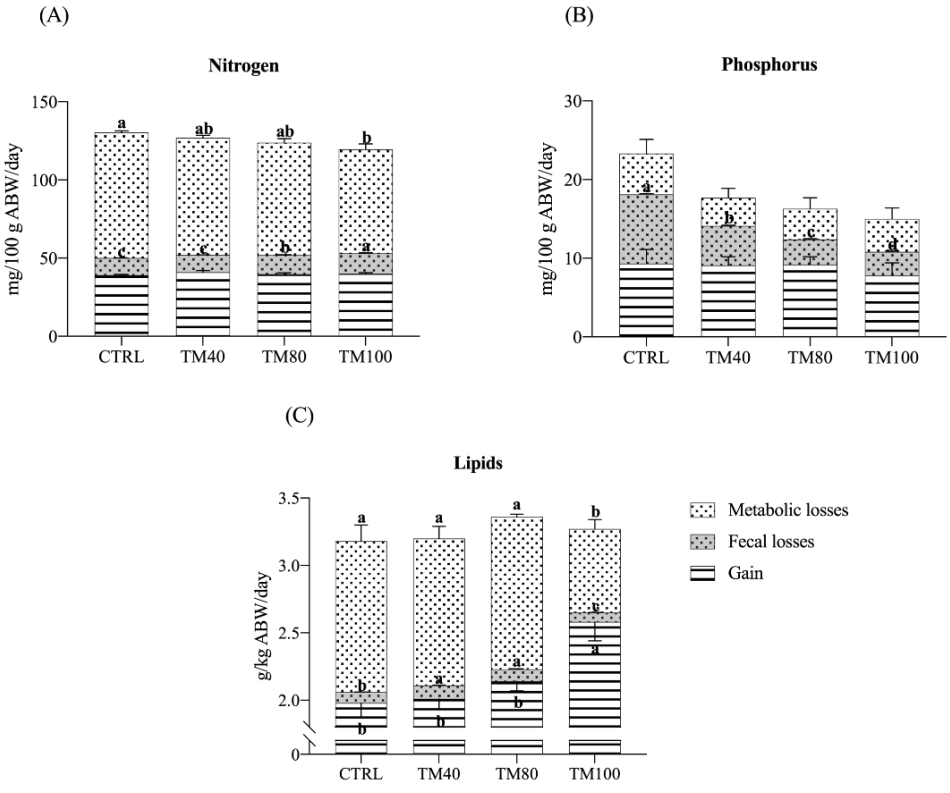

Figure 4.2. Daily nitrogen (A), phosphorous (B), and lipids (C) balance of European sea bass fed experimental diets. Bars represent means ± SD; n = 3 (pools of 5 fish/replicate). Labeled means without a common superscript letter differ significantly, p < 0.05.

Table 4.4. Apparent digestibility coefficients and nutrient balances of European sea bass fed experimental diets.

	CTRL	TM40	TM80	TM100	*p* value
ADC, %					
Dry matter	79.0 ± 0.4^{a}	76.4 ± 0.5^{b}	78.3 ± 0.3^{a}	78.9 ± 0.3^{a}	<0.01
Protein	91.7 ± 0.1^{a}	91.3 ± 0.4^{a}	90.1 ± 0.3^{ab}	89.1 ± 0.9^{b}	<0.01
Lipids	97.4 ± 0.2^{b}	97.0 ± 0.4^{b}	97.3 ± 0.2^{b}	98.0 ± 0.1^{a}	0.02
Energy	87.5 ± 0.6^{a}	85.7 ± 0.4^{b}	86.6 ± 0.3^{ab}	87.9 ± 0.4^{a}	<0.01
Phosphorus	62.4 ± 1.0^{c}	71.8 ± 0.6^{b}	78.6 ± 1.0^{a}	80.1 ± 0.5^{a}	<0.01
Nitrogen balance, mg/100 g ABW/day					
Digestible N intake	119.4 ± 1.4^{a}	115.9 ± 1.5^{a}	111.5 ± 3.1^{ab}	106.5 ± 2.7^{b}	0.02
N gain	39.1 ± 0.4	40.9 ± 1.2	39.7 ± 0.8	40.0 ± 0.7	0.49
N retention efficiency, % DN	32.8 ± 0.2^{b}	35.3 ± 1.0^{ab}	35.6 ± 0.6^{ab}	37.6 ± 1.7^{a}	0.04
Fecal N losses	10.9 ± 0.1^{c}	11.1 ± 0.1^{c}	12.2 ± 0.4^{b}	13.1 ± 0.3^{a}	<0.01
Metabolic N losses	80.3 ± 1.1^{a}	74.9 ± 1.7^{ab}	71.9 ± 2.6^{ab}	66.5 ± 3.4^{b}	0.02
Total N losses	91.2 ± 1.2	86.0 ± 1.8	84.1 ± 2.9	79.6 ± 3.7	0.08
Phosphorus balance, mg/100 g ABW/day					
Digestible P intake	14.5 ± 0.2^{a}	12.7 ± 0.2^{bc}	13.2 ± 0.4^{b}	12.0 ± 0.3^{c}	<0.01
P gain	9.3 ± 1.8	9.1 ± 1.1	9.2 ± 1.0	7.8 ± 1.6	0.86
Phosphorus retention efficiency, % DP	64.2 ± 12.1	71.6 ± 9.0	70.6 ± 9.6	64.5 ± 12.6	0.94
Fecal P losses	8.8 ± 0.1^{a}	5.0 ± 0.1^{b}	3.2 ± 0.1^{c}	3.0 ± 0.1^{d}	<0.01
Metabolic P losses	5.2 ± 1.8	3.6 ± 1.2	3.9 ± 1.4	4.2 ± 1.5	0.89
Total P losses	13.9 ± 1.7^{a}	8.6 ± 1.2^{b}	7.5 ± 1.5^{b}	7.2 ± 1.4^{b}	0.04
Lipid balance, g/kg ABW/day					
Digestible L intake	3.1 ± 0.04	3.1 ± 0.04	3.3 ± 0.1	3.2 ± 0.1	0.29
L gain	2.0 ± 0.1^{b}	2.0 ± 0.1^{b}	2.1 ± 0.1^{b}	2.6 ± 0.1^{a}	0.01
Lipids retention efficiency, % DL	63.8 ± 3.8^{b}	65.0 ± 2.8^{b}	65.5 ± 0.3^{b}	80.6 ± 2.7^{a}	<0.01
Fecal L losses	0.08 ± 0.001^{b}	0.10 ± 0.001^{a}	0.09 ± 0.003^{a}	0.07 ± 0.002^{c}	<0.01
Metabolic L losses	1.1 ± 0.1^{a}	1.1 ± 0.1^{a}	1.1 ± 0.02^{a}	0.6 ± 0.1^{b}	<0.01
Total L losses	1.2 ± 0.1^{a}	1.2 ± 0.1^{a}	1.2 ± 0.02^{a}	0.7 ± 0.1^{b}	<0.01
Energy balance, kJ/kg ABW/day					
Digestible E intake	327.5 ± 3.8	322.0 ± 4.0	334.6 ± 9.5	318.2 ± 8.1	0.41
E gain	123.4 ± 2.3^{b}	127.3 ± 5.2^{b}	128.6 ± 3.6^{b}	148.6 ± 6.7^{a}	0.02
Energy retention efficiency, % DE	37.7 ± 1.2^{b}	39.5 ± 1.8^{b}	38.5 ± 0.8^{b}	46.7 ± 1.1^{a}	<0.01
Metabolizable Energy	307.5 ± 3.6	303.3 ± 3.8	316.7 ± 8.8	301.6 ± 3.7	0.39
Fecal E losses	46.9 ± 0.6^{b}	53.9 ± 0.7^{a}	51.5 ± 1.5^{a}	43.9 ± 1.1^{b}	<0.01
Branchial + urinary E losses	20.0 ± 0.3^{a}	18.7 ± 0.4^{ab}	17.9 ± 0.4^{ab}	16.6 ± 0.9^{b}	0.02
Total E losses	250.9 ± 6.7^{a}	248.7 ± 7.8^{a}	257.5 ± 8.8^{a}	213.4 ± 3.7^{b}	0.01
Total heat production	184.0 ± 5.9^{a}	176.1 ± 6.9^{a}	188.1 ± 6.8^{a}	153.0 ± 2.6^{b}	<0.01

Values are means ± SEMs; ADC: n = 4 (pools of faeces from 18 fish/replicate); Nutrient balance: n = 3 (pools of 5 fish/replicate). Labeled means without a common superscript letter differ significantly, $p < 0.05$. ADC, apparent digestibility coefficient; DE, digestible energy; DL, digestible lipids; DN, digestible nitrogen; DP, digestible phosphorus; E, energy; L, Lipids; N, nitrogen; P, phosphorus. The ADCs of the experimental diets were calculated according to Maynard et al. (1979): dry matter ADC (%) = 100 × [1 - (dietary Cr_2O_3 level / feces Cr_2O_3 level)] and nutrients or energy ADC (%) = 100 × [1 - (dietary Cr_2O_3 level / feces Cr_2O_3 level) × (feces nutrient or energy level / dietary nutrient or energy)]; Average body weight (ABW) = (final body weight + initial body weight) / 2; Digestible N, L, P or E intake = (dry feed consumption × N, L, P (%) or E (kJ/g) in the diet × ADC N, L, P or E) / ABW / days; N, L, P or E gain = (final carcass N, L, P or E content - initial carcass N, L, P or E content) / ABW / days; N, L, P or E retention efficiency (NRE, LRE, PRE, or ERE) = (N, L, P or E gain / digestible N, L, P or E intake) ×100; Fecal N, L, P or E losses = crude N, L, P or E intake × [1 - (ADC N, L, P or E / 100)]. Metabolic N, L or P losses = digestible N, L or P intake − N, L or P gain; Branchial and urinary E losses = non-fecal N losses × 25 kJ/N; Total N, L, P or L losses = crude N, L or P intake − N, L or P gain; Metabolizable energy (ME) = digestible E intake - branchial and urinary E losses; Total heat loss = E intake – E gain; Total heat production = ME - energy gain.

4.3.2. Growth performance and blood biochemistry

As shown in Table 4.5, fish almost tripled their initial weight, and no mortality was registered during the entire growth trial. All groups grew at the same rate and a significant decrease of FCR from 1.2 in fish fed CTRL to 1.0 in those fed TM100 was observed. Final K remained similar among dietary treatments. The whole-body lipids and energy content increased in fish fed TM100 with a concomitant decrease of moisture. This group also showed a statistically significant increase of HSI that was positively correlated with whole-body lipid content (0.59; $p = 0.04$) and lipid gain (0.59; $p = 0.04$). The VSI also displayed a tendency to increase in fish fed TM100 fish, being this biometric index negatively correlated with metabolic (−0.64; $p = 0.03$) and total lipid (−0.64; $p = 0.03$) losses (Supplementary Table S3; Appendix). Regarding blood analysis, plasma levels of total cholesterol and NEFA increased with the replacement of FM by *d*TM, regardless of the inclusion level. Plasma triglycerides were only significantly affected by TM100, resulting in the highest values (Figure 4.3). Cholesterol and/or triglycerides showed positive correlations with HSI and VSI (Supplementary Table S3; Appendix). Concerning the fish-in:fish-out ratio (Fi:Fo), the calculated values were reduced by 20% in fish fed TM100 in comparison to those fed CTRL or the two other experimental groups (Figure 4.4).

Table 4.5. Growth performance, somatic indexes and whole-body composition of European sea bass fed experimental diets.

	CTRL	TM40	TM80	TM100	*p* value
Growth performance					
Initial body weight, g	55.6 ± 0.01	55.7 ± 0.03	55.7 ± 0.03	55.7 ± 0.02	0.31
Final body weight, g	159.4 ± 3.9	161.0 ± 3.7	159.2 ± 3.5	162.9 ± 2.2	0.86
Final K	1.3 ± 0.01	1.3 ± 0.01	1.3 ± 0.01	1.3 ± 0.02	0.09
DGI, %/day	2.4 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.5 ± 0.04	0.87
VFI, %/day	1.7 ± 0.02^{a}	1.7 ± 0.02^{a}	1.6 ± 0.1^{a}	1.5 ± 0.04^{b}	<0.01
FCR	1.2 ± 0.02^{a}	1.1 ± 0.03^{a}	1.1 ± 0.02^{a}	1.0 ± 0.03^{b}	0.01
PER	1.8 ± 0.03	1.9 ± 0.1	1.9 ± 0.04	2.0 ± 0.1	0.13
Somatic indexes					
HSI, %	1.2 ± 0.03^{b}	1.1 ± 0.1^{b}	1.1 ± 0.03^{b}	1.5 ± 0.01^{a}	0.02
VSI, %	8.1 ± 0.2	8.1 ± 0.3	7.9 ± 0.3	9.0 ± 0.3	0.06
Whole body composition, g/100 g WW					
Moisture	66.3 ± 0.2^{a}	66.6 ± 0.5^{a}	66.3 ± 0.1^{a}	63.9 ± 0.9^{b}	0.02
Protein	16.9 ± 0.1	17.4 ± 0.3	17.1 ± 0.03	17.0 ± 0.2	0.34
Lipids	11.4 ± 0.6^{b}	11.6 ± 0.3^{b}	12.2 ± 0.3^{b}	14.1 ± 0.7^{a}	0.02
Energy, kJ/g WW	7.7 ± 0.1^{b}	7.9 ± 0.2^{b}	8.0 ± 0.1^{b}	8.8 ± 0.3^{a}	0.02
Phosphorus	0.6 ± 0.1	0.6 ± 0.04	0.6 ± 0.1	0.6 ± 0.1	0.81
Ash	4.2 ± 0.5	3.9 ± 0.2	4.1 ± 0.3	4.1 ± 0.1	0.89

Values are means ± SEMs; growth performance: n = 75 (25 fish/replicate); somatic indexes and whole-body composition: n = 15 (5 fish/replicate). Labeled means without a common superscript letter differ significantly, $p < 0.05$. DGI, daily growth index; FCR, feed conversion ratio; HSI, hepatosomatic index; K, condition factor; PER, protein efficiency ratio; VFI, voluntary feed intake; VSI, viscerosomatic index; WW, wet weight. K = [final body weight / (final body length)^{3}] × 100; DGI = 100 × [(final body weight)$^{1/3}$ - (initial body weight)$^{1/3}$] / days; VFI = 100 × dry feed intake / average body weight / day; FCR = dry feed intake / weight gain; PER = weight gain / crude protein intake; HSI = 100 × liver weight / body weight; VSI = 100 × weight of viscera / body weight.

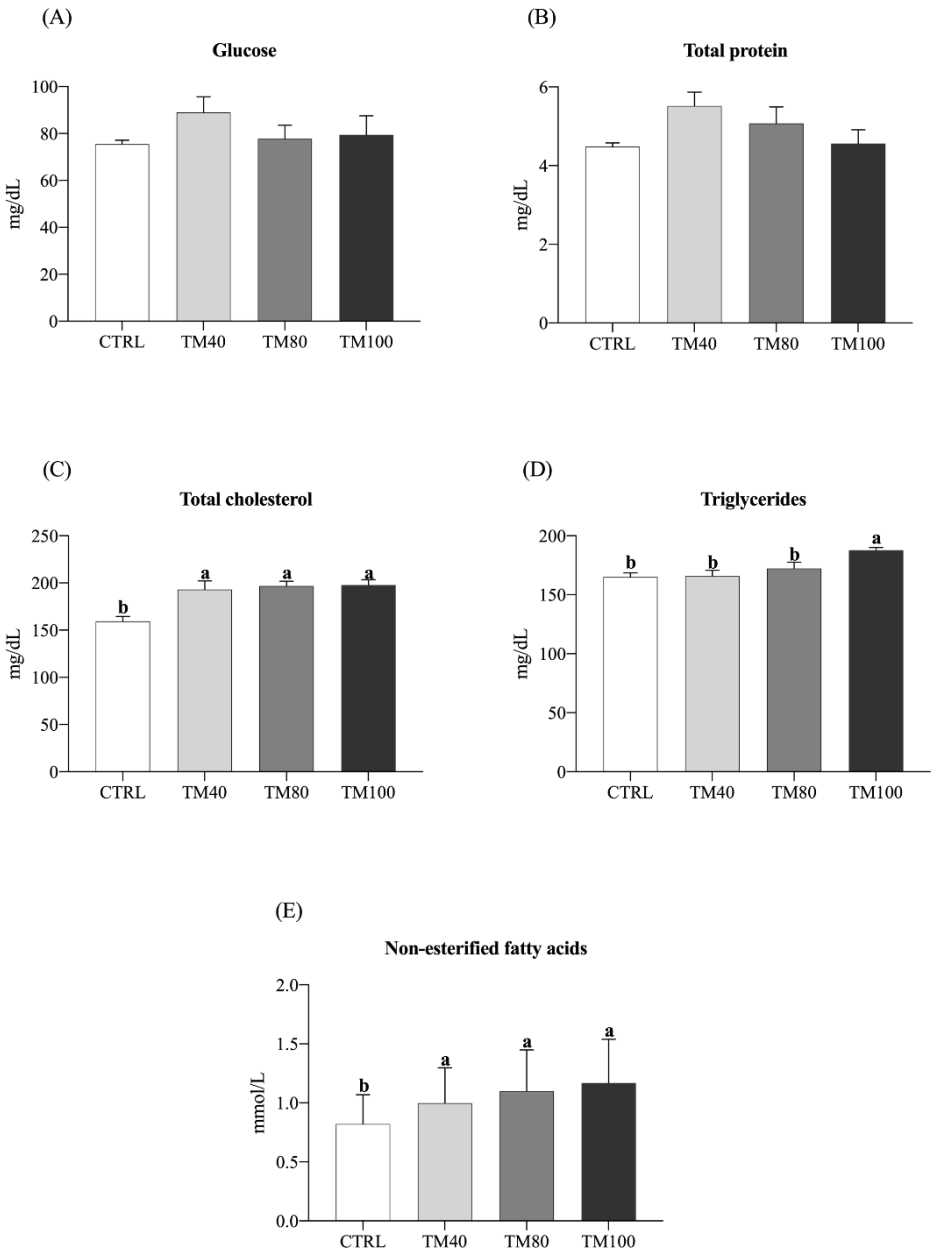

Figure 4.3. Plasma glucose (A), total protein (B), total cholesterol (C), triglycerides (D) and non-esterified fatty acids (E) of European sea bass fed experimental diets. Bars represent means ± SEM; n = 15 (5 fish/replicate). Labelled means without a common superscript letter differ significantly, $p < 0.05$.

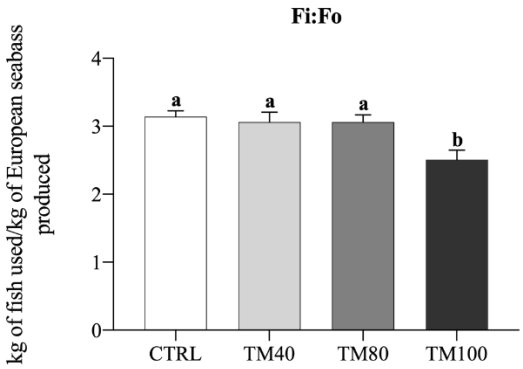

Figure 4.4. Fi:Fo ratio. Bars represent means ± SD; n = 3 (pools of 5 fish/replicate). Labelled means without a common superscript letter differ significantly, $p < 0.05$. Fi:Fo was calculated according to Tacon and Metian (2008), assuming yield from forage fish of 24% for FM and 4.8% for FO.

4.3.3. Muscle total lipids and fatty acids analyses

Despite the increase of lipids in TM100 diet, the muscle total lipids content was similar among groups (Table 4.6). The muscle saturated (SFA) and monounsaturated FA (MUFA) profile reflected the dietary FA profile (Table 4.2). Up to 80% of FM replacement by *d*TM, the muscle PUFA profile also reflected the dietary FA profile. On the other hand, and despite the highest percentage of PUFA in TM100 diet, muscle PUFA levels in fish fed TM100 were comparable to those of all other treatments. The relative percentage of muscle EPA, DHA, and n-3 LC-PUFA of fish fed TM40 and TM80 was similar to those fed CTRL, despite their dietary percentage being decreased with the *d*TM inclusion. Although fish fed TM100 had the lowest relative percentage of EPA and DHA, when expressed in wet weight (g/100 g WW), both muscle EPA and EPA + DHA final contents were similar among all fish. As observed in the experimental diets, the percentage of muscle n-6 LC-PUFA increased with *d*TM inclusion and consequently the n-3/n-6 ratio decreased.

Table 4.6. Muscle total lipid content and fatty acid composition of the European sea bass fed experimental diets.

	CTRL	TM40	TM80	TM100	*p* value
Total lipids, g/100 g WW	2.5 ± 0.1	2.5 ± 0.1	2.6 ± 0.3	2.8 ± 0.2	0.73
Fatty acids, g/100 g total fatty acids					
14:00	4.2 ± 0.1	4.1 ± 0.04	4.0 ± 0.3	3.6 ± 0.04	0.05
16:00	17.8 ± 0.03	17.6 ± 0.1	17.6 ± 0.2	17.8 ± 0.1	0.74
18:00	3.5 ± 0.06	3.7 ± 0.05	3.7 ± 0.05	3.9 ± 0.02	0.08
Σ SFA[1]	27.4 ± 0.1	27.3 ± 0.1	27.1 ± 0.4	26.9 ± 0.1	0.81
16:1n-7	6.1 ± 0.2^{a}	5.6 ± 0.02^{a}	5.6 ± 0.2^{a}	4.5 ± 0.03^{b}	0.01
18:1n-9	17.5 ± 0.5^{b}	17.3 ± 0.1^{b}	18.0 ± 0.5^{b}	22.4 ± 0.1^{a}	0.01
18:1n-7	2.9 ± 0.1^{a}	2.7 ± 0.02^{ab}	2.6 ± 0.1^{ab}	2.2 ± 0.004^{b}	0.04
20:1n-9	2.0 ± 0.2	1.9 ± 0.02	1.7 ± 0.2	1.2 ± 0.003	0.08
22:1n-11	1.3 ± 0.2	1.2 ± 0.01	1.1 ± 0.2	0.5 ± 0.004	0.07
Σ MUFA[2]	31.7 ± 0.3	30.6 ± 0.1	30.8 ± 0.3	32.2 ± 0.2	0.05
18:2n-6	4.6 ± 0.04^{d}	7.1 ± 0.1^{c}	9.9 ± 0.005^{b}	13.5 ± 0.2^{a}	<0.01
18:3n-3	1.1 ± 0.01	1.1 ± 0.01	1.1 ± 0.02	1.1 ± 0.01	0.23
18:4n-3	1.3 ± 0.1^{a}	1.2 ± 0.01^{a}	1.2 ± 0.1^{a}	0.9 ± 0.003^{b}	0.01
20:4n-6	0.9 ± 0.02^{a}	1.0 ± 0.02^{a}	0.9 ± 0.01^{a}	0.7 ± 0.01^{b}	<0.01
20:5n-3, EPA	10.2 ± 0.2^{a}	10.0 ± 0.05^{a}	9.9 ± 0.2^{a}	7.9 ± 0.1^{b}	<0.01
22:5n-3	1.8 ± 0.04^{a}	1.8 ± 0.02^{a}	1.8 ± 0.02^{a}	1.3 ± 0.2^{b}	<0.01
22:6n-3, DHA	11.6 ± 0.3^{a}	12.6 ± 0.1^{a}	11.7 ± 0.1^{a}	9.0 ± 0.2^{b}	<0.01
EPA + DHA	21.8 ± 0.5^{a}	12.7 ± 0.1^{b}	11.7 ± 0.1^{b}	10.3 ± 0.1^{c}	<0.01
Σ PUFA[3]	35.0 ± 0.6^{b}	37.9 ± 0.1^{a}	39.7 ± 0.5^{a}	37.3 ± 0.2^{ab}	<0.01
Σ n-3 LC-PUFA[4]	27.1 ± 0.6^{a}	27.7 ± 0.2^{a}	26.7 ± 0.4^{a}	21.1 ± 0.3^{b}	<0.01
Σ n-6 LC-PUFA[5]	6.4 ± 0.1^{d}	9.0 ± 0.1^{c}	11.7 ± 0.02^{b}	15.2 ± 0.2^{a}	<0.01
Σ n-3/Σ n-6	4.2 ± 0.1^{a}	3.1 ± 0.05^{b}	2.3 ± 0.04^{c}	1.4 ± 0.03^{d}	<0.01
Σ n-6/Σ n-3	0.2 ± 0.01^{d}	0.3 ± 0.005^{c}	0.4 ± 0.01^{b}	0.7 ± 0.02^{a}	<0.01
Fatty acids, g/100 g WW					
20:5n-3, EPA	0.22 ± 0.01	0.20 ± 0.01	0.21 ± 0.02	0.20 ± 0.01	0.46
22:6n-3, DHA	0.25 ± 0.01^{a}	0.24 ± 0.01^{a}	0.25 ± 0.02^{a}	0.18 ± 0.01^{b}	<0.01
EPA + DHA	0.47 ± 0.02	0.44 ± 0.01	0.46 ± 0.04	0.38 ± 0.01	0.38

Values are means ± SEMs; Total lipids: n = 15 (5 fish/replicate); Fatty acids: n = 6 (2 pools/replicate). Labeled means without a common superscript letter differ significantly, $p < 0.05$. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LC-PUFA, long-chain PUFA; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; WW, wet weight. [1] Includes 12:0, 13:0, 15:0, 17:0, 20:0, 20:0, 22:0, 24:0. [2] Includes 14:1n-5, 16:1, 17:1n-7, 20:1n-7, 20:1n-11, 22:1n-9, 24:1n-9. [3] Includes 16:2n-4, 16:3n-4, 16:4n-1, 18:2n-4, 18:3n-4, 18:3n-6, 18:4n-1, 20:2n-6, 20:3n-3, 20:4n-3, 20:3n-6, 20:3n-[3] 22:2n-6, 21:5n-3; 22:4n-6. [4] Includes 20:3n-3, 20:4n-3, 21:5n-3. [5] Includes 18:3n-6, 20:3n-6, 22:4n-6.

4.3.4. Intestine and muscle histological analysis

The morphology of anterior intestine was well-preserved in all fish, with no signs of *villus* fusion or enterocyte vacuolization. Intestine CSA varied between 11 and 15 mm^2. The OLL and ICL varied between 32 and 41 μm, and 58 and 69 μm, respectively. The lamina propria width varied between 27 and 30 μm. The *villus* length and width ranged from 1749 to 2001 μm and 161 to 180 μm, respectively. The number of AGC and NGC per *villus* ranged from 147 to 192, and 70 to 113, respectively. The morphometric parameters analyzed in the anterior intestine did not vary significantly among dietary treatments, with the exception of submucosa thickness that increased concomitantly with dietary *d*TM inclusion level: the submucosa thickness increased from 27 μm in fish fed CTRL to 29, 30, and 33 μm in those fed TM40, TM80 and TM100, respectively (Supplementary Table S4; Appendix). White muscle CSA varied between 459 and 496 mm^2, total number of fibers ranged from 104 to 108 thousand, and fiber density from 217 to 228 (Supplementary Table S5; Appendix). Fiber diameter varied between 65 and 67 μm, with 5–6% of the fibers having below 20 μm and 2–3% above 140 μm. None of the morphometric parameters analyzed in muscle had significant differences among dietary treatments.

4.3.5. Muscle instrumental colour and texture analysis

Texture and colour parameters were similar among dietary treatments (Supplementary Table S6; Appendix). Hardness ranged from 0.9 to 1 N, adhesiveness from −0.01 to −0.004 J, springiness from 1.2 to 1.6, cohesiveness from 0.4 to 0.5, chewiness from 0.5 to 0.8 J, and resilience was 0.4 irrespectively of the dietary treatment. In the muscle, L^* varied from 41 to 42, a^* from −0.5 to −0.2, b^* from −0.7 to 0.1, H° from 183 to 250 and C° from 1 to 1.2.

4.3.6. Gene expression

The relative gene expression profile of key selected markers in liver and muscle is shown in Supplementary Tables S7 and S8 (Appendix), with arbitrarily assigned values of 1 for hormone sensitive lipase (*hsl*) in liver of fish fed CTRL, and calpain 2 (*capn2*) in muscle of fish fed CTRL. Among all the analyzed genes, up to three markers of hepatic lipid metabolism and four markers of muscle growth were differentially expressed with the FM replacement, and the corresponding log_2 fold-changes (*d*TM/CTRL) were graphically represented. In liver, the expression of elongation of very long-chain FA protein 6

(*elovl6)* was significantly down-regulated in fish fed TM100, but not in those fed TM40 and TM80 (Figure 4.5A). Conversely, FA desaturase 2 (*fads2)* was markedly up-regulated in fish fed TM100, remaining almost unaltered in those fed TM40 and TM80 (Figure 4.5B). A strong nutritional regulation was also found for cholesterol 7-alpha-monooxygenase (*cyp7a1*) with a significant down-regulation in all *d*TM groups (Figure 4.5C). A similar trend was observed for the expression of stearoyl-CoA desaturase 1b (*scd1b*), although this down-regulation was not statistically significant (Figure 4.5D). The expression of lipolytic peroxisome proliferator-activated receptor α (*ppar*α) also evidenced a non-significant down-regulation trend for fish fed TM100 (Figure 4.5E). The expression of e*lovl6*, *cyp7a1* and *ppar*α was negatively correlated with plasma cholesterol levels (−0.64 ($p < 0.01$), −0.46 ($p < 0.01$), and −0.37 ($p = 0.02$), respectively) (Supplementary Table S9; Appendix). Myoblast determination protein 2 (*myod2*) was down-regulated in TM80 and TM100 (Figure 4.6A), whereas a significant down-regulation of muscle atrophy F-box (*mafbx/atrogin-1*) was only found in fish fed TM100 (Figure 4.6B). Contrarily, the expression of myostatin (*mstn*) (Figure 4.6C) and myoblast fusion factor (*mymk*) (Figure 4.6D) tended to increase with FM replacement level, but this increase was only statistically significant in TM100.

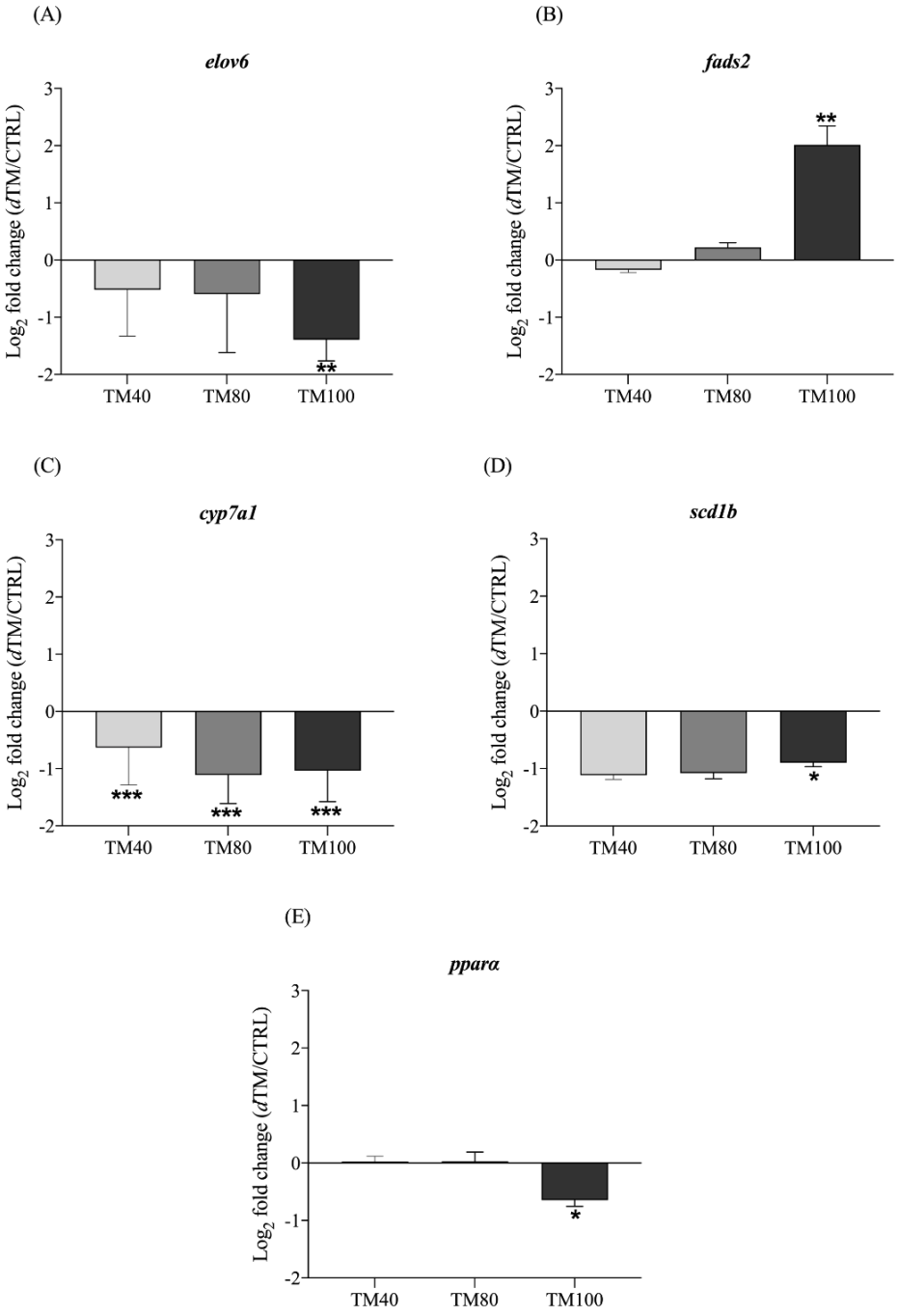

Figure 4.5. Fold-changes (dTM/CTRL) of differentially expressed genes in liver tissue: *elovl6* (A), *fads* (B), *cyp7a1* (C), *scd1b* (D) and *pparα* (E). The asterisks indicate statistically significant differences (*p < 0.05; **p < 0.01; t-student) between European sea bass fed *d*TM and CTRL diets. Values >1 indicate up-regulated genes in *d*TM fish; values < 1 indicate down-regulated genes in *d*TM fish; n = 9 (3 fish/replicate).

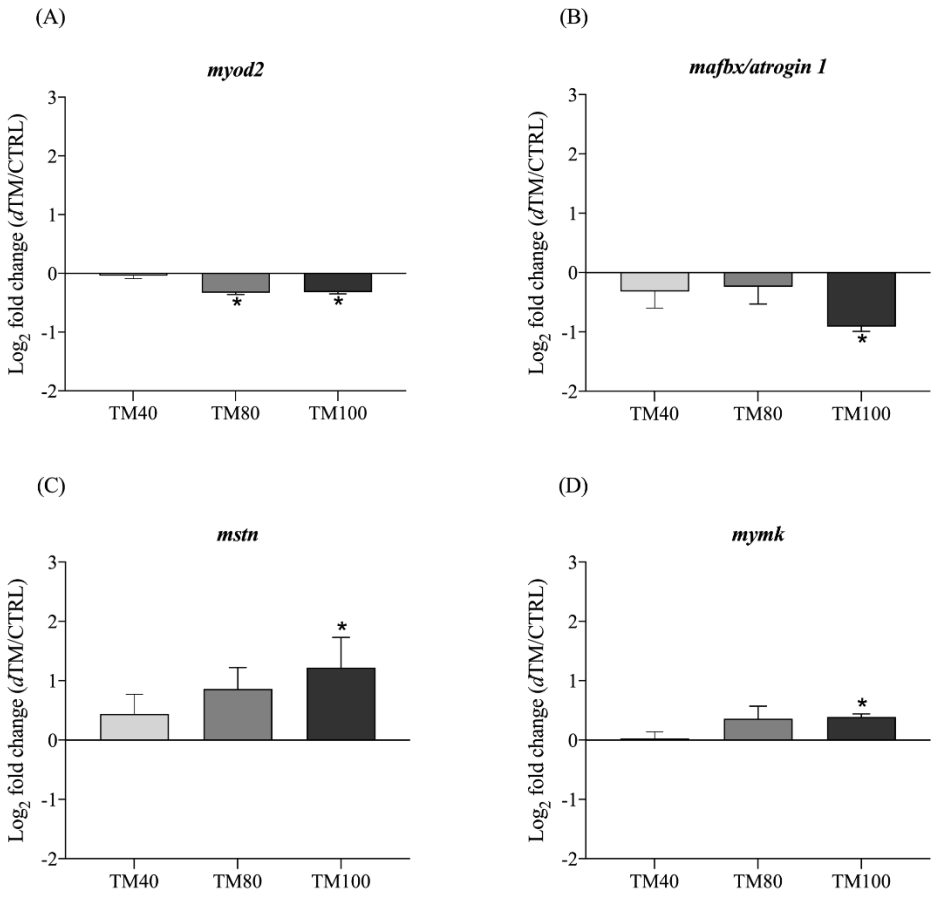

Figure 4.6. Fold-changes (*d*TM/CTRL) of differentially expressed genes in muscle tissue*: myod2* (A), *mstn* (B), *mafbx*/*atrogin 1* (C), and *mymk* (D). The asterisks indicate statistically significant differences (*p < 0.05; t-student) between European sea bass fed *d*TM and CTRL diets. Values > 1 indicate up-regulated genes in *d*TM fish; values <1 indicate down-regulated genes in *d*TM fish. n = 9 (3 fish/replicate).

4.4. Discussion

Since the use of IM in aquafeeds was authorized by EU in 2017, increasing efforts have been made to identify the best insect species to substitute FM, and its highest dietary inclusion level (Gasco et al., 2020). Despite the current high market prices of insect meal compared to conventional protein sources (Arru et al., 2019), the insect production is a fast-growing sector, and several companies or start-ups have been recently founded in Europe. This may lead to increased price competitiveness in the near future. The present study clearly shows that the total substitution of FM by *d*TM can be achieved in European sea bass without impairing growth performance or condition factor and even improving FCR after 10 weeks of feeding. To the best of our knowledge, the maximum replacement of FM by full-fat IM, successfully tested in European sea bass, varied between 36% and 50%, in trials of similar duration (Gasco et al., 2016; Abdel-Tawwab et al., 2020). Skalli and Robin (2004) pointed out that growth impairment at

higher FM replacement levels is primarily due to nutrient deficiencies in n-3 LC-PUFA, which are fixed for marine fish close to 0.7% on DM basis (Skalli and Robin, 2004). In fact, a previous study in sea bass with 71% FM replacement by TM did not fulfil this requirement, impairing growth (Gasco et al., 2016), whilst all experimental diets tested in the present study contained 3.8–4.3% DM n-3 LC-PUFA, probably due to the high quality of FO, and did not affect growth performance. In blackspot sea bream and gilthead sea bream (*Sparus aurata*), it was possible to replace FM by full-fat TM up to 49% and 74%, respectively, without affecting growth performance or FCR, after 18–23 weeks of feeding (Iaconisi et al., 2017; Piccolo et al., 2017). In red sea bream, the total replacement of FM by *d*TM was successfully achieved without affecting FCR and even increasing growth after 4 weeks of feeding (Ido et al., 2019). Contrarily, the use of defatted black soldier fly (*Hermetia illucens)* to replace FM at levels above 18% in turbot (*Psetta maxima*) and 35% in meagre (*Argyrosomus regius*), for 7–9 weeks, did not affect FCR, but impaired growth performance (Kroeckel et al., 2012; Guerreiro et al., 2020). Thus, optimal FM substitution levels could vary among fish, insect species, rearing conditions, processing methods of IM, and duration of the experimental trials making comparisons difficult (Osimani et al., 2016; Iaconisi et al., 2019). Therefore, standardization of insect protein production is needed to assure the correct formulation of diets not only for fish, but also for other animal species.

In the present study, up to 80% of FM replacement did not alter N intake, N retention or metabolic N losses. However, this trade-off was apparently altered in fish fed TM100; these fish conserved a higher N retention compared to control by reducing metabolic N losses. Digestibility disturbances with the use of IM are attributed to the chitin, which has been negatively correlated with protein digestibility in *in vitro* digestibility studies (Marono et al., 2015). Although chitinolytic activity was reported in some fish species (Gutowska et al., 2004; Krogdahl et al., 2005; Kawashima et al., 2016), there are evidences that high dietary chitin levels have negative impacts on fish growth and nutrient utilization (Alegbeleye et al., 2012; Kroeckel et al., 2012). In the present study, this negative impact seems to have apparently been mitigated by the low chitin content (4.8% DM) in *d*TM. Moreover, the integrity of anterior intestine was well preserved in all fish, without major morphological changes, with the exception of submucosa thickening in fish fed *d*TM. Li et al. (2020) have observed a higher degree of submucosa cellularity in the anterior intestine of seawater-phase Atlantic salmon (*Salmo salar*) fed a *H. illucens* based diet, but the submucosa of mid and posterior intestine remained unaltered after 8 weeks of feeding such diets to freshwater-phase fish (Li et al., 2019). According to Sitjà-Bobadilla et al. (2005), the increase in thickness of submucosa is usually due to infiltrations of

granular eosinophil cells, but this could not be confirmed in the present study. In contrast to the present results, in rainbow trout (*Oncorhynchus mykiss*), the replacement of FM by 25–50% full-fat or 100% defatted *H. illucens* reduced *villus* length in the anterior intestine after 12–14 weeks of feeding (Dumas et al., 2018; Cardinaletti et al., 2019). These controversial results may be associated to different species, feeds and processing methods used for IM production, leading to different nutritional profiles.

It was also observed that P ADC increased from 62% in fish fed CTRL to 72–80% in those fed the *d*TM diets. This higher P digestibility resulted in a pronounced reduction of total P losses, which suggests that the use of *d*TM as a FM replacer in European sea bass could help reducing P output into the environment. Likewise, Wang et al. (2017) demonstrated in Nile tilapia that FM replacement by increasing levels of *M. domestica* from 18% to 100% resulted in lower concentrations of total phosphate in the water. This metabolic feature might be part of the adaptive response of fish facing a reduced dietary P supply. Indeed, P deficiencies are a common nutritional disturbance of FM replacement by alternative proteins in aquafeeds (Sugiura et al., 2004; Prabhu et al., 2014). In the case of IM, this is a relevant issue because the mineral content of this feedstuff is markedly lower than FM or other conventionally FM substitutes, such as plant proteins. Indeed, ash content was reduced herein from 11.7% in CTRL diet to 6.5% in TM100 diet.

With total FM replacement, the increase of dietary lipids and energy content was concomitant with an improved digestibility of lipids that would favor increased whole-body lipid content by the end of the growth trial. This trend was not found in previous European sea bass studies where FM was replaced by IM from 25% to 71% (Gasco et al., 2016; Magalhães et al., 2017; Abdel-Tawwab et al., 2020). Likewise, in rainbow trout, Rema et al. (2019) did not observe any impact of *d*TM on lipid digestibility and retention. Regardless of this, our data of tissue body fat depots suggest an enhanced flux of lipids toward liver and secondly mesenteric fat when FM is totally replaced by *d*TM. This assumption is supported by data on muscle lipid content, and circulating lipid levels that were positively correlated with HSI and VSI. This is not surprising since European sea bass is a fish with low to moderate lipid deposition rates in the fillet (Ballester-Lozano et al., 2016). Although lipid content of liver has not been assessed, the strong and positive correlation between circulating triglycerides and HSI, and the moderate and positive correlation between final whole-body lipids content, lipid gain and HSI may indicate liver steatosis which is a sign of deficiencies in minerals and n-3 LC-PUFA (Ballester-Lozano et al., 2015). In contrast, enhanced deposition rate in mesenteric fat depots is highly informative of P deficiencies (Sugiura et al., 2004). In any case, both mineral and lipid

metabolism seem to be highly sensitive to FM replacement by IM, which might be exacerbated at long-term through the production cycle, especially in the case of lipid-enriched diets (see below).

Liver plays a major role in fish lipid metabolism, and its gene expression profile is greatly influenced by dietary composition and feeding levels (Monroig et al., 2018). In the present study, all diets were formulated with 12–13% of FO and the n-3 LC-PUFA levels (3.8–4.3% DM) were above recommended level for marine fish species (approximately 0.7% DM) (Skalli and Robin, 2004). When revisiting the gene expression profile of gilthead sea bream fed isolipidic diets with a maximal replacement of FO by vegetable oils or semisynthetic diets formulated to be deficient in n-3 LC-PUFA, the most responsive hepatic enzymes of lipid metabolism are *scd1* and *elovl6*, and secondly *fads2* (Benedito-Palos et al., 2016). This will contribute to mitigate the signs of deficiencies in n-3 LC-PUFA, though it is very important to limit hepatic lipogenesis to avoid the lipotoxic effects of excessive fat accumulation (Perera et al., 2019). Indeed, *scd1* is the rate limiting enzyme in the synthesis of MUFA, especially oleic acid (OA, 18:1n-9) and palmitoleic acid (16:1n-7) from stearoyl-CoA and palmitoyl-CoA, respectively. Likewise, *elovl6* is responsible for the elongation of SFA and MUFA of 12, 14 and 16 carbons to form 18-carbon FA (Weiss-Hersh et al., 2020). Therefore, the up-regulation of *scd1b* and *elovl6* enhances the biosynthesis of MUFA, which in turn increases the unsaturation index of FA membrane phospholipids. This is reinforced by the recent observation of Perera et al. (2019) that *scd1a* is epigenetically regulated in gilthead sea bream by broodstock nutrition with alpha-linolenic acid (ALA, 18:3n-3) enriched diets. In the present study, no changes in ALA were found among diets, whilst both OA and linoleic acid (LA, 18:2n-6) were 3–7 times higher in TM100 than in the CTRL diet. The observed down-regulation of *elovl6* in fish fed TM100, accompanied by a global decreasing trend of *scd1b* expression with FM replacement, support a reduced *de novo* lipogenic activity. In this scenario, the simultaneous up-regulated expression of *fads2* in fish fed TM100 will serve to further desaturate PUFA, but the capacity of *de novo* synthesis of n-3 LC-PUFA is limited in marine fish and European sea bass in particular, due to the lack of *elovl2* and reduced delta 5 desaturase activity (Monroig et al., 2018). Thus, although all dietary treatments provided European sea bass recommended n-3 LC-PUFA levels, TM100 diet may compromise the muscle n-3 LC-PUFA content at long term.

Cholesterol metabolism is also affected by FM replacement, and the hypocholesterolemic effect of plant proteins as FM replacers has been observed in a wide range of fish species, including gilthead sea bream (Gómez-Requeni et al., 2004; Benedito-Palos et al., 2016), European sea bass (Messina et al., 2013), rainbow

trout (Romarheim et al., 2008) and salmon (Hartviksen et al., 2014). Likewise, feeding trials conducted with semisynthetic diets highlighted that FO replacement with vegetal oils acts lowering triglycerides and cholesterol in gilthead sea bream (Ballester-Lozano et al., 2015). In the same study, diets formulated for deficiencies in phospholipids and vitamins also showed a hypocholesterolemic effect, whereas P deficiency acted as a hypercholesterolemic factor. Since this same trend was found herein with FM replacement by *d*TM, the availability of bioactive P becomes again a limiting factor for the successful replacement of FM, though signs of growth and health impairment were mostly masked at short-term. The precise mechanism remains unknown, but it would be favored by the down-regulated expression of hepatic *cyp7a1*, the rate limiting enzyme of the bile acid synthesis pathway (Jelinek et al., 1990). This assumption was supported by the negative correlation between circulating cholesterol and the expression level of hepatic *cyp7a1*, as *cyp7a1* deficiency has been reported to lead to hypercholesterolemia (Pullinger et al., 2002; Erickson et al., 2003; Qayyum et al., 2018). The ultimate physiological consequences of these findings remain elusive, but intriguingly the suppression of the hepatic *cyp7a1* expression in mice fed cholesterolemic diets mainly involves the activation of inflammatory cytokines (Henkel et al., 2011).

Growth of fish is intrinsically related to protein deposition in muscle, which is regulated by systemic and local signaling pathways involving the GH/IGF system (Mommsen, 2001). In this study, no differences in the expression of *gh* receptors or *igfs* transcripts were observed among diets, which is in line with growth performance results. Nonetheless, *myod2* plays a pivotal role in myoblast proliferation and differentiation (Valente et al., 2013), and its down-regulated expression in fish fed TM80 and TM100 should be indicative of some muscle growth derangement. Moreover, *mstn* is a negative regulator of muscle growth through inhibition of proliferation and differentiation of myogenic progenitor cells (Bonnieu et al., 2007), and its up-regulation coupled to the down-regulated expression of *myod2* highly supports muscle growth inhibition at the transcriptional level. The extent to which this negative feature is counter-regulated by compensatory growth mechanisms remains uncertain, but the expression of key transcripts of the ubiquitin proteasome pathway, with a main role in the protein turnover of skeletal muscle (Foletta et al., 2011), remained unaltered (muscle RING-finger protein-1; *murf1*) or were even down-regulated (*mafbx/atrogin-1*) with the total FM replacement. Furthermore, a recent study in zebrafish demonstrated that *mymk* plays a pivotal role in myoblast fusion and consequently in muscle growth (Shi et al., 2018), and interestingly the expression of this growth-promoting factor was consistently up-regulated with the total FM replacement. The net result at short-term would be, thereby, the preservation of

muscle growth with the use of IM as a main protein source in European sea bass. This was supported by the lack of changes in skeletal muscle cellularity and instrumental texture properties. To our best knowledge, the muscle cellularity of fish fed IM diets was never assessed before, but previous studies in different farmed fish species have evidenced the preservation of textural properties with high levels of FM replacement by IM (Lock et al., 2016; Borgogno et al., 2017; Iaconisi et al., 2017; Wang et al., 2017; Secci et al., 2019; Bruni et al., 2020).

In conclusion, the results of the present study demonstrate that FM replacement by *d*TM is largely feasible in European sea bass without detrimental effects on growth performance, nutrient utilization, intestinal integrity, and flesh nutritional and textural quality. Indeed, FM replacement by *d*TM resulted in fair levels of EPA and DHA in muscle of European sea bass (0.38–0.46 g/100 g WW), which are above those recommended by EFSA (2010) for human consumption to decrease the risk of cardiovascular diseases (0.25 g of EPA plus DHA per 100 g of fish). Also, most of the possible negative effects on P and lipid metabolism are largely mitigated at this high replacement level. Nonetheless, further research is needed to fully validate such nutritional approach at farm level throughout the production cycle, giving special attention to the total energy content of the diet since lipid-energized diets can exacerbate the disturbance of lipid metabolism. It is also important to mention that the reduction of P emissions to the environment, coupled with the reduction in the Fi:Fo ratio, prove that *d*TM is an environmentally sustainable alternative to FM.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by IATS-CSIC and CSIC Review Boards, European (2010/63/EU) Animal Directives, and Spanish Laws (Royal Decree RD53/2013) on the handling of experimental animals.

Author contributions

LV, JP-S, JC-G, and EM conceived and designed the study. AB, BO, LP, TS, MM, and SF conducted research, analyzed data, and performed statistical analysis. AB, JP-S,

and LV wrote the article and had primary responsibility for final content. All authors read and approved the final manuscript.

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Conflict of interest

EM was employed by the company SORGAL – Sociedade de Óleos e Rações, S.A., S. João de Ovar, Portugal. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 5.

Partial and total fishmeal replacement by defatted *Tenebrio molitor* larvae meal do not alter short- and mid-term regulation of food intake in European sea bass (*Dicentrarchus labrax*)

by

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Abstract

Understanding the underlying mechanisms responsible for regulation of food intake in fish is crucial for proper formulation of aquafeeds and consequently ensure the best growth performance of fish. Over the last decade, the study of the impact of insect meal-based diets on growth performance and voluntary feed intake of several fish species has emerged, but little is known about their influence on anorexigenic potential. This is the first study evaluating short- and mid-term responses of European sea bass (*Dicentrarchus labrax*) fed diets not only with partial, but also with total fishmeal (FM) replacement by defatted *Tenebrio molitor* larvae meal (*d*TM). A fishmeal-based diet was used as a control (CTRL) and two other diets were formulated to include 20% and 40% of *d*TM, replacing 50% and 100% of fishmeal (FM), respectively. The short-term response was evaluated after the first exposure to the experimental diets, whereas the mid-term response was evaluated after 7 days of feeding. In both short- and mid-term trials, food intake was registered. Hepatic and plasmatic metabolites and the expression of hypothalamic and telencephalic neuropeptides involved in food intake were assessed 2 and 24 h post-feeding. No differences occurred in food intake levels, neither in the short- nor mid-term. In the short-term, plasmatic glucose levels increased in fish fed TM100, regardless post-feeding sampling time. In the mid-term, fish fed TM100 had the highest levels of triglycerides in liver and the lowest levels of α-amino-acids in plasma, irrespective of post-feeding sampling time. At central level, dietary treatment did not alter the expression of neuropeptide Y (*npy*), agouti-related protein 2 (*agrp2*), pro-opio melanocortin a (*pomca*) or cocaine- and amphetamine-related transcript 2 (*cartpt2*) in hypothalamus and telencephalon. The obtained results suggest that partial and total FM replacement by *d*TM does not influence short- and mid-term regulation of food intake in European sea bass. Nonetheless, changes observed in hepatic and plasmatic metabolite levels of fish fed TM100 may indicate alterations of intermediary metabolism in the long-term.

Keywords: AgRP/NPY; appetite; aquafeeds; insect meal; POMC/CART.

5.1. Introduction

In fish, as in mammals, feeding behaviour is regulated by complex regulatory mechanisms carried out in the central nervous system, as response to both internal and external signals, such as food availability or its nutritional composition (Bertucci et al., 2019). Changes in circulating nutrient levels are detected by neuronal populations located in specific regions of the brain, such as hypothalamus and telencephalon, through specific nutrient sensors which receive metabolic information; or through hormonal receptorsreceive endocrine signals from peripheral hormones (Delgado et al., 2017; Soengas, 2021). Two neuronal populations integrate the information from the levels of circulating metabolites resulting in changes in mRNA abundance of orexigenic peptides' neuropeptide Y (NPY) and agouti-related peptide (AgRP), which stimulate appetite, and anorexigenic peptides' pro-opio melanocortin (POMC) and cocaine- and amphetamine-related transcript (CART), which supress appetite. These two populations of neurons inhibit each other, signalling other higher-order neurons and ultimately resulting in increased or decreased food intake (Soengas et al., 2018). Food intake could be regulated immediately after exposure to the dietary treatment for the first time (short-term regulation), after several days of feeding (mid-term regulation), or after longer feeding periods (weeks, months, or years) reflecting the energy balance of the animal (long-term regulation) (Soengas, 2014). Understanding the orexigenic and anorexigenic mechanisms responsible for feeding behaviour in fish is pivotal for ensuring the most appropriate dietary formulations for aquaculture industry, especially when new ingredients were suggested as alternatives to the limited marine resources, such as fishmeal (FM) and fish oil (FO) (FAO, 2020).

Since the insect protein from seven insect species (*Hermetia illucens, Musca domestica, Tenebrio molitor, Alphitobius diaperinus, Acheta domesticus, Gryllodes sigillatus* and *Gryllus assimilis*) was authorized by the European Union for their use in aquafeeds (European Commission, 2017), the interest in such sustainable ingredients has clearly emerged to both the scientific community and the aquaculture industry. In just a few years, the insect's production industry has risen in Europe, and the *H. illucens* and *T. molitor* are the main insect species produced (Pippinato et al., 2020). Consequently, they have been the most studied insect species used as FM substitute in diets for both freshwater and marine fish species with great importance for Mediterranean aquaculture, such as European sea bass (*Dicentrarchus labrax*) (Nogales-Mérida et al., 2018). In 2019, the European Union produced 84.430 tonnes of European sea bass, contributing with 32% of the worldwide production (FAO, 2022). Since sea bass is a carnivorous fish species, its protein requirement is high, ranging from 45% to 55%, depending on the stage

of development (NRC, 2011). Thus, when diets for this fish species are formulated, it is of paramount importance to use ingredients with a well-balanced essential amino acids (EAA) profile and high content of digestible protein. The potential of *H. illucens* and *T. molitor* larvae meal (HI and TM, respectively), both full-fat and defatted (*d*-), as protein sources in diets for European sea bass was previously assessed by Basto et al. (2020). The nutrient digestibility of these insect meals (IM) evidenced that *d*TM is the most promising alternative to FM in diets for this fish species, not only due to its higher digestible protein content but also due to its higher amount of digestible EAA when compared to HI, *d*HI and TM. To the best of our knowledge, the impact of dietary inclusion of *d*TM on the underlying mechanisms involved in the regulation of food intake of European sea bass was only evaluated in our previous study (Basto et al., 2021a). This study demonstrated that the central homeostatic regulation of food intake in fish was not affected by diets with up to 36% *d*TM inclusion, corresponding to 80% FM replacement by *d*TM. However, fish were simply evaluated after 10 weeks of feeding, possibly overlooking changes in mechanisms involved in the short-and mid-term regulation of food intake. Unravelling such short-term responses is essential since many of the regulatory mechanisms involved in food intake regulation occur at short times (Conde-Sieira and Soengas, 2017; Soengas et al., 2018; Soengas, 2021). Moreover, the impact of totally replacing FM by *d*TM on underlying mechanisms of regulation of food intake was also never studied before. Therefore, we aimed to test the hypothesis that short- and mid-term responses of homeostatic mechanisms involved in food intake regulation of European sea bass are not altered when this species is fed diets with partial or total FM replacement by *d*TM, thus supporting its use as ingredient in aquafeeds for this species.

5.2. Materials and methods

The study was directed by accredited scientists in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGAV Portugal, following FELASA category C recommendations) and conducted according to the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals for scientific purposes.

5.2.1. Experimental diets

Based on the known nutritional requirements of European sea bass (National Research Council, 2011), three isoproteic (47% on a dry matter basis; DM), isolipidic (20% DM) and isoenergetic (24% DM) diets were formulated and extruded by SPAROS Lda. (Portugal). A practical dietary formulation with 40% FM was used as control (CTRL)

and two other experimental diets were formulated with 20% and 40% of *d*TM, resulting in 50% and 100% FM replacement (TM50 and TM100, respectively). All experimental diets were supplemented with DL-Methionine; TM50 and TM100 were supplemented with monocalcium phosphate; TM100 was supplemented with L-Lysine, L-Threonine and L-Tryptophan. Ingredients, chemical composition, and fatty acid (FA) profile of experimental diets are presented on Table 5.1.

5.2.2. Fish husbandry

European sea bass juveniles were obtained from a commercial fish farm and transported for the Fish Culture Experimental Unit of CIIMAR (Porto, Portugal). Fish were held in quarantine for 2 weeks and hand fed with a commercial diet (AQUASOJA – 50% crude protein, 20% lipids). After quarantine period, fish were individually weighed (g) and nine homogeneous groups of 24 fish (70 ± 5 g; CV = 7%) were distributed in fiberglass tanks of 160 L where they were maintained until the experimental trials began. Fish were fed a commercial diet by automatic feeders until visual apparent satiation, as previously described in Basto et al. (2021b). During quarantine, acclimation period to the experimental tanks, and short- and mid-term trials fish were held in a seawater recirculation aquaculture system (RAS) at 22 ± 1 °C, salinity of 35‰, flow rate at 6 L min^{-1}, oxygen level > 90% ± 1 saturation, and 12 L:12D photoperiod). Levels of total ammonium ($NH4^{+}$), nitrite ($NO2^{-}$), and nitrate ($NO3^{-}$), as well as pH were daily monitored to ensure levels within the recommended ranged for marine fish species ($NH_4^{+} \leq 0.05$ mg L^{-1}; $NO_2^{-} \leq 0.5$ mg L^{-1}; $NO_3^{-} \leq 50$ mg L^{-1}; $7.5 \leq pH \leq 8.5$).

Table 5.1. Ingredients, chemical composition, and fatty acid profile of *d*TM and experimental diets.

	*d*TM	CTRL	TM50	TM100
Ingredients (%)				
Fishmeal [1]		40.0	20.0	-
Defatted *Tenebrio molitor* larvae meal		-	20.5	40.4
Soy protein concentrate [2]		10.5	10.5	10.5
Soybean meal [3]		13.0	13.0	13.0
Rapeseed meal [4]		5.0	5.0	5.0
Wheat meal [5]		16.2	15.2	14.3
Fish oil [6]		14.0	13.3	12.5
Vitamin and mineral premix [7]		1.0	1.0	1.0
Vitamin C		0.1	0.1	0.1
Vitamin E		0.1	0.1	0.1
Monocalcium phosphate		-	1.0	2.0
L-Lysine		-	-	0.2
L-Threonine		-	-	0.2
L-Tryptophan		-	-	0.1
DL-Methionine		0.1	0.2	0.3
Chemical composition (% DM)				
Dry matter	97.8	93.1	92.6	92.5
Protein	71.0	46.9	47.3	47.2
Lipids	12.1	19.7	19.8	19.0
Gross energy (kJ g^{-1} DM)	24.3	23.2	23.5	24.0
Ash	4.8	10.2	8.1	6.3
Phosphorus	0.8	1.2	1.2	1.0
Fatty acids (% DM)				
14:0	0.3	0.9	0.9	0.9
16:0	1.9	2.3	2.5	2.8
18:0	0.5	0.5	0.6	0.6
Σ SFA[8]	2.9	4.1	4.4	4.9
16:1	0.2	0.8	0.7	0.7
18:1n-9 (OA)	4.0	2.4	2.5	2.6
20:1n-9	0.02	0.6	0.6	0.5
24:1n-9	-	0.1	0.1	0.1
Σ MUFA[9]	4.3	4.5	4.5	4.4
18:2n-6 (LA)	4.5	1.0	1.6	2.2
18:3n-3 (ALA)	0.2	0.3	0.3	0.2
18:4n-3	0.004	0.4	0.4	0.4
20:3n-3	-	0.1	0.1	0.1
20:4n-6	0.004	1.3	1.3	1.1
20:5n-3 (EPA)	-	1.3	1.2	1.0
22:5n-3	-	0.2	0.2	0.2
22:6n-3 (DHA)	-	1.5	1.3	1.1
EPA + DHA	-	2.8	2.5	2.1
DHA / EPA	-	1.2	1.1	1.0
Σ PUFA[10]	4.6	6.2	6.3	6.4
Σ PUFA n-3	0.2	3.8	3.4	3.0
Σ PUFA n-6	0.004	1.3	1.3	1.1
Σ PUFA n-3/Σ PUFA n-6	40.1	2.9	2.7	2.6

The abbreviations for the experimental diets stand for: CTRL – control diet; TM50 and TM100 – diets with 50 and 100% fishmeal replacement by insect meal; [1] Peruvian fishmeal super prime: 71% crude protein (CP), 11% crude fat (CF), Exalmar, Peru; [2] Soy protein concentrate: 65% CP, 0.7% CF, ADM Animal Nutrition, The Netherlands; [3] Soybean meal 48: dehulled solvent extracted soybean meal: 48% CP, 2% CF, Cargill, Spain; [4] Rapseed meal: 36% CP, 3% CF, PREMIX Lda., Portugal; [5] Wheat meal: 10% CP, 1% CF, Casa Lanchinha, Portugal; [6] Sardine oil, Sopropêche, France; [7] Vitamin and mineral premix: WISIUM, ADM Portugal S.A., Portugal; [8] Includes 12:0, 15:0, 20:0, 21:0, 22:0 and 24:0; [9] Includes: 14:1, 17:1, 18:1n-7 and 22:1n-9; [10] Includes 18:3n-6 and 20:2.

5.2.3. Short-term trial

After 1-month acclimation period to the tanks, fish were fasted for 24 h before the short-term feeding trial began to ensure that the basal levels of metabolites involved in the regulation of food intake were achieved (Conde-Sieira and Soengas, 2017). Fish were then fed a single meal, distributed by automatic feeders (Basto et al., 2021b). Each diet was fed to triplicate groups of 24 fish (n = 72 fish per dietary treatment) and food intake was registered. After 2 and 24 h of feeding each experimental diet, all fish were lightly anesthetized in the tank with 2-phenoxyethanol (200 μL L^{-1}) and n = 12 fish per dietary treatment were sampled. Blood was collected with heparinized syringes from the caudal vein, immediately centrifuged (10,000 g for 5 min at 4 °C) and the resulting supernatant plasma was collected and stored at −80 °C until metabolite analysis. After that, fish were sacrificed by decapitation, and hypothalamus and telencephalon were collected and immediately frozen in dry ice and stored at −80 °C until RNA extraction. Liver was also collected and stored at −80 °C until metabolite analysis.

5.2.4. Mid-term trial

After the short-term trial, each experimental diet was distributed to the remaining fish by automatic feeders (Basto et al., 2021b), three times a day, for seven consecutive days. After feeding each diet for seven days, and after 24 h fasting period, fish were lightly anesthetized in the tank with 2-phenoxyethanol (200 μL L^{-1}) and n = 12 fish per dietary treatment were sampled, both 2 and 24 h after feeding. At the end of the experiment, food intake was registered and blood, hypothalamus, telencephalon, and liver were collected as described in the short-term trial.

5.2.5. Metabolite levels analysis

Liver samples were homogenized in 7.5 vols of ice-cooled 0.6 M perchloric acid and neutralized with 1 M potassium bicarbonate. The homogenate was centrifuged (10,000 g for 4.5 min at 4 °C), and the supernatant used to assay tissue metabolites. Plasma was also treated with 0.6 M perchloric acid and neutralized with 1 M potassium bicarbonate prior to be centrifuged at 13,500 g for 4.5 min at 4 °C. Levels of glucose, lactate, triglyceride, total cholesterol, and non-esterified fatty acids (NEFA) in deproteinized plasma and liver were determined enzymatically using commercial kits (1,001,190, 1,001,330, 1,001,313 and 1,001,090, Spinreact, Spain, and 434–91,795

NEFA-HR (2) R1 and 436–91,995 NEFA-HR (2) R2, Wako Chemicals, Germany, respectively), adapting manufacturer's instructions to a microplate format. Liver glycogen was measured according to Keppler and Decker (1974) method. Total α-amino acids of liver and plasma were assessed using the colorimetric ninhydrin method (Moore, 1968) with alanine as standard.

5.2.6. mRNA relative abundance analysis by RT-qPCR

Total RNA was extracted from hypothalamus and telencephalon using TRIzol reagent (Life Technologies, USA) and treated with RQ1DNAse (Promega, USA) according to the manufacturer's recommendations. RNA purity was tested by optical density (OD) absorption ratio (OD 260 nm/280 nm) using a NanoDrop 2000c (Thermo, Vantaa, Finland), and only samples with an OD 260 nm/280 nm ratio > 1.8 were used for analysis. Synthesis of cDNA was performed with 2 µg of total RNA using Superscript II reverse transcriptase and random hexamers (Promega, USA), following the standard protocol. Relative expression of genes was determined by real-time quantitative PCR (RT-qPCR) using the CFX96 Connect Real-Time PCR Detection System (Bio-Rad Laboratories Inc., USA). Analyses were performed on 1 µL cDNA using MAXIMA SYBR Green qPCR Mastermix (Life Technologies, USA), in a total PCR reaction volume of 15 µL, containing 50–500 nM of each primer. The expression of *agrp2*, *npy*, *cartpt2* and *pomca* was measured using previously described sequences of primers for the same fish species (Basto et al., 2021a), which are shown in the Table 5.2. A fragment of each sequence containing the amplicon was amplified by conventional PCR and run on a 1.2% agarose gel; the corresponding bands were cut from the gel, purified with the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, USA) and sequenced in a SeqStudio Genetic Analyser (Applied Biosystems-Thermofisher, USA) in Servicio de Determinación Estructural, Proteómica y Genómica (CACTI-Universidade de Vigo). The obtained sequences satisfactorily matched the reference GenBank sequences. Thermal cycling was initiated with incubation at 95 °C for 15 min using hot start iTaq DNA polymerase activation; 40 steps of PCR were performed, each one consisting of heating at 95 °C for 15 s for denaturing, and at specific annealing temperature for 30 s and extension at 72 °C for 30 s. Following the final PCR cycle, melting curves were systematically monitored (55 °C temperature gradient at 0.5 °C/s from 55 to 95 °C) to ensure that only one fragment was amplified. Each sample was analysed in duplicate and samples without reverse transcriptase or without cDNA were run as negative controls. Standard curves for each target and reference genes, *β-actin (actb)* and *elongation factor 1-α 1* (*eef1α1*), were done using a 1.5-fold serial dilution and the relative quantification of

each target transcripts was calculated using the respective standard curve and normalized to the geometric mean of two housekeeping genes, according to Del Vecchio et al. (2021). The expression of *actb* and ***eef1α1*** was measured using previously described primers for European sea bass (Azeredo et al., 2015; Basto et al., 2021a). Efficiency of primers was evaluated, and only values between 90% and 105% were accepted (Table 5.2).

5.2.7. Statistical analysis

Data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov and Levene's tests, respectively, and log-transformed whenever required. Differences were tested by a two-way ANOVA, with post-feeding sampling time and dietary treatment as main factors. A significance level of 95% ($P < 0.05$) was considered followed by a post-hoc Tukey HSD test, using Statistica™ 13.5.0.17 (TIBCO Software Inc., USA). All data with statistically significant differences were submitted to a principal component analysis (PCA) to evaluate the linear combinations of the original variables that best separate the groups (discriminant functions), using XLSTAT 2018 ® system software (Addinsoft, USA).

Table 5.2. Oligonucleotide sequences used to evaluate relative mRNA levels of genes by RT-qPCR.

Gene	Primer sequence 5'-3'	Annealing temperature (°C)	PCR Efficiency (%)	Database	Accession number
agrp2	F: GGGCAGAGGACACAAAGAAA	56	Hypothalamus: 92	GeneBank	HE660087
	R: TGTGACTTTCCTGTGGTGGA		Telencephalon: 94		
β-actin	F: TCCTGCGGAATCCACGAGA	57	Hypothalamus: 92	GeneBank	AY148350
	R: AACGTCGCACTTCATGATGCT		Telencephalon: 97		
cartpt2	F: CCGAACCTGACCAGCGAGAA	62	Hypothalamus: 100	GeneBank	MZ441181
	R: GCTCCCCGACATCACACGTT		Telencephalon: 102		
eef1a1	F: CGTTGGCTTCAACATCAAGA	55	Hypothalamus: 102	GeneBank	AJ866727
	R: GAAGTTGTCTGCTCCCTTGG		Telencephalon: 104		
npy	F: ACGGAGGGATACCCGGTGAA	60	Hypothalamus: 94	GeneBank	AJ005378
	R: GCTGAGTAGTACTTGGCCAGCTC		Telencephalon: 96		
pomca	F: CCGGTCAAAGTCTTCACCTC	57	Hypothalamus: 101	GeneBank	AY691808
	R: ACCTCCTGTGCCTTCTCCTC		Telencephalon: 104		

agrp2, agouti-related protein 1; c*art2,* cocaine- and amphetamine-related transcript protein-like 2; ***ef1α,*** elongation factor 1α; *npy,* neuropeptide Y; *pomca,* pro-opio melanocortin a.

5.3. Results

3.1. Food intake

The results of food intake of European sea bass fed the experimental diets for the first time (short-term response) and after seven days of feeding (mid-term response) are shown in Figure 5.1A and Figure 5.1B, respectively. The dietary inclusion of *d*TM did not significantly affect fish food intake, neither in the short- nor mid-term.

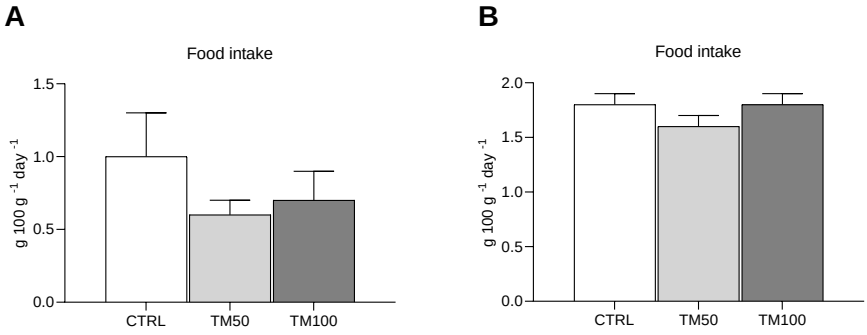

Figure 5.1. Food intake of European sea bass fed experimental diets for the first time, i.e. short-term trial (A) and after seven days of feeding, i.e. mid-term trial (B). Bars represent means ± standard deviation (SD). Short-term trial: n = 72 fish per dietary treatment (24 fish per 3 tanks). Mid-term trial: n = 36 fish per dietary treatment (12 fish per 3 tanks).

5.3.2. Liver and plasma metabolites

The post-feeding metabolites levels in liver and plasma of European sea bass after short- and mid-term exposure to the experimental diets are shown in Figure 5.2 and Figure 5.3, respectively, and the *F*- and *P*-values of the two-way ANOVA applied to these parameters are presented in Table 5.3. In the short- and mid-term response, glycogen levels in the liver of European sea bass did not change either with the dietary treatment or the post-feeding sampling time (Figure 5.2A and Figure 5.3A). After the short-term exposure to the experimental diets, glucose levels were significantly higher in the liver of fish fed TM100 when compared to those fed CTRL and TM50, regardless the post-feeding sampling time (Figure 5.2B). After the mid-term exposure to the experimental diets, fish fed TM50 had significantly higher levels of glucose in liver than those fed CTRL and TM100, 24 h after feeding (Figure 5.3B). Besides, sea bass fed CTRL had significantly higher levels of glucose in liver 2 h after feeding when compared to the 24 h sampling (Figure 5.3B); the opposite was observed for fish fed TM50, which had

significantly lower levels of glucose 2 h after feeding when compared to 24 h (Figure 5.3B). In plasma, glucose levels were not affected by dietary treatment but were significantly higher 2 h after feeding when compared to 24 h, either in the short- or mid-term response (Figure 5.2C and Figure 5.3C, respectively). Lactate levels remained unaltered, both in liver and plasma of European sea bass after feeding the experimental diets for the first time and after 7 consecutive days of feeding (Figure 5.2D-E and Figure 5.3 D-E, respectively). Both in the short- and mid-term response, triglycerides levels in liver were significantly higher 2 h after feeding than 24 h, regardless the dietary treatment (Figure 5.2F and Figure 5.3F, respectively). Moreover, in the mid-term response fish fed TM100 had the highest levels of triglycerides in liver, irrespectively of the post-feeding sampling time (Figure 5.3F). Plasma triglycerides were not affected by the dietary treatment, or the post-feeding sampling time, neither in the short- nor in the mid-term (Figure 5.2G and Figure 5.3G, respectively); Cholesterol and NEFA levels in liver also did not vary with *d*TM inclusion and post-feeding sampling time, neither in the short- nor in the mid-term (Figure 5.2H and Figure 5.2J and Figure 5.3H and Figure 5.3J**,** respectively); in plasma, both cholesterol and NEFA levels were significantly higher 2 h after feeding when compared to 24 h, regardless the dietary treatment (Figure 5.2I and Figure 5.2K and Figure 5.3I and Figure 5.3K**,** respectively). In the short-term response, 2 h after feeding, the levels of α-amino acids were significantly lower in the liver of fish fed TM100 when compared to those fed CTRL (Figure 5.2L); in plasma, the levels of α-amino acids did not change with dietary treatment and post-feeding sampling time (Figure 5.2M**)**. Contrarily, in the mid-term response, the levels of α-amino acids in liver did not change with dietary treatment and post-feeding sampling time (Figure 5.3L). However, 2 h after feeding, the α-amino acids levels in plasma of fish fed TM100 were significantly lower than those fed CTRL and TM50 (Figure 5.3M).

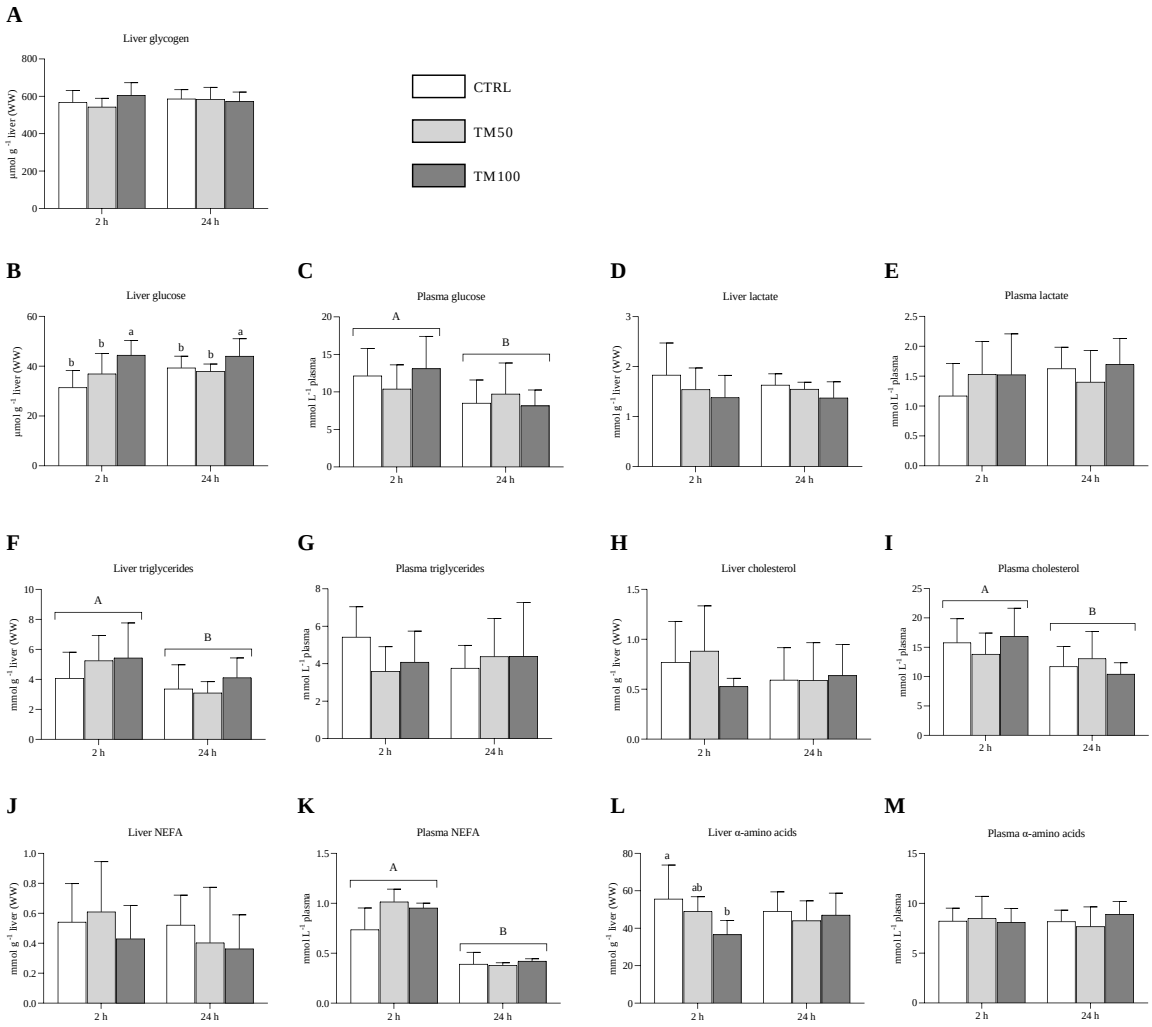

Figure 5.2. Post-feeding metabolite levels in liver and plasma of European sea bass 2 and 24 h after feeding the experimental diets for the first time (short-term trial). Bars represent means ± SD of the results obtained from n = 12 fish per dietary treatment sampled at different post-feeding times. Different uppercase letters indicate significant differences ($P < 0.05$) between post-feeding sampling times, regardless dietary treatments. Different lowercase letters indicate significant differences ($P < 0.05$) among dietary treatments within the same post-feeding sampling time.

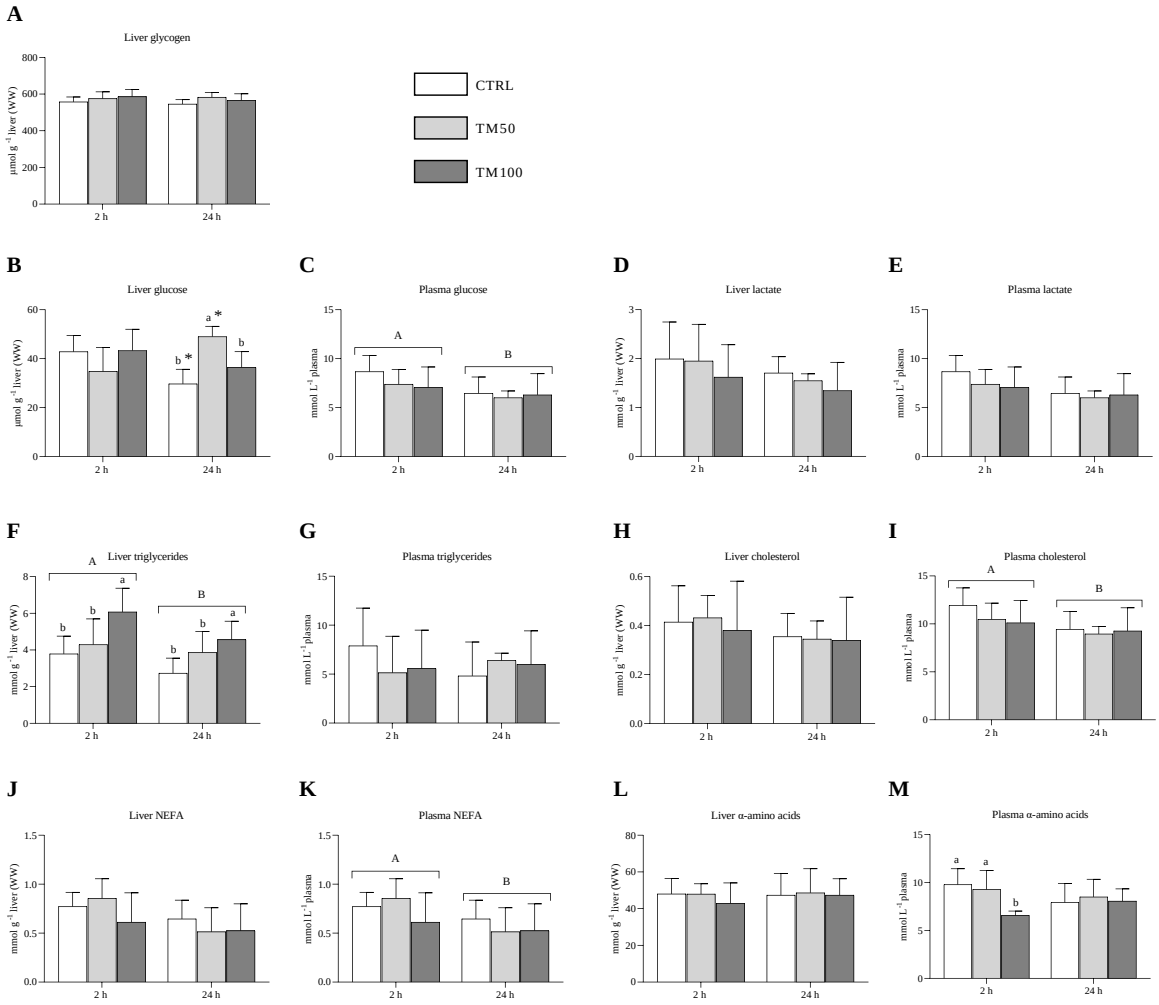

Figure 5.3. Post-feeding metabolite levels in liver and plasma of European sea bass 2 and 24 h after feeding the experimental diets for seven days (mid-term trial). Bars represent means ± SD of the results obtained from n = 12 fish per dietary treatment sampled at different post-feeding times. Different uppercase letters indicate significant differences ($P < 0.05$) between post-feeding sampling times, regardless dietary treatments. Different lowercase letters indicate significant differences ($P < 0.05$) among dietary treatments within the same post-feeding sampling time. The asterisk (*) indicates significant differences ($P < 0.05$) between post-feeding sampling times within the same dietary treatment.

5.3.3. mRNA relative abundance of neuropeptides in hypothalamus and telencephalon

The results of post-feeding mRNA relative abundance of neuropeptides involved in the regulation of food intake in hypothalamus and telencephalon of European sea bass after short- and mid-term exposure to the experimental diets are presented in Figure 5.4 and Figure 5.5, respectively. The expression of *pomca* in telencephalon is not presented because its mRNA relative abundance was very limited. In hypothalamus, the mRNA relative abundance of *npy* and *cartpt2* did not change with *d*TM dietary inclusion or

post-feeding sampling time, neither at short- nor at mid-term (Figure 5.4A and Figure 5.4C, and Figure 5.5A and Figure 5.5C, respectively). The expression of *agrp2* in hypothalamus was significantly lower 2 h after feeding when compared to its expression 24 h after feeding, either after short- or mid-term exposure to the experimental diets (Figure 5.4B and Figure 5.5B, respectively). Still in hypothalamus, either at short- or mid-term, the opposite pattern was observed for the expression of *pomca* (Figure 5.4D and Figure 5.5D, respectively). In telencephalon, the expression of neuropeptides did not vary, neither with *d*TM dietary inclusion nor with post-feeding sampling time, after either short or mid-term exposure to the experimental diets (Figure 5.4E-G and 5.5E-G, respectively). The *F*- and *P*-values of the two-way ANOVA applied to mRNA relative abundance of neuropeptides assessed in the two brain tissues are shown in Table 5.3**.**

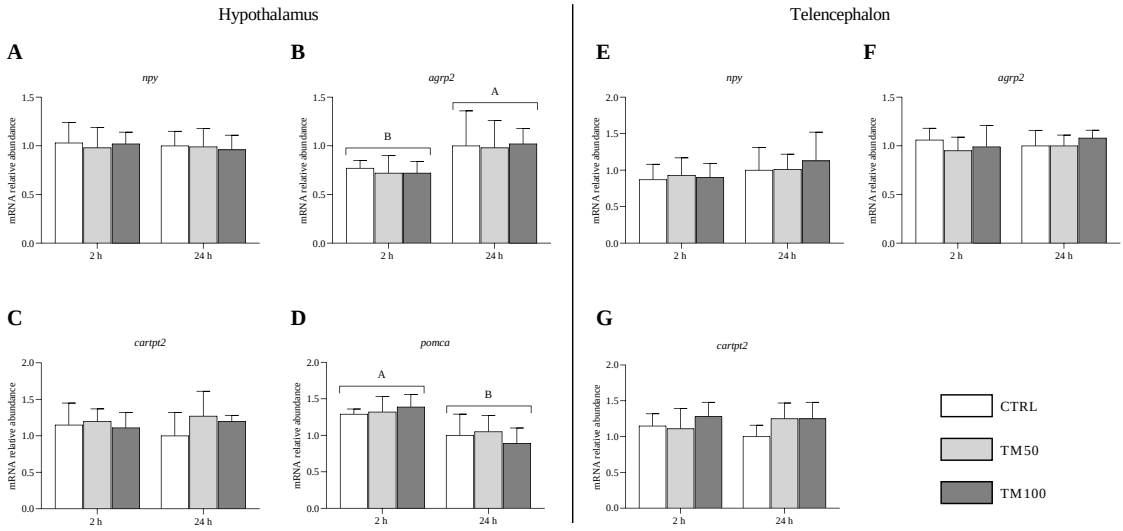

Figure 5.4. Post-feeding mRNA relative abundance of neuropeptides involved in the regulation of food intake in hypothalamus and telencephalon of European sea bass 2 and 24 h after feeding the experimental diets for the first time (short-term trial). Bars represent means ± SD of results obtained from n = 6 fish per dietary treatment sampled at different post-feeding times. Different uppercase letters indicate significant differences ($P < 0.05$) between post-feeding sampling times, regardless dietary treatments.

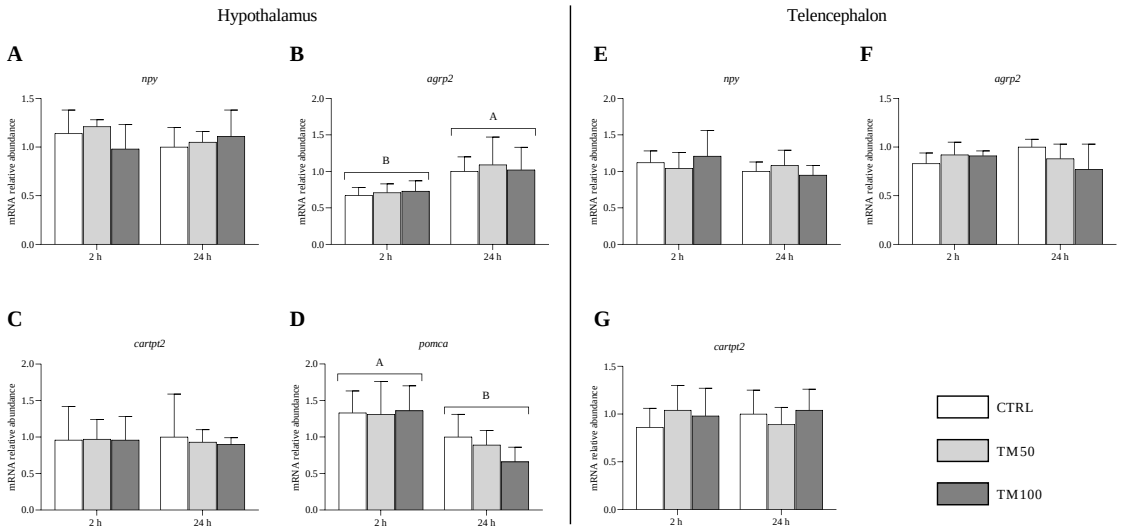

Figure 5.5. Post-feeding mRNA relative abundance of neuropeptides involved in the regulation of food intake in hypothalamus and telencephalon of European sea bass 2 and 24 h after feeding the experimental diets for seven days (mid-term trial). Bars represent means ± SD of results obtained from n = 6 fish per dietary treatment sampled at different post-feeding times. Different uppercase letters indicate significant differences ($P < 0.05$) between post-feeding sampling times, regardless dietary treatments.

Table 5.3. *F*- and *P*-values obtained after two-way ANOVA of parameters assessed in liver, plasma, hypothalamus and telencephalon of European sea bass fed experimental diets for the first time and after seven consecutive days of feeding.

	1st day of feeding						7th day of feeding					
	Dietary treatment (D)		Post-feeding time (T)		D × T		Dietary treatment (D)		Post-feeding time (T)		D × T	
	F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Liver metabolites												
Glycogen	0.89	0.42	0.35	0.56	2.04	0.14	3.31	0.05	0.65	0.43	0.67	0.52
Glucose	11.60	**< 0.001**	2.86	0.10	2.69	0.08	2.18	0.12	0.79	0.38	13.25	**< 0.001**
Lactate	2.72	0.08	2.87	0.07	1.82	0.15	2.07	0.14	3.51	0.07	0.05	0.96
Cholesterol	1.03	0.36	1.86	0.18	1.92	0.16	0.21	0.81	0.01	0.92	1.16	0.32
Triglycerides	0.10	0.90	10.28	**0.002**	2.94	0.06	18.54	**< 0.001**	11.57	**0.001**	1.04	0.36
NEFA	2.19	0.12	2.70	0.11	0.29	0.75	0.09	0.92	0.83	0.37	3.01	0.06
α-amino acids	4.47	**0.02**	0.02	0.90	3.36	**0.04**	0.03	0.97	0.64	0.43	0.74	0.48
Plasma metabolites												
Glucose	0.15	0.86	12.56	**0.001**	2.09	0.13	1.11	0.34	7.83	**0.01**	0.67	0.52
Lactate	1.02	0.37	1.80	0.19	1.90	0.16	0.83	0.44	0.24	0.63	0.08	0.92
Cholesterol	0.03	0.97	14.45	**< 0.001**	2.69	0.08	1.11	0.34	7.83	**0.01**	0.67	0.52
Triglycerides	0.53	0.60	0.14	0.71	2.43	0.10	0.18	0.84	0.24	0.63	2.10	0.13
NEFA	0.74	0.49	32.01	**< 0.001**	0.57	0.74	2.08	0.14	8.27	**0.01**	1.41	0.25
α-amino acids	0.37	0.70	0.001	0.97	1.22	0.30	4.89	**0.01**	0.65	0.42	4.52	**0.02**
Hypothalamus neuropeptides												
npy	0.09	0.91	0.17	0.69	0.10	0.91	2.31	0.14	0.01	0.92	0.55	0.59
agrp2	0.12	0.89	12.14	**0.002**	0.07	0.93	0.23	0.80	18.25	**< 0.001**	0.11	0.89
cartpt2	1.30	0.29	0.01	0.94	0.63	0.54	0.20	0.82	0.51	0.49	0.05	0.95
pomca	0.15	0.87	25.47	**< 0.001**	1.14	0.33	0.72	0.50	19.39	**< 0.001**	1.04	0.37
Telencephalon neuropeptides												
npy	0.29	0.75	2.32	0.14	0.30	0.75	0.59	0.57	4.69	0.05	0.08	0.93
agrp2	1.12	0.34	0.83	0.37	1.21	0.31	0.55	0.59	0.06	0.81	2.99	0.08
cartpt2	3.02	0.07	0.14	0.72	3.32	0.05	0.80	0.47	0.10	0.76	0.26	0.78

agrp2, agouti-related protein 1; *cartpt2*, cocaine- and amphetamine-related transcript protein-like 2; *npy*, neuropeptide Y; *pomca*, pro-opio melanocortin a. *P*-values in bold (P<0.05) indicate statistical significant differences at a significance level of 95%.

5.4. Discussion

In recent years, several efforts have been made to assess the impact of IM as alternative to the limited available FM used as main protein source in diets for aquaculture with most efforts related to growth and nutritional requirements (Nogales-Mérida et al., 2018; Gasco et al., 2020; Alfiko et al., 2022). Most of the available studies evaluated the effect of IM-based diets on fish food intake, but only our previous study (Basto et al., 2021a) and those of Pulido-Rodriguez et al. (2021) and Zarantoniello et al. (2020) assessed the impact of IM on the underlying mechanisms responsible for homeostatic regulation of food intake. Zarantoniello et al. (2020) observed that total FM replacement by full-fat HI resulted in increased mRNA abundance of the neuropeptide *npy* after 15 days of feeding. However, they tested the experimental diets in fish larvae with 5 to 20 days after fertilization and used the whole individual to assess the expression of genes instead of evaluating them in the appropriate brain regions involved in regulation of food intake, namely hypothalamus and telencephalon (Soengas et al., 2018). Moreover, the amount of feed supplied to fish larvae during the entire trial was fixed (2% of larvae weight) and, from day 5 to day 10 after fertilization, fish larvae also were fed on the rotifer *Brachionus plicatilis* thus further limiting the relevance of the results from a mechanistic point of view. In our previous study, we demonstrated that dietary inclusion of 36% *d*TM (80% FM replacement) did not alter mRNA abundance of hypothalamic neuropeptides *npy*, *agpr2*, *cartpt2* and *pomca* after 10 weeks of feeding (Basto et al., 2021a). These results allowed us to suggest that appetite-regulatory mechanisms were not affected by long-term partial replacement of FM by *d*TM meal. Likewise, in gilthead sea bream (*Sparus aurata*), Pulido-Rodriguez et al. (2021) demonstrated that the expression of *npy* at central level also remained unchanged after being fed a diet with 32% full-fat HI prepupae meal, replacing 47% vegetable proteins (VP), for 21 weeks.

The few available studies assessing the mechanisms involved in food intake regulation in fish fed IM-rich diets were conducted after long-term feeding periods, and the impact of IM on the short- and mid-term regulation food intake remained unknown. Moreover, none of the available studies focused on a brain region - telencephalon - essential for fish food intake regulation (Soengas et al., 2018). Most studies have demonstrated that mechanisms regulating food intake operate in the first hours after fish being exposed to different levels of nutrients (Librán-Perez et al., 2014; Bonacic et al., 2017; Comesana et al., 2018; Conde-Sieira et al., 2018; Velasco et al., 2020). Nevertheless, the present study clearly showed that short- and mid-term partial replacement of FM by *d*TM meal induced no changes in food intake of European sea bass

even in diets completely devoid of fish meal. Therefore, the mechanisms involved in homeostatic regulation of food intake were apparently not affected by FM replacement since mRNA abundance of neuropeptides was similar among diets. However, the hypothalamic expression of *agrp2* increased whereas the hypothalamic expression of *pomca* decreased between sampling times, which could be reflecting a typical response of feeding circadian rhythms of fish. In fact, several studies have demonstrated that feeding cycles are determined endogenously to anticipate and respond to predictable changes of food availability (Soengas et al., 2018). It is important to emphasize that this is the first time that the impact of IM replacement on mechanisms regulating food intake is evaluated, not only in fish hypothalamus, but also in telencephalon. More importantly, considering that most regulatory central mechanisms operate under short- and mid-term time periods (Delgado et al., 2017; Soengas et al., 2018), the present results are more important from a physiological point of view than those previously obtained in a long-term study which are more relevant in terms of production.

The central mechanisms involved in the homeostatic regulation of food intake in fish are also affected by changes in circulating levels of metabolites through different sensing systems for nutrients including fatty acids (Conde-Sieira and Soengas, 2017). The *d*TM meal was particularly rich in palmitic (16:0; PA), oleic (18:1n-9; OA), and linoleic (18:2n-6; LA) acids, resulting in a concomitant increase of these FA in the experimental diets; the LA varied the most, increasing from 1.0% in CTRL to 2.2% in TM100. In contrast, the limited or even absence of n-3 long-chain polyunsaturated FA (n-3 LC-PUFA) in *d*TM, particularly eicosapentaenoic (20:5n-3; EPA) and docosahexaenoic acids (22:6n-3; DHA), resulted in decreased content of these group of FA in the experimental diets. To the authors' best knowledge, the hypothalamic FA sensing mechanisms and the modulation of neuropeptides involved in food intake regulation have not been previously assessed in European sea bass. However, in other fish species it was previously described that hypothalamus detects changes in circulating levels of OA, LA and α-linolenic acid (18:3 n-3; ALA), resulting in the activation of anorexigenic potential, ultimately leading to alterations of food intake (Conde-Sieira and Soengas, 2017). In rainbow trout (*Oncorhynchus mykiss*), either an intraperitoneal injection (IP) (Librán-Perez et al., 2012), intracerebroventricular injection (ICV) (Librán-Perez et al., 2014), or oral administration (Velasco et al., 2016) of OA have all activated hypothalamic FA sensing systems, resulting in modulation of neuropeptide mRNA abundance and consequently decreased food intake. Luo et al. (2020) observed that not only ICV administration of OA, but also ICV of LA and ALA inhibited food intake of Chinese perch (*Siniperca chuatsi*), which was consistent with the activation of hypothalamic FA sensing systems.

Contrarily, stearic acid (18:0; SA) seems to have no impact on the mechanisms involved in the regulation of food intake or FA sensing system of this species (Luo et al., 2020). In Senegalese sole (*Solea senegalensis*), likewise in rainbow trout, the FA sensing systems appeared to be sensitive to increased levels of OA and ALA, but not to IP injection of SA or EPA (Conde-Sieira et al., 2015). Thus, the previously mentioned studies proved that hypothalamus is capable of detecting changes in specific FA (OA, LA and ALA) when directly administrated (IP and ICV injection or orally) to fish. Yet, little is known about the impact of a complete diet, containing macronutrients at different levels, on the central mechanisms responsible for the regulation of food intake in fish.

Bonacic et al. (2017) studied the impact of feeding Senegalese sole diets with 75% FO replaced by vegetable oils (VO), at different lipid levels (8% or 18%, at the expense of carbohydrates) on food intake and on the expression of appetite–regulating genes after 13 weeks. Contrarily to results obtained by Conde-Sieira et al. (2015), neither the voluntary feed intake (VFI) nor the expression of neuropeptides in the brain of Senegalese sole were affected by the dietary treatments, which had variable levels of OA, LA and ALA (Bonacic et al., 2017). Besides, the VFI decreased, whilst the expression of *cart1a* and *cart1b* increased in fish fed experimental diets with the highest levels of both EPA and DHA, which also had the highest n-3 LC-PUFA/n-6 LC-PUFA ratios (Bonacic et al., 2017). Roy et al. (2020a) also observed that supplementation of plant-based diets with EPA and DHA resulted in decreased VFI of rainbow trout after 9 weeks of feeding. Nonetheless, authors observed an increase of *npyB* and a decrease of *pomcA* in hypothalamus of rainbow trout 6 h after feeding high levels of EPA and DHA (Roy et al., 2020a). These contradictory results between VFI and neuropeptides expression may suggest that EPA and DHA have a different role in homeostatic and hedonic regulation of food intake. Hedonic or reward-based regulation of food intake is driven by pleasure and/or sensory perception, resulting in increased consumption of highly palatable foods independently of the energy balance status (Lutter and Nestler, 2009; Tulloch et al., 2015). Thus, high levels of EPA and DHA may be responsible for the upregulation of orexigenic neuropeptides and downregulation of those with anorexigenic potential in the short-term, but VFI still decreases in the long-term due to alterations in fish metabolism. In fact, in a previous study of Roy et al. (2020b), it was demonstrated that rainbow trout can discriminate between diets containing low or medium levels of EPA and DHA (0.2 and 5% of total FA, respectively) and high EPA and DHA levels (20% of total FA) and preferred the high-level dietary treatment which could refer to a hedonic response under the presence of these FA in the diet. On the other hand, differences between low and medium levels were not discriminated by fish, suggesting a dose-dependent response (Roy et al.,

2020b). In the present study, despite the different FA profile among dietary treatments, the food intake and expression of neuropeptides responsible for homeostatic regulation of feeding behaviour in European sea bass remained unchanged, either in the short- or mid-term. These correspond well to previous long-term findings (Basto et al., 2021a) and suggest that increased dietary content of OA and LA, and decreased content of EPA and DHA were not enough to induce and anorexigenic response in hypothalamus and telencephalon of European sea bass. This is further supported by the absence of changes both in food intake and in mRNA abundance of main neuropeptides involved in the central regulation of food intake. These results therefore strengthen the hypothesis that both partial and total FM replacement by *d*TM does not impair homeostatic regulation of European sea bass food intake.

The present results would also indicate that presumed differences in the amino acidic composition of *d*TM diets compared with CTRL may also not influence the homeostatic control of food intake, since only differences in glutamine + glutamate were expected as reported in a previous study using similar experimental diets (Basto et al., 2021a, Basto et al., 2021b). Furthermore, it was previously reported that the amino acids with higher influence on the sensing mechanisms involved in food intake regulation are valine and leucine (Comesana et al., 2018) and no significant differences were detected for these amino acids content in diets with similar formulations to the ones used in the present study (Basto et al., 2021a, Basto et al., 2021b). However, it cannot be discarded that the variations in the amino acidic composition of *d*TM diets compared with control, even being small, could influence parameters related with the hedonic regulation of food intake that operates at short term, since there are not previous studies underlying this aspect. Thus, further studies underlying the effect of insect-based diets on parameters involved in the hedonic regulation of food intake in fish would be of high interest.

In the present study, despite no major impact on food intake, hepatic and plasmatic metabolites suggest alterations in intermediary metabolism of European sea bass fed *d*TM that may compromise growth performance and/or health status of fish at long-term. In the short-term, the dietary inclusion of *d*TM led to increased glucose levels in liver (regardless post-feeding time), whereas in the mid-term the hepatic glucose levels were higher in fish fed TM50 at 24 h post-feeding. It is well known that when glucose levels are high, this metabolite can be stored as glycogen (glycogenesis) or converted into lipids (lipogenesis), such as FA or triglycerides (Polakof et al., 2012). Since no changes in glycogen nor NEFA levels were observed, it can be inferred that the increased hepatic glucose levels of fish fed *d*TM may have contributed to the increased triglycerides levels at short- and mid-term, even if differences lacked statistical significance. Nonetheless, to

validate this hypothesis would be important to deepen knowledge about the effect of such diets on different intermediary metabolic pathways, of European sea bass fed *d*TM, such as glycolysis and lipogenesis, through metabolomic and enzymatic tools.

5.5. Conclusions

The present results demonstrate that both the partial and total replacement of FM by *d*TM do not alter food intake of European sea bass. The mRNA abundance of neuropeptides involved in the homeostatic regulation of appetite remained unaffected in the short- and mid-term scenario, both in hypothalamus and (for the first time in fish) in telencephalon. However, observed changes in hepatic and plasmatic metabolites may have detrimental effects on growth performance and/or health status of fish, at the long-term. Thus, further studies are necessary to elucidate such possible impact and provide further support for the use of dTM as a relevant ingredient in the formulation of more sustainable aquafeeds for European sea bass.

CRediT authorship contribution statement

Ana Basto: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Luisa M.P. Valente:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **José L. Soengas:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Marta Conde-Sieira:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision.

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Chapter 6.

Total fishmeal replacement by defatted *Tenebrio molitor* larvae meal induces alterations in intermediary metabolism of European sea bass (*Dicentrarchus labrax*)

by

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Lay summary

Insect meal has been increasingly considered as novel protein source in diets for different animal models, including fish. European sea bass is one of the most important fish species in Europe, particularly in Mediterranean. Fishmeal is considered a premium protein source in diets for aquaculture fish. In this connection, this study evaluated the impact of partial and total fishmeal replacement by an insect meal (defatted *Tenebrio molitor* larvae meal) on intermediary metabolism of European sea bass. After 16-week of feeding, the results obtained demonstrated that selected insect meal can be successfully used to replace 50% of fish in diets for European sea bass, without adversely affecting liver health or intermediary metabolism of nutrients. On the other hand, total fishmeal replacement by insect meal may compromise fish health in the long-term.

Abstract

The replacement of fishmeal (FM) by insect meal (IM) in aquafeed formulation has been thoroughly studied lately, but little is known about their impact on nutrient metabolism of fish. This study evaluated the impact not only of partial, but also total FM replacement by IM on intermediary metabolism of European sea bass (*Dicentrarchus labrax*). A fishmeal-based diet was used as a control (CTRL) and two other diets were formulated to include 20% and 40% of defatted *Tenebrio molitor* larvae meal (*d*TM), replacing 50% (TM50) and 100% (TM100) of fishmeal (FM), respectively. After a 16-week feeding trial, a multidisciplinary approach including assessment of histological, biochemical, molecular, and enzymatic parameters was adopted to investigate hepatic and plasmatic responses to the different dietary formulations. The results obtained demonstrated that *d*TM can be successfully used to replace 50% of FM in diets for European sea bass, without adversely affecting liver health or intermediary metabolism of nutrients. As for TM100, although no signs of steatosis were observed in the liver, the activity of glycolytic and lipogenic genes and enzymes increased when compared to CTRL diet ($P < 0.05$), resulting in higher levels of plasmatic non-esterified fatty acids and triacylglycerides ($P < 0.05$), which in the long-term may compromise fish health, thus precluding such a high degree of substitution for use in practical diets for European sea bass.

Keywords: animal nutrition; hepatic genes and enzymes; insect meal; intermediary metabolism; metabolites

6.1. Introduction

Over the last decades, the outstanding efforts made to find alternative protein sources to replace fishmeal (FM) in aquafeeds have caused particular interest in fish physiological responses to the new dietary formulations (Aragão et al., 2022). In this regard, the evaluation of the organs involved in regulation of feed intake, digestion, absorption, and metabolism is of paramount importance to understand the feasibility of using new protein sources in aquafeeds formulations. Fish intestine plays a key role in the peripherical regulation of feed intake, digestion and absorption of nutrients and immunity response (Ray and Ringø, 2014; Blanco et al., 2021). After nutrient absorption the liver plays a primary role in the metabolism of dietary nutrients and its morphology and composition is highly dependent on the diet. Therefore, this organ must be the main target when it is intended to deepen knowledge about metabolic responses of fish to new dietary ingredients (Randazzo et al., 2021). The evaluation of hepatosomatic index (HSI) can give an indication of possible metabolic disorders in fish, such as hepatic steatosis (i.e., increased liver size due to lipids accumulation), which is the most common consequence of alterations in the metabolism of different nutrients in fish and may result in growth and health impairments of the animals (Asaoka et al., 2013; Jia et al., 2020). However, such hypothesis must be validated through different methodologies. Histological techniques are the most conventional ones used to identify morphological modifications and lipid deposition in liver, with Oil Red O being not only the most intuitive, but also the most accurate staining for that purpose (Levene et al., 2010; Riva et al., 2018). Additionally, new dietary formulations can also trigger metabolic alterations in the liver by the activation of a set of metabolic-related genes and enzymes with key roles in different pathways (e.g., glycolysis, gluconeogenesis, lipogenesis, lipolysis, β-oxidation, and amino acid anabolism and catabolism), providing information on metabolic responses of fish to new aquafeeds formulations, even in the absence of clear histo-morphological evidence (Viegas et al., 2014; Zhang et al., 2020).

Since the inclusion of insect meal (IM) from seven insect species in aquafeeds was approved by the European Union (EC Regulation No. 2017/893; European Commission, 2017), the interest in this protein source as alternative to FM has emerged (Alfiko et al., 2022; Tran et al., 2022). Generally, insects have high protein content (up to 75%), with a well-balanced essential amino acids profile; they are rich in minerals (e.g., iron and zinc) and vitamins (e.g., vitamin B_{12}) and have biologically active compounds, such as chitin, antimicrobial peptides, and short-medium fatty acids (e.g., lauric acid) with beneficial effects on intestinal health of different fish species (Randazzo et al., 2021; Weththasinghe et al., 2021). On the other hand, and despite their high fat content, insects

have limited amounts or even absence of n-3 long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic and docosahexaenoic acids (EPA and DHA, respectively) (Nogales-Mérida et al., 2018). Since these nutrients are essential for carnivorous marine fish species (NRC, 2011), this may cause constraints when IM is used to replace high levels of FM in diets for such species. *Hermetia illucens* (HI) and *Tenebrio molitor* (TM) are the main insect species produced in Europe and have been the most studied as FM alternatives, in diets for both freshwater, and marine fish species with great importance for Mediterranean aquaculture like European sea bass (*Dicentrarchus labrax*) (Nogales-Mérida et al., 2018; Pippinato et al., 2020). Previous results of the *in vivo* nutrients' digestibility of HI and TM, both full-fat and defatted (-*d*), in European sea bass juveniles demonstrated that *d*TM is the most promising alternative to FM in diets for this fish species for having both the highest digestible protein (641 mg g^{-1} DM) and digestible essential amino acids (296 mg g^{-1} DM) content (Basto et al., 2020). The inclusion of *d*TM did not alter feed intake and its regulatory mechanisms in European sea bass (Basto et al., 2021a, 2022a). However, when *d*TM was included at high levels (as total FM replacement) in diets for sea bass, induced alterations in hepatic and plasmatic metabolites, suggesting alterations in intermediary metabolism of fish that may compromise growth performance and/or health status of fish at long-term (Basto et al., 2021b; Basto et al., 2021a; Basto et al., 2022).

In this connection, the present study aimed to explore for the first time the impact of partial and total substitution of FM by *d*TM in a comprehensive approach, focusing on the underlying mechanisms involved in nutrients' metabolism of European sea bass, through histological, biochemical, molecular, and enzymatic techniques.

6.2. Materials and Methods

6.2.1. Ethics statement

Experimental trial and sampling procedures were directed by accredited researchers (following FELASA category C recommendations), conducted according to the guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU from the European Union), and approved by the Ethical Committee of CIIMAR, overseen by the National Competence Authority (Direção Geral de Alimentação e Veterinária - DGAV).

6.2.2. Experimental diets

According to the nutritional requirements of European sea bass (NRC, 2011), three isoproteic, isolipidic and isoenergetic diets (47% protein, 20% lipids and 24 kJ g $^{-1}$ on a dry matter basis, respectively) were formulated and extruded by SPAROS Lda. (Portugal). A control diet (CTRL) was formulated with 40% FM and 14% FO and two other experimental diets were formulated to replace 50% and 100% of FM by *d*TM, on protein basis (TM50 and TM100, respectively). Experimental diets with *d*TM inclusion (TM50 and TM100) were supplemented with monocalcium phosphate. All experimental diets were supplemented with DL-Methionine. L-Lysine, L-Threonine and L-Tryptophan were also added to TM100 diet. Feed ingredients and proximate composition of experimental diets are presented on Table 6.1. Experimental diets are the same in this and our previous study (Basto et al., 2022).

Table 6.1. Ingredients, chemical composition, and fatty acid profile of *d*TM and experimental diets.

	***d*TM**	**CTRL**	**TM50**	**TM100**
Ingredients (%)				
Fishmeal [1]		40.0	20.0	-
Defatted *Tenebrio molitor* larvae meal		-	20.5	40.4
Soy protein concentrate [2]		10.5	10.5	10.5
Soybean meal [3]		13.0	13.0	13.0
Rapeseed meal [4]		5.0	5.0	5.0
Wheat meal [5]		16.2	15.2	14.3
Fish oil [6]		14.0	13.3	12.5
Vitamin and mineral premix [7]		1.0	1.0	1.0
Vitamin C		0.1	0.1	0.1
Vitamin E		0.1	0.1	0.1
Monocalcium phosphate		-	1.0	2.0
L-Lysine		-	-	0.2
L-Threonine		-	-	0.2
L-Tryptophan		-	-	0.1
DL-Methionine		0.1	0.2	0.3
Chemical composition (% DM)				
Dry matter	97.8	93.1	92.6	92.5
Protein	71.0	46.9	47.3	47.2
Lipids	12.1	19.7	19.8	19.0
Gross energy (kJ g^{-1} DM)	24.3	23.2	23.5	24.0
Ash	4.8	10.2	8.1	6.3
Phosphorus	0.8	1.2	1.2	1.0

The abbreviations for the experimental diets stand for: CTRL – control diet; TM50 and TM100 – diets with 50 and 100% fishmeal replacement by insect meal; [1] Peruvian fishmeal super prime: 71% crude protein (CP), 11% crude fat (CF), Exalmar, Peru; [2] Soy protein concentrate: 65% CP, 0.7% CF, ADM Animal Nutrition, The Netherlands; [3] Soybean meal 48: dehulled solvent extracted soybean meal: 48% CP, 2% CF, Cargill, Spain; [4] Rapseed meal: 36% CP, 3% CF, PREMIX Lda., Portugal; [5] Wheat meal: 10% CP, 1% CF, Casa Lanchinha, Portugal; [6] Sardine oil, Sopropêche, France; [7] Vitamin and mineral premix: WISIUM, ADM Portugal S.A., Portugal.

6.2.3. Experimental trial and fish sampling

The trial was conducted in fish holding facilities of CIIMAR (Porto, Portugal). After a three-weeks quarantine period, homogeneous groups of 15 fish (69 ± 8 g; CV = 8%) were established and distributed into 12 circular fiberglass tanks (160 L), in a seawater recirculation aquaculture system (RAS). The system was supplied with continuous water

flow (6 L min^{-1}) and maintained at 22 ± 1 °C, salinity of 35‰, oxygen level > 90% ± 1 saturation, and 12L:12D photoperiod. Levels of total ammonium, nitrite and nitrate ($NH4^+$, $NO2^-$ and $NO3^-$, respectively) as well as pH were daily supervised to guarantee levels within the recommended ranged for marine fish species (NH_4^+ ≤ 0.05 mg L^{-1}; NO_2^- ≤ 0.5 mg L^{-1}; NO_3^- ≤ 50 mg L^{-1}; 7.5 ≤ pH ≤ 8.5). Experimental diets were tested in quadruplicate tanks and fish were fed by automatic feeders three times a day, seven days a week, as previously described by Basto et al. (2021b). At the end of 16 weeks of feeding trial, and after a 48-h fasting period, three fish/tank (i.e., *n* = 12 fish/treatment) were anesthetized (2-phenoxyethanol, 300 µL L^{-1}) before being individually weighed and measured. Blood was sampled from the caudal vein using heparinized syringes, centrifuged (5,000 *g* for 5 min at 4 °C) and the resulting plasma supernatant was stored at -80 °C for further quantification of metabolite levels. Then, fish were euthanized by spinal cord section, viscera and liver were collected and weighed, and two portions of liver (≈ 150 mg each) were sampled and immediately frozen in dry ice and stored at -80 °C until mRNA relative abundance, metabolite levels and enzymatic activity analysis. Another portion of liver (≈ 1 cm^3) was also collected and immediately frozen in isopentane, cooled by dry ice, and stored at -80°C for further histological evaluation.

6.2.4. mRNA relative abundance analysis by RT-qPCR

Total RNA was extracted from liver using TRIzol reagent (Life Technologies, USA) and treated with RQ1DNAse (Promega, USA) following the manufacturer's instructions. RNA purity (A260/A280 ratio) was evaluated by spectrophotometry, using a NanoDrop 2000c (Thermo, Vantaa, Finland), and only samples with A260/A280 ratio > 1.8 were used. Subsequently, superscript II reverse transcriptase and random hexamers (Promega, USA) were used to synthesize cDNA from 2 µg of total RNA. The relative expression of genes was assessed by real-time quantitative PCR (RT-qPCR), using the CFX96 Connect Real-Time PCR Detection System (Bio-Rad Laboratories Inc., USA). Reactions were carried out with 50–500 nM of each primer (forward and reverse), MAXIMA SYBR Green qPCR Master Mix (Life Technologies, USA) and 1 µL cDNA, in a total PCR reaction volume of 15 µL. Thermal cycling conditions were 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 s, 56-64 °C for 30 s (annealing temperatures ate present in Table 6.2), and 72 °C for 30 s. At the end of each run, a post-amplification dissociation curve (55 °C temperature gradient at 0.5 °C/s from 55 to 95 °C) was obtained to ensure reaction specificity. Efficiency ranged between 90 and 105% (Table 6.2), and each unknown sample was run in duplicate, as well as negative controls without reverse transcriptase or without cDNA. Data were analysed using the arithmetic mean of β-actin *(actb),* elongation

factor 1α 1 (*eef1α1*), and ribosomal 18s RNA (*18s*) as housekeeping genes, according to Pfaffl (2001). The expression of housekeeping and target genes was measured using previously described sequences of primers for the same fish species (Viegas et al., 2013; Viegas et al., 2014; Azeredo et al., 2015; Rimoldi et al., 2015; Viegas et al., 2015; Cadiz et al., 2018; Basto et al., 2021a; Betancor et al., 2021), which are shown in Table 6.2. For *acly*, *got1*, *fbp1* and *hk1* new primers were designed using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) and sequenced as previously described by Basto et al. (2022), to ensure that sequences satisfactorily matched the reference GenBank sequences.

Table 6.2. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR).

Gene	Forward	Reverse	Annealing temperature (ºC)	GeneBank acession number
acly	AGAGACGGCAGTAGCCAAAA	ATTGGCCACCAGTTCATCAT	56.0	*
18s	CCGCTTTGGTGACTCTAGATAACC	CAGAAAGTACCATCGAAAGTTGATAGG	59.0	AY831388.1
actb	TCCTGCGGAATCCACGAGA	AACGTCGCACTTCATGATGCT	60.9	AY148350.1
c-alat	TGAAGGAGGGGGTCAAGAAA	AGGGTAAGAACACAGAGCCA	57.4	JX073702
cpt1a	TGCCAAGAGGTCATCCAGAGTTCT	AGTCCACATCATCCGCCAGAGA	64.2	KF857302
eef1	CGTTGGCTTCAACATCAAGA	GAAGTTGTCTGCTCCCTTGG	55.5	AJ866727
fas	CGTCAAGCTCTCCATCCCTG	GGTGGTGTCTAGGCAGTGTC	59.6	MF566098
fbpase	GCTTTGACCCACTGGATGGT	GGCGGGATCAAGCATAAAGC	58.4	*
g6pase	TGAGACCCGGTTTTATGGAG	CATGCAGACCACCAGCTCTA	57.0	AM987970
g6pdh	GAGATGGTGCAGAACCTCATGG	CCACAGAAGACATCCAGGATGAG	62.0	JX073705
gdh	CCATCAGCCAGGGAGGAATC	TGCTCATGTAAGACGCCTCG	58.7	KF857576
gck	ATCGTCAGGGAACTCACACC	GAGTTCAGGCTTGCTTCACC	58.3	AM986860
asat1	CTGGGTGGTACAGGTGCTTT	AGCGTTGTGATTTTCCCAAG	55.8	*
gp	TTCCCAGACAAAGTCGCTGT	GTCTTGTGAGGTCCCATGC	58.2	*
gs	GACAAGGAGGCAGGTGAGAG	GAAGACGTGAGCACAGTGGA	59.2	*
hoad	CGGAAGTGGTCAGATGGGTG	TCGTCGCTTGTATCCACCAG	59.0	KF857303
hk	CACCAATGCGTGCTACATGG	GTCGACGTCCCTGTCAAACT	58.1	*
pepck	GCGCCATCAACACTAAAGGT	TTGTGCACTCTGTCCTCCAG	57.2	DV217087
pk	CTGTTTCCTGTGGAGGCAGT	CAGCACAGCATTTGAAGGAG	55.8	AM981422

acly, ATP citrate lyase; *18s*, ribosomal RNA 18s; *actcb*, β-actin; *c-alt*, cytosolic alanine aminotransferase; *cpt1a*, carnitine palmitoyltransferase 1a; *eef1a1*, elongation factor 1α; *fas*, fatty acid synthase; *fbpase*, fructose-1,6-biphosphatase; *g6pase*, glucose-6-phosphatase; *g6pdh*, glucose-6-phophate-dehydrogenase; *gdh*, glutamate dehydrogenase; *gk*, glucokinase; *got1*, aspartate aminotransferase; *gp*, glycogen phosphorylase; *gs*, glycogen synthase; *hadh*, hydroxyacyl-Coenzyme A dehydrogenase; *hk*, hexokinase; *pepck*, phosphoenolpyruvate carboxylase; *pk*, pyruvate kinase.

6.2.5. Assessment of metabolite levels

For metabolite levels assessment, liver samples were homogenized and deproteinized by ultrasonic disruption in 7.5 vols of ice-cooled 0.6 M perchloric acid and neutralized with 1 M potassium bicarbonate. The homogenate was centrifuged at 10,000 *g* for 4.5 min at 4 °C, and the supernatant was used to assess tissue metabolite levels. Similarly, plasma samples were also deproteinized (0.6 M perchloric acid), neutralized (1 M potassium bicarbonate), and supernatant was collected after being centrifuged at 13,500 *g* for 4.5 min at 4 °C. Glucose, lactate, triglycerides (TAG), total cholesterol, and non-esterified fatty acids (NEFA) levels were determined enzymatically using commercial kits (1001190, 1001330, 1001313 and 1001090, Spinreact, Spain, and 434–91795 NEFA-HR (2) R1, and 436–91995 NEFA-HR (2) R2, Wako Chemicals, Germany, respectively), adapting manufacturer's instructions to a microplate format. Total α-amino acid levels were assessed through the colorimetric ninhydrin method (Moore, 1968), with alanine as standard. Liver glycogen level was assessed using the Keppler and Decker (1974) method.

6.2.6. Assessment of enzyme activities

For enzymatic activity assessment, liver samples (1:9 w/v) were homogenized by ultrasonic disruption in ice-cooled buffer containing 50 mM Tris (pH 7.6), 5 mM EDTA, 2 mM 1,4-dithiothreitol, and a protease inhibitor cocktail (Merck KGaA, Germany). The homogenate was centrifuged at 9,000 *g* for 11 min at 4 °C, and the supernatant was used for enzyme assays. Enzyme activities were run in 96-well microplates and determined using a microplate reader Infinite 200 Pro (Tecan, Männedorf, Switzerland). Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm or, in the case of carnitine palmitoyl transferase 1a (Cpt1L; *EC 2.3.1.21*) activity, of 5,5-dithiobis-(2-nitrobenzoic acid)-CoA complex at 412 nm. To ensure that enzyme activities were assessed at maximum rates, optimal substrate concentrations were determined through preliminary tests, adapting to European sea bass the previously developed methods for rainbow trout (*Oncorhynchus mykiss*) (Polakof et al., 2007a, 2007b, 2008a, 2008b, 2008c; Librán-Pérez et al. 2012, 2013a, 2013b). The reactions were started by the addition of supernatant (10-25 µL), omitting the substrate in control wells (final volume 180-295 µL) and allowing the reactions to proceed at 37°C for preestablished time periods (10-45 min). The results of enzymatic activity are expressed per protein level, which was assayed in triplicate in homogenates, according to the

bicinchoninic acid method (Smith et al., 1985), with bovine serum albumin as standard (Merck KGaA, Germany). Hexokinase (Hk; *EC* 2.7.1.1) and glucokinase (Gck; *EC* 2.7.1.2) activities were assessed in a Tris-HCl buffer (80 mM, pH 7.0) containing 10.2 mM KCl, 37.5 mM $MgCl_2$, 11.5 mM KH_2PO_4, 20 mM $NaHCO_3$, 4 mM EDTA, 2.6 mM DTT, 2 mM $NADP^+$, 7 mM ATP, 0.13 U mL^{-1} glucose 6-phosphate dehydrogenase, 0.13 U mL^{-1} 6-phosphogluconate dehydrogenase, and 1.2 M (Hk) or 12 mM (Gck) D-glucose (omitted for controls). Pyruvate kinase (Pk; *EC* 2.7.1.40) activity was assessed in an imidazole buffer (50 mM, pH 7.4) containing 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM ADP, 0.15 mM NADH, 21 U mL^{-1} lactate dehydrogenase, and 2 mM phospho(enol)pyruvate (omitted for controls). Phosphoenolpyruvate carboxykinase cytosolic form (Pepck1; *EC* 4.1.1.32) activity was assessed in a Tris-HCl buffer (50 mM, pH 7.5) containing 1 mM $MnCl_2$, 20 mM $NaHCO_3$, 1.5 mM phospho(enol)pyruvate, 0.3 mM NADH, 1.7 U mL^{-1} malate dehydrogenase, and 0.5 mM 2'-deoxyguanosine-5-diphosphate (omitted for controls). Fructose 1,6-bisphosphatase (Fbpase; *EC* 3.1.3.11) activity was assessed in an imidazole buffer (85 mM, pH 7.7) containing 5 mM $MgCl_2$, 0.5 mM $NADP^+$, 2.5 U mL^{-1} 6-phosphoglucose isomerase, 0.8 U mL^{-1} glucose 6-phosphate dehydrogenase, and 7 mM fructose 1,6-bisphosphate (omitted for controls). Glucose 6-phosphatase (G6pase; *EC* 3.1.3.9) activity was assessed in an imidazole buffer (100 mM, pH 6.5) containing 25 mM D-glucose 6-phosphate (omitted for controls). Glycogen synthase liver form (Gsase; *EC* 2.4.1.11) activity was assessed in an imidazole buffer (50 mM, pH 7.5) containing 150 mM KCl, 15 mM $MgCl_2$, 2 mg mL^{-1} glycogen, 1.5 mM phospho(enol)pyruvate, 0.3 mM NADH, 4.6 mM D-glucose 6-phosphate, 1.4 U mL^{-1} lactate dehydrogenase, 1.4 U mL^{-1} pyruvate kinase, and 100 mM uridine diphosphoglucose (omitted for controls). Glycogen phosphorylase liver form (Gpase; *EC* 2.4.1.1) activity was assessed in a phosphate buffer (50 mM, pH 7.0) containing 27 mM $MgSO_4$, 24.2 mM EDTA, 2.5 mM AMP, 0.5 mM $NADP^+$, 1.7 U mL^{-1} phosphoglucomutase, 6.8 U mL^{-1} glucose 6-phosphate dehydrogenase, 10 µM α-D-glucose 1,6-bisphosphate, and 40 mg mL^{-1} glycogen (omitted for controls). Glucose-6-phosphate-dehydrogenase (G6pdh; *EC* 1.1.1.49) activity was assessed in an imidazole buffer (78 mM, pH 7.7) containing 5 mM $MgCl_2$, 0.5 mM $NADP^+$, and 20 mM D-glucose 6-phosphate (omitted for controls). ATP citrate lyase (Acly; *EC* 4.1.3.8) activity was assessed in a Tris-HCl buffer (50 mM, pH 7.8) containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM sodium citrate, 10 mM β-mercaptoethanol, 5 mM ATP, 0.3 mM NADH, 7 U mL^{-1} malate dehydrogenase, and 8 mM Coenzyme A (omitted for controls). Fatty acid synthase (Fas; *EC* 2.3.1.85) activity was assessed in a phosphate buffer (0.1 mM K_2HPO_4 and 0.1 mM KH_2PO_4, pH 6.5) containing 0.1 mM NADPH, 25 µM acetyl-CoA, and 40 µM malonyl-CoA (omitted for controls). Carnitine palmitoyl transferase 1 (Cpt1L; *EC* 2.3.1.21) activity was assessed in a Tris-HCl buffer (75 mM, pH 8.0) containing 1.5 mM EDTA, 0.25 mM

DTNB, 35 μM palmitoyl-CoA, and 5.5 mM L-carnitine (omitted for controls). Aspartate aminotransferase 1 (Ast1; *EC* 2.6.1.1) activity was assessed in an imidazole buffer (50 mM, pH 7.8) containing 10 mM α-ketoglutarate, 0.05 mM pyridoxal 5'-phosphate, 0.3 mM NADH and 1.5 mM L-aspartate (omitted for controls). Alanine transaminase (Aat1; *EC* 2.6.1.2) activity was assessed in an imidazole buffer (50 mM, pH 7.8) containing 10 mM α-ketoglutarate, 0.025 mM pyridoxal 5'-phosphate, 0.2 mM NADH, 21 U mL^{-1} lactate dehydrogenase and 14 mM L-alanine (omitted for controls). Glutamate dehydrogenase (Gdh; *EC* 1.4.1.2) activity was ssessed in an imidazole buffer (50 mM, pH 7.8) containing 250 mM ammonium acetate, 0.10 mM NADH, 1 mM ADP and 0.35 mM α-ketoglutarate (omitted for controls).

6.2.7. Histological analysis

Frozen sections of liver (12 μm) were obtained with a cryostat and stained with Oil Red O, using a commercial kit (010303, DIAPATH S.p.A., Italy) according to manufacturer's instructions. To evaluate the accumulation of lipid droplets degree each section was totally examined at a 200× magnification under a light microscope (Olympus BX51, 194 GmbH, Germany) coupled with a camera (Olympus DP50, GmbH, Germany), using an imaging software (Olympus cellSens Dimension Desktop). A semi-quantitative approach was used, according to the following five scores and criteria: Score 0 – absence of lipid droplets; Score 1 (low) – on average, < 1/3 of the hepatocyte cytoplasm shows lipid droplets; Score 2 (moderate) – on average, 1/3 < × < 2/3 of the hepatocyte cytoplasm shows lipid droplets; Score 3 (high) – on average, > 2/3 of the hepatocyte cytoplasm shows lipid droplets; Score 4 (extreme) – 3/3 of the hepatocyte cytoplasm shows lipid droplets.

6.2.8. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 26.0 (IBM corporation, USA). All variables were tested for normality and homogeneity of variances using Kolmogorov-Smirnov and Levene's tests, respectively, followed by one-way ANOVA. When one-way ANOVA showed significance ($P < 0.05$), groups were compared using Tukey's HSD multiple comparison test.

6.3. Results

6.3.1. Growth performance, somatic indexes and histological evaluation of liver

After 16 weeks of feeding trial, the dietary inclusion of *d*TM did not affect fish growth nor HSI (Figure 6.1A-B). In general, European sea bass hepatocytes showed moderate accumulation of lipid droplets (Score 2), and no significant differences were observed among dietary treatments (Figure 6.2).

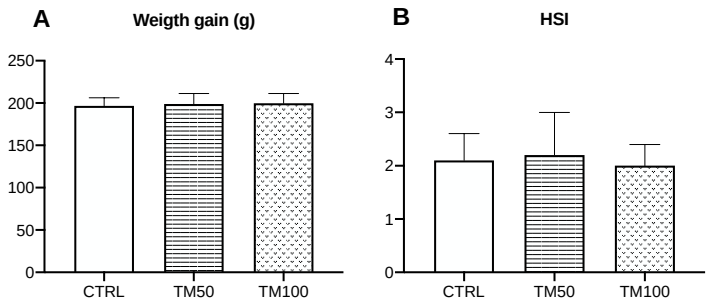

Figure 6.1. Weight gain (A) and hepatosomatic index (B) of European sea bass fed experimental diets. Bars represent means ± standard deviation of the mean (n = 60 fish per treatment for weight gain and n = 24 fish per treatment for hepatosomatic index).

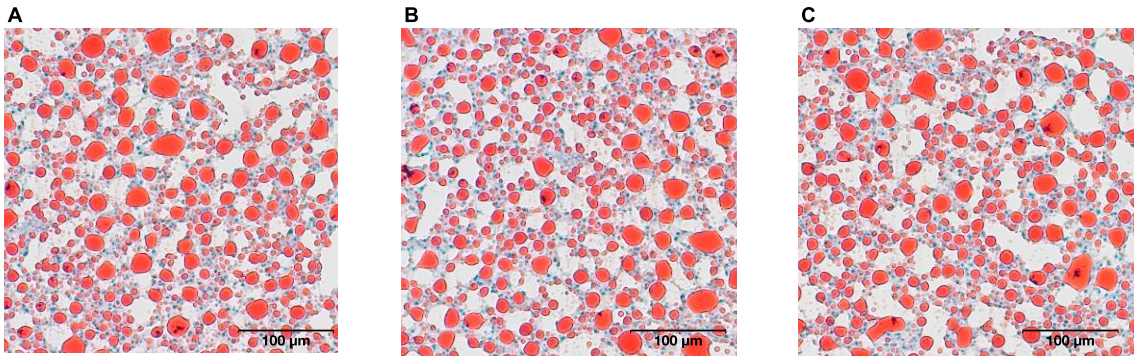

Figure 6.2. Fat depots of histological liver sections of European sea bass fed CTRL (A), TM50 (B) and TM100 (C), stained by Oil Red O.

6.3.2. Liver and plasma metabolite levels

The hepatic levels of glycogen, glucose, lactate, total α-amino acid, NEFA, triglyceride, and cholesterol did not change with dietary inclusion of *d*TM (Table 6.3). Plasmatic levels of NEFA and TAG significantly increased in fish fed TM50 or TM100 diets when compared to those fed CTRL diet, whereas plasmatic levels of glucose, lactate, total α-amino acid, and cholesterol remained similar among dietary treatments (Table 6.3).

Table 6.3. Liver and plasma metabolite levels of European sea bass fed experimental diets.

	CTRL	TM50	TM100	*p*-value
Liver (mmol g^{-1})				
Glycogen	34.8 ± 2.7	36.4 ± 2.6	34.7 ± 3.7	0.126
Glucose	31.3 ± 0.2	31.2 ± 0.7	32.8 ± 1.0	0.083
Lactate	1.3 ± 0.2	1.4 ± 0.2	1.2 ± 0.2	0.690
Total α-amino acids	23.8 ± 2.4	21.5 ± 1.8	23.8 ± 2.8	0.750
NEFAs	0.3 ± 0.04	0.3 ± 0.1	0.3 ± 0.1	0.972
TAGs	3.2 ± 0.2	3.1 ± 0.3	3.5 ± 0.3	0.775
Cholesterol	0.3 ± 0.03	0.3 ± 0.1	0.3 ± 0.1	0.964
Plasma (mmol L^{-1})				
Glucose	6.3 ± 0.4	6.4 ± 0.7	6.9 ± 0.5	0.706
Lactate	9.3 ± 0.8	7.9 ± 0.8	8.8 ± 1.1	0.592
Total α-amino acids	5.0 ± 0.3	5.1 ± 0.3	6.1 ± 0.6	0.203
NEFAs	0.1 ± 0.01 ^{b}	0.2 ± 0.02 ^{a}	0.2 ± 0.02 ^{a}	0.016
TAGs	3.7 ± 0.1 ^{b}	5.6 ± 0.6 ^{a}	5.6 ± 0.5 ^{a}	0.019
Cholesterol	7.9 ± 0.4	8.5 ± 0.8	8.6 ± 0.7	0.749

The abbreviations stand for: NEFA, non-esterified fatty acids; TAGs, triacylglycerides. Values are presented as mean ± standard deviation of the mean (n = 12 fish per treatment). Values in the same row without a common superscript letter differ significantly ($p < 0.05$).

6.3.3. Metabolic-related genes and enzymes in liver

Although the mRNA abundance of glycolysis-related genes (*gck, hk1* and *pklr*) did not significantly vary with dietary inclusion of *d*TM (Figure 6.3A-C), the activity of Gck significantly increased in fish fed TM100 when compared to those fed CTRL and TM50 (Figure 6.3A). Similarly to results of mRNA abundance, the activity of Hk and Pk remained similar between fish fed CTRL and *d*TM diets (Figure 6.3B-C). Amongst the gluconeogenesis-related genes and their encoded enzymes, only *g6pc1*/G6pase significantly decreased in fish fed TM100 when compared to those fed CTRL diet (Figure 6.3D), whereas *pck1*/Pepck1 and *fbp1*/Fbpase remained similar among all dietary treatments (Figure 6.3E-F). The mRNA abundance of glycogenolysis-related gene *pygl* did not change with *d*TM dietary inclusion, but the activity of Gpase was significantly lower in fish fed TM100 than in fish fed CTRL or TM50 diets (Figure 6.3G). The glycogenesis-related gene *gys2* and its encoded enzyme Gsase remained similar among dietary treatments (Figure 6.3H). The mRNA abundance of pentose phosphate pathway-related gene *g6pd* also did not change with dietary inclusion of *d*TM, but the activity of its encoded enzyme G6pdh significantly increased in fish fed TM100 when compared to those fed CTRL and TM50 (Figure 6.3I). Likewise, total FM replacement by *d*TM also resulted in increased activity of the lipogenic enzyme Acly compared with controls, whereas mRNA relative abundance of *acly* did not vary among dietary treatments (Figure 6.3J). The mRNA abundance of *fasn* and the activity of its encoded enzyme Fas significantly increased in fish fed and TM100 when compared to those fed CTRL (Figure 6.3K). The β-oxidation-related gene *cpt1a* showed no significant differences whereas its encoded enzyme Cpt1L displayed lower activities in fish fed TM50 or TM100 when

compared with fish fed with CRTL diet (Figure 6.3L). The mRNA abundance of *hadh* did not vary significantly with dietary inclusion of *d*TM (Figure 6.2M). Concerning amino acid metabolism-related genes and enzymes, the mRNA abundance of *got1* and its encoded enzyme Ast1 remained similar among dietary treatments (Figure 6.3N). On the other hand, both mRNA abundance of *gpt* and the activity of its encoded enzyme Aat1 significantly decreased in fish fed TM100 diets when compared to those fed CTRL diet (Figure 6.3O). Finally, the mRNA abundance of *glud1* was not altered by feeding experimental diets, but the activity of its encoded enzyme Gdh was significantly lower in fish fed TM100 than in those fed CTRL diet (Figure 6.3P).

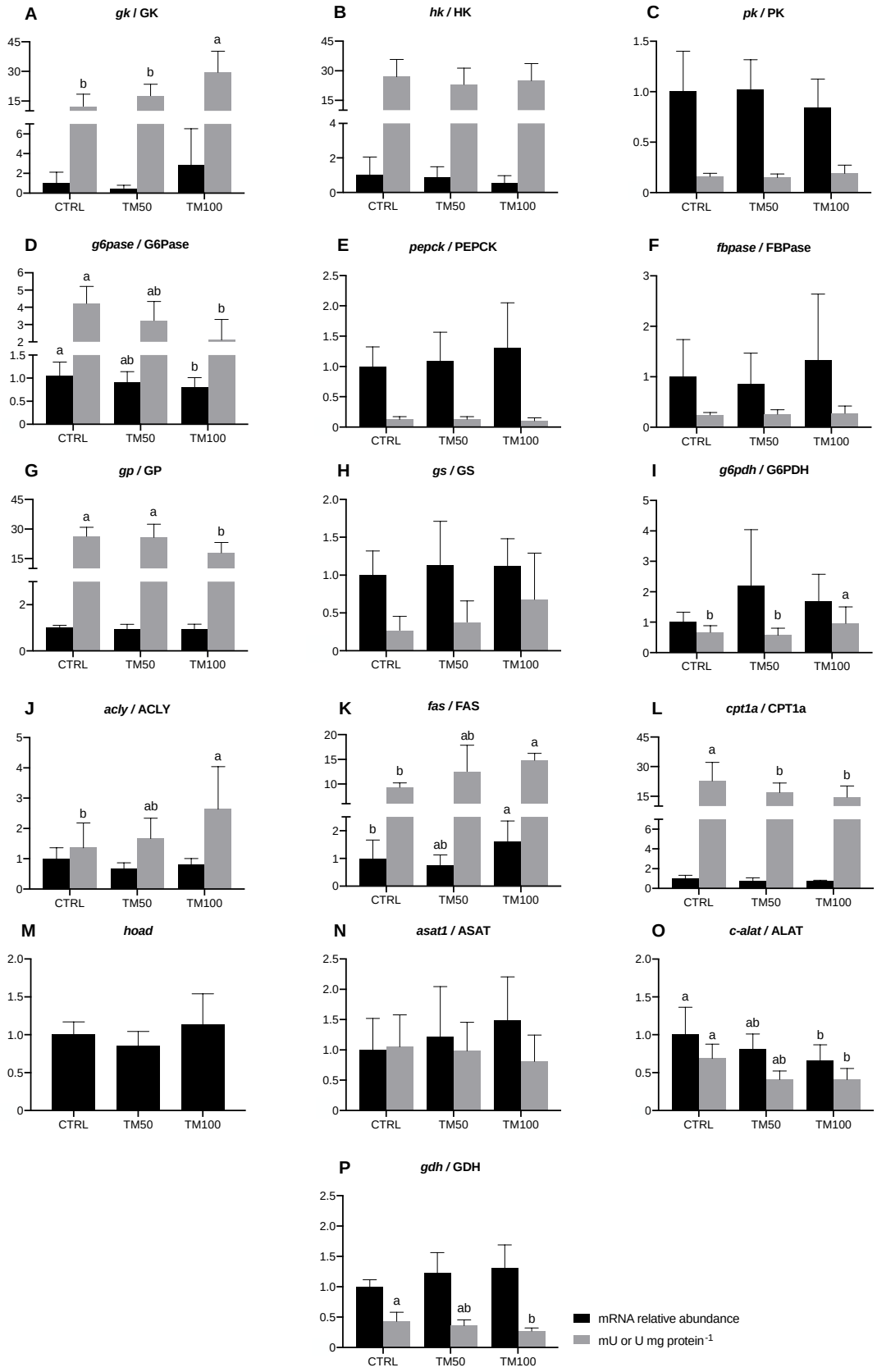

Figure 6.3. Expression and activity of key genes and enzymes of intermediary metabolism of European sea bass fed experimental diets. Bars represent means ± standard deviation of the mean (n = 12 fish per treatment).

6.4. Discussion

In previous studies, we demonstrated that partial (up to 80%) and total dietary FM replacement by *d*TM neither affected homeostatic regulation of feed intake nor growth performance of European sea bass, but hepatic and plasmatic metabolites suggested alterations in intermediary metabolism when FM was totally replaced by *d*TM (Basto et al., 2021b; Basto et al., 2021a; Basto et al., 2022). Therefore, we aimed to thoroughly characterize those metabolic changes. The present study showed that a diet devoid of FM and with a high IM inclusion (TM100) induced the glycolytic, glycogenolytic, pentose phosphate and lipogenic pathways, whereas inhibited the gluconeogenesis and FAs β-oxidation.

In the present study, partial (50%) and total FM replacement by *d*TM also did not affect weight gain of European sea bass after 16 weeks of feeding trial. However, fish fed TM50 and TM100 diets displayed higher levels of NEFA and TAG in plasma than those of fish fed the control diet. Since liver is the main organ involved in intermediary metabolism in fish, we assessed changes in the mRNA abundance and activities of main enzymes involved in glucose, lipid, and amino acid metabolism to ascertain the origin of the changes in plasma.

It is well known that plasmatic glucose levels are maintained in homeostasis with those present in liver due to the existence of specific diffusion through glucose transporters (Polakof et al., 2012). In the liver, excess of glucose may be stored as glycogen through the action of Gsase enzyme (glycogenesis), or catabolized through the glycolytic pathway, followed by tricarboxylic acid (TCA) cycle and respiratory chain for energy production, or through the pentose phosphate pathway resulting in the production of NADPH and ribose 5-phosphate, needed for *de novo* lipid biosynthesis (lipogenesis) or nucleotide synthesis, respectively (Polakof et al., 2012). On the other hand, under hypoglycaemic conditions, glucose requirements can be satisfied by glycogen depletion to glucose through the action of Gpase (glycogenolysis), or gluconeogenesis from non-carbohydrate sources through the action of Pepck and Fbpase (Polakof et al., 2012). In the present study, we observed that total FM replacement by *d*TM led to an increase in the use of glucose in liver, as supported by increased Gck activity. The lack of changes in Hk relates to the more important role of Gck in liver of fish (Polakof et al., 2012).

The increased availability of glucose 6-phosphate resulting from the increased activity of Gck can be used through three different pathways (Enes et al., 2009; Polakof et al., 2012). First, this glucose is not apparently used through glycolysis since no changes occurred in the mRNA abundance and activity of the glycolytic enzyme Pk. The lack of

changes observed in parameters indicative of glycolytic capacity occurred in parallel with the lack of changes observed in parameters related to gluconeogenesis, such as the mRNA abundance and activity of the enzymes Fbpase and Pepck1. As a second possibility, the increased availability of glucose 6-phosphate could have been used to synthesize glycogen through glycogenesis. However, no main changes occurred in glycogen levels as well as in mRNA abundance and activity of the glycogenic enzyme Gsase (though a decrease occurred in the activity of the glycogenolytic enzyme Gpase). The third pathway through which increased availability of glucose 6 phosphate could have been used is glucose release to the plasma. This capacity is however inhibited in liver, based on changes displayed by the responsible enzyme G6pase in terms not only of changes in mRNA abundance but also of enzyme activity. Finally, the increased glucose 6-phosphate levels resulting from Gck activity could have been used through pentose phosphate pathway involved in the production of reducing power in the form of NADPH. This pathway seems to be activated since increased activity of the main regulatory enzyme of the pathway, i.e., G6pdh increased in fish fed TM100. As a whole, changes occurring in glucose metabolism in liver indicate that feeding TM100 result in increased use of glucose, which is not being use to provide energy to hepatic cells (no changes in glycolytic/gluconeogenic capacities), to store glycogen (no changes in glycogen levels as well as in glycogenic/glycogenolytic capacities) or to be released into plasma (no changes in levels of plasma glucose and the activity of the liver enzyme G6pase). The increased use of glucose is therefore apparently directed to the pentose phosphate pathway thus providing NADPH, which is usually associated with increased capacity for lipid synthesis.

The increased capacity of liver to synthesize lipid, based on changes in glucose metabolism described above, must be supported by changes in the mRNA abundance and/or activity of enzymes involved in lipid metabolism. Acly represents the starting point of lipogenesis, which is initiated by conversion of citrate into acetyl-CoA and oxaloacetate through the action this enzyme. Afterwards, occurs the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase, and the final step of lipogenesis is characterized by the action of Fas under malonyl-CoA (Zhang et al., 2020). In its turn, malonyl-CoA inhibits Cpt1L, the first rate-limiting enzyme of β-oxidation. In these processes, significant amounts of NADPH are required for reducing power, and it is supplied by the action of dehydrogenases of the pentose phosphate pathway, such as G6pdh (Weil et al., 2013), as commented above. Accordingly, the mRNA abundance and the activity of the lipogenic enzyme Fas, as well as the activity of the lipogenic enzyme Acly clearly increased in fish fed with TM100 compared with controls. A rise in mRNA abundance of lipogenic enzymes also occurred in liver of rice field eel (*Monopterus albus*) in which FM was partially

replaced by HI (Hu et al., 2020) whereas in gilthead sea bream (*Sparus auratus*) a 30% replacement of FM by TM did not affect liver lipogenic capacity (Mastroraki et al., 2022). This increased lipogenic capacity matches clearly with the increased activity of G6pdh mentioned above. Furthermore, we also assessed in liver the activity and/or mRNA abundance of enzymes involved in lipolysis in liver. While no changes occurred for mRNA abundance of *hadh* (transcript of the Hadh lipolytic enzyme), a clear decrease occurred in the activity of Cpt1, one of the enzymes involved in fatty acid oxidation in liver. This is indicating that not only lipogenic pathway is activated but also that lipolytic pathway is inhibited. Guerreiro et al. (2020) observed that 52% FM substitution by partially defatted HI in meagre (*Argyrosomus regius*) decreased mRNA abundance of *hadh*, which is involved in FAs β-oxidation. Considering that no changes occurred in liver lipid levels, as demonstrated by both biochemical and histological measurements, the present results suggest that a fast mobilization of synthesized lipids occur in liver, and these lipids are exported into plasma to be used in other tissues. This would help to explain why NEFA and TAG levels increase in plasma (but not in liver) of fish fed TM100 diet. These results are in line with those obtained in our previous studies in the same species and may explain the increased whole-body fat content observed when FM was totally replaced by *d*TM (Basto et al., 2021b; Basto et al., 2022). In other studies, increased levels of lipid occurred in liver such as in zebrafish (*Danio rerio*) fed with a diet in which FM was replaced by HI (Zarantoniello et al., 2020) pointing to differences in the speed of FA mobilization between species and IM used. Besides, when *d*TM was used as single protein source in diets for European sea bass for 10 weeks, it was observed a down-regulation trend of peroxisome proliferator-activated receptor-α (*pparα*) (Basto et al., 2021b), which is a nuclear hormone receptor that regulates β-oxidation of fatty acids (Li et al., 2020), highlighting that total FM replacement by *d*TM inhibits β-oxidation pathway, in a way comparable to that herein observed.

According to Engelking (2015), Ast1, Aat1 and Gdh are considered the most relevant enzymes involved in amino acid metabolism. Aat1 and Ast1 are responsible for transamination of alanine and aspartate to pyruvate and glutamate, respectively. Gdh is a mitochondrial enzyme that reversibly converts glutamate to α-ketoglutarate and NH_4^+. Except for Ast1, changes observed in the other enzymes after feeding diets are comparable, with decreased activity (also mRNA abundance in the case of *gpt*) in fish fed TM100 diet compared with controls. This reduced activity clearly indicates a reduced capacity of the liver to oxidize amino acid both for fuelling purposes and/or to synthesize glucose through gluconeogenesis, which is one of the main pathways in which amino acids are involved in the metabolism of teleost liver (Polakof et al., 2012). Since amino

acids are a primary source of carbon for gluconeogenesis, such decline of Aat1 activity contributes to explain the reduction of gluconeogenesis in fish fed TM100, as previously observed through the reduced activity of G6Pase. Comparable decreases in the oxidative capacity of amino acids occurred in gilthead sea bream (*Sparus aurata*) and tench (*Tinca tinca*) fed with a diet with a partial replacement of FM by TM (Fabrikov et al., 2020). However, in other studies no changes occurred in the capacity for oxidizing amino acids. Thus, Mastoraki et al. (2020) evaluated the effect of 30% FM replacement by different IM on amino acids metabolism of European sea bass and concluded that partially defatted HI and full-fat TM or *Musca domestica* did not affect the activity of Ast1, Aat1 and Gdh. In gilthead sea bream, 50% of FM replacement by full-fat TM or HI also did not change the activity of Ast1, Aat1, and Gdh (Fabrikov et al., 2021), and a similar lack of changes also occurred when 30% of FM was replaced by TM (Mastoraki et al., 2022). In mammals, serum activities of Ast1, Aat1 and their ratio are commonly evaluated as biomarkers for liver health (Gwaltney-Brant, 2016). Thus, our findings suggest that partial or complete replacement of FM by *d*TM might have a protective effect in the liver of European sea bass. Similar results were obtained by Belghit et al. (2019) when FM was totally replaced by partially defatted HI in diets for sea-water phase of Atlantic salmon (*Salmo salar*). Regarding Gdh, Skiba-Cassy et al. (2016) suggested that dietary excess of methionine in diets for rainbow trout triggered amino acid deamination and an increase in the activity of this enzyme. In this regard, the observed decrease of Gdh activity in the liver of fish fed TM100 may suggest the deficit of some amino acid. Besides, since the activity of Aat1 decreased in fish fed TM100, lower concentrations of glutamate were expected, and thus a decreased activity of Gdh. However, further studies are needed to better understand the impact of different dietary formulations on either Ast1, Aat1 or Gdh.

6.5. Conclusions

Despite no signs of steatosis were observed in the liver of fish fed TM100, this dietary treatment induced a clear increase in the liver capacity to use glucose and a decrease in the capacity of using amino acids. These changes result in redirecting liver metabolism to an enhanced lipogenic capacity reflected in high levels of plasmatic NEFA and TAG, which in the long-term may compromise fish health. Thus, despite 100% replacement of FM by *d*TM render comparable growth and weight gain, the important number of metabolic changes described above preclude to consider such a level of replacement for the practical formulation of diets for European sea bass. In the future, it would be important to deepen knowledge about the effect of such diet on oxidative stress and immunological condition of fish. Contrarily to the current findings, Chemello et al.

(2020) demonstrated that total FM replacement by partially defatted TM did not alter activity of Ast1, Aat1, Gdh, G6pdh, and Fas in the liver of rainbow trout. However, it is important to point out that in the study conducted by Chemello et al. (2020) a total FM replacement was achieved with the inclusion of 20% IM, whereas in the present study to totally substitute FM it was necessary to include 40% of *d*TM. Thus, the results obtained by Chemello et al. (2020) are not completely comparable with those herein obtained.

In contrast to fish fed with TM100, no significant changes occurred in any of the parameters assessed in liver of fish fed TM50 compared with fish fed the control diet supporting that glucose, lipid, and amino acid metabolism in liver are not altered by 50% replacement of FM by *d*TM. In rainbow trout, the activity of different enzymes involved in intermediary metabolism of nutrients were also not influenced up to 50% of FM replacement by full-fat TM or HI (Melenchón et al., 2020; Melenchón et al., 2022). Therefore, *d*TM can be successfully used to replace 50% of FM in aquafeeds for European sea bass without negatively affecting fish hepatic health and intermediary metabolism of nutrients. This is important for considering such level of replacement in the formulation of practical diets for European sea bass.

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Declaration of conflicts of 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.

Authors contribution

Ana Basto: Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization. **Luísa M. P. Valente:** Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition. **Vera Sousa:** Investigation, Formal Analysis, Validation. **Marta Conde-Sieira:** Conceptualization, Methodology, Formal analysis, Writing – Review & Editing, Supervision. **José L. Soengas:** Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition.

Data Availability

None of the data were deposited in an official repository. The data that support the study findings are available from the authors upon request.

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Chapter 7.

Nutritional, organoleptic and sensory quality of market-sized European sea bass (*Dicentrarchus labrax*) fed defatted *Tenebrio molitor* larvae meal as main protein source

by

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Abstract

This study evaluated the impact of replacing 50 and 100% fishmeal protein by defatted (*d*-) *Tenebrio molitor* (TM) in diets for market-sized European sea bass. Growth performance, nutrient utilization and fillet nutritional, organoleptic and sensory quality traits were assessed after 16 weeks of feeding. The substitution of 50% fishmeal protein by *d*TM significantly improved feed efficiency without affecting fish growth performance. Muscle fat and n-3 PUFA content remained similar among fish, resulting in EPA and DHA levels above the recommended daily intake of 250 mg for human consumption. Despite some differences in the muscle histomorphometric and instrumental texture measurements, consumers could not perceive differences between muscle samples of fish fed the control or *d*TM diets. All samples were characterized by their soft and pleasant texture, and *d*TM samples were also associated with a “juicy texture”.

Keywords: consumer acceptance, fatty acid quality indexes, insect meal, muscle color and texture, omega-3 fatty acids, onehealth.

7.1. Introduction

Fish have a pivotal role in human nutrition by contributing to a balanced healthy diet. They are rich protein sources that provide consumers with at least 17% of the global animal protein intake. Fish are also a unique source of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs), such as eicosapentaenoic (EPA, C20:5n-3) and docosahexaenoic (DHA, C22:6n-3), which have an important role in promoting the neurodevelopment and reducing the risk of chronic pathologies (i.e., cardiovascular diseases (CVD), diabetes and obesity) in human (EFSA, 2015; FAO and WHO, 2011). The omega-3 flesh content is strongly dependent on the composition of fish diets. But in recent years, aquafeeds have been steadily reducing marine ingredients inclusion level due to sustainability reasons, compromising fish nutritional value for human (Cottrell et al., 2020; Naylor et al., 2021).

European sea bass (*Dicentrarchus labrax*) is a fish species greatly appreciated by consumers, being one of the main marine fish species produced in the Mediterranean region (FAO, 2022). It is a high-trophic-level species, requiring diets with high protein content (45–55%, dry mater (DM) basis) (NRC, 2011) mainly provided by either marine and/or vegetable ingredients. However, the limited availability of fishmeal (FM), the environmental and social effects associated to soybean meal production, and also the feed-food competition is forcing the growing aquaculture industry to find alternative ingredients for aquafeeds (Naylor et al., 2021). Insects have been pointed out as a promising substitute to the conventional protein sources used in aquafeeds, due to their valuable nutritional profile, prominent levels of antioxidant and antimicrobial compounds and sustainable features (Gasco et al., 2020; Tran et al., 2022). Insects have high protein content (up to 70% DM), with a well-balanced essential amino acid (EAA) profile and are also rich sources of some vitamins and minerals; they also grow fast, reproduce easily, have low feed conversion ratios (FCR) and small need of arable land and water (Gasco et al., 2020; Tran et al., 2022). Additionally, the use of insect meal (IM) in aquafeeds was authorized by European Commission (2017) raising the interest of both the scientific community and aquaculture sector in this protein source. However, insects have limited content or even absence of EPA and DHA. This may cause constraints when incorporated at high levels in aquafeeds, particularly in fish species like European sea bass. Marine fish have a limited capacity to biosynthetize n-3 LC-PUFAs due to a low activity of key endogenous enzymes like the Δ5-desaturase (Monroig et al., 2018). Thus, exceptional care must be taken when using IM to substitute high levels of FM or both FM protein and fish oil (FO) in diets for marine fish species, to avoid compromising nutrient utilization, fish performance and fillet quality (Cottrell et al., 2020), contributing to the onehealth concept.

Hermetia illucens (HI) and *Tenebrio molitor* (TM) have been the most studied insect species as FM substitutes, in diets for both freshwater, and marine fish species (Nogales-Mérida et al., 2018). Basto et al. (2020) have recently tested the in vivo digestibility of these IMs (both full-fat and defatted) in European sea bass juveniles, concluding that defatted TM (*d*TM) is the most promising alternative to FM, for having both the highest digestible protein (641 mg g^{-1} DM) and digestible EAA (296 mg g^{-1} DM) content, which were able to meet European sea bass requirements. Full-fat TM has been successfully included (25–50%, corresponding to a 64–71% FM protein replacement) in diets for European sea bass juveniles without detrimental impacts on fish growth (Antonopoulou et al., 2019; Gasco et al., 2016; Mastoraki et al., 2020; Reyes et al., 2020). Moreover, the growth performance of European sea bass fed a diet totally devoid of FM, but including 60% *d*TM and 12% sardine oil, was not impaired after 10 weeks of feeding; although the recommended daily intake (RDI) for human consumption of EPA + DHA (0.25 g per 100 g of fish) (EFSA, 2010) was not compromised, fillet DHA content was reduced (Basto et al., 2021). We must, however, note that all these short-termed studies were performed during juvenile stages (< 30 g fish) emphasizing the need for an assessment of the impact of IM dietary inclusion on the quality of market-sized fish.

Sensory attributes of fish, such as appearance, colour, texture and taste, among others, play a key role in consumer' perception of fish freshness and quality and consequently in its purchase decision (Carlucci et al., 2015). All these properties can be affected by fish dietary formulations and should hence be taken into consideration when novel ingredients are included in aquafeeds. The effect of IM on consumer-liking and perception of sensory attributes of fish filets was previously assessed in salmonids (Belghit et al., 2019; Borgogno et al., 2017; Bruni et al., 2020a; Sealey et al., 2011), but never evaluated in marine fish species such as European sea bass. The use of 15% HI as total FM protein replacement in Atlantic salmon (*Salmo salar*) resulted in filets very well accepted by consumers (Belghit et al., 2019; Bruni et al., 2020a), but in rainbow trout (*Oncorhynchus mykiss*), the dietary inclusion of 36–40% HI as 50% FM protein replacement led to significant changes in intensity of aroma, flavour and texture that were perceived by trained panellists (Borgogno et al., 2017; Sealey et al., 2011).

In this context, the present study had a dual objective: to explore the impact of dietary inclusion of *d*TM on a) growth performance, nutrient utilization, and flesh quality traits of market-sized European sea bass; and b) to further assess fish global acceptance and perception of sensory properties (appearance, odour, texture and taste) by consumers.

7.2. Materials and methods

7.2.1. Ethical statement

This study was directed by accredited scientists in compliance with Directive 2010/63/EU of the European Parliament. The experiment was approved by CIIMAR Ethical Committee for managing animal welfare (ORBEA-CIIMAR) and by the Portuguese Veterinary Authority (1005/92, DGAV Portugal).

7.2.2. Experimental diets

Based on the known nutritional requirements of European sea bass (NRC, 2011), a FM protein-based diet (40% FM) with 47% of crude protein was used as control diet (CTRL). Two other isoproteic, isolipidic and isoenergetic diets were formulated to replace 50% and 100% of the FM protein by *d*TM (TM50 and TM100, respectively). All experimental diets were extruded by SPAROS Lda. (Portugal) and supplemented with DL-methionine. The TM50 and TM100 diets were further supplemented with monocalcium phosphate, and TM100 with l-lysine, L-threonine and L-tryptophane. Feed ingredients, chemical composition, and FA profile of *d*TM and experimental diets are detailed in Table 7.1. To determine the apparent digestibility coefficients (ADC) of diets, 1% chromium oxide (Cr_2O_3) was added to each diet, as inert marker. Experimental diets are the same in this and our previous study (Basto et al., 2022).

7.2.3. Fish husbandry

European sea bass juveniles were obtained from a commercial fish farm (Acuinuga – Acuicultura y Nutrición de Galicia S. L., Spain) and transported to the experimental facilities of CIIMAR (Portugal). Fish were held in quarantine for a 3-week period and hand-fed with a commercial diet (AQUASOJA, Portugal – 50% crude protein and 20% crude fat on DM basis). During quarantine period, all fish were held in a recirculation aquaculture system (RAS) at 22 ± 1 °C, 35 ± 0.5 ‰, 6 L min^{-1}, oxygen level > 90% ± 1 saturation, and 12 L/12D photoperiod. Levels of total ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-), as well as pH were daily monitored to ensure levels within the recommended ranged for marine fish species ($NH_4^+ \leq 0.05$ mg L^{-1}; $NO_2^- \leq 0.5$ mg L^{-1}; $NO_3^- \leq 5$ mg L^{-1}; $7.5 \leq pH \leq 8.5$).

Table 7.1. Ingredients, chemical composition, and fatty acid profile of *d*TM and experimental diets.

	*d*TM	CTRL	TM50	TM100
Ingredients (%)				
Fishmeal [1]		40.0	20.0	-
Defatted *Tenebrio molitor* larvae meal (*d*TM)		-	20.5	40.4
Soy protein concentrate [2]		10.5	10.5	10.5
Soybean meal [3]		13.0	13.0	13.0
Rapeseed meal [4]		5.0	5.0	5.0
Wheat meal [5]		16.2	15.2	14.3
Fish oil [6]		14.0	13.3	12.5
Vitamin and mineral premix [7]		1.0	1.0	1.0
Vitamin C		0.1	0.1	0.1
Vitamin E		0.1	0.1	0.1
Monocalcium phosphate		-	1.0	2.0
L-Lysine		-	-	0.2
L-Threonine		-	-	0.2
L-Tryptophan		-	-	0.1
DL-Methionine		0.1	0.2	0.3
Chemical composition (% DM)				
Dry matter	97.8	93.1	92.6	92.5
Protein	71.0	46.9	47.3	47.2
Lipids	12.1	19.7	19.8	19.0
Gross energy (kJ g^{-1} DM)	24.3	23.2	23.5	24.0
Digestible protein:digestible energy (mg kJ^{-1} DM)	-	21.4	21.0	20.2
Ash	4.8	10.2	8.1	6.3
Phosphorus	0.8	1.2	1.2	1.0
Acid detergent fibre (ADF)	12.7	3.1	4.9	5.4
Chitin	5.5	-	-	-
Fatty acids (% DM)				
C14:0	0.3	0.9	0.9	0.9
C16:0	1.9	2.3	2.5	2.8
C18:0	0.5	0.5	0.6	0.6
Σ SFA[8]	2.9	4.1	4.4	4.9
C16:1	0.2	0.8	0.7	0.7
C18:1n-9 (OA)	4.0	2.4	2.5	2.6
C20:1n-9	0.02	0.6	0.6	0.5
C24:1n-9	-	0.1	0.1	0.1
Σ MUFA[9]	4.3	4.5	4.5	4.4
C18:2n-6 (LA)	4.5	1.0	1.6	2.2
C18:3n-3 (ALA)	0.2	0.3	0.3	0.2
C18:4n-3	0.004	0.4	0.4	0.4
C20:3n-3	-	0.1	0.1	0.1
C20:4n-6	0.004	1.3	1.3	1.1
C20:5n-3 (EPA)	-	1.3	1.2	1.0
C22:5n-3	-	0.2	0.2	0.2
C22:6n-3 (DHA)	-	1.5	1.3	1.1
EPA + DHA	-	2.8	2.5	2.1
DHA / EPA	-	1.2	1.1	1.0
Σ PUFA[10]	4.0	4.3	4.3	4.2
Σ n-3 PUFA	0.1	2.7	2.3	2.0
Σ n-6 PUFA	3.8	1.6	1.9	2.2
Σ n-3 PUFA / Σ n-6 PUFA	0.04	2.9	2.6	2.7

[1] Peruvian fishmeal super prime: 71% crude protein (CP), 11% crude fat (CF), Exalmar, Peru; [2] Soy protein concentrate: 65% CP, 0.7% CF, ADM Animal Nutrition, The Netherlands; [3] Soybean meal 48: dehulled solvent extracted soybean meal: 48% CP, 2% CF, Cargill, Spain; [4] Rapseed meal: 36% CP, 3% CF, PREMIX Lda., Portugal; [5] Wheat meal: 10% CP, 1% CF, Casa Lanchinha, Portugal; [6] Sardine oil, Sopropêche, France; [7] Vitamin and mineral premix: WISIUM, ADM Portugal S.A., Portugal; [8] Includes C12:0, C15:0, C20:0, C21:0, C22:0 and C24:0; [9] Includes C14:1, C17:1, C18:1n-7 and C22:1n-9; [10] Includes C18:3n-6 and C20:2. The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of the fishmeal protein replaced by defatted *Tenebrio molitor* larvae meal.

7.2.4. Digestibility trial

Nine homogeneous groups of 10 fish were individually weighed (70 ± 4 g; CV = 6%) and randomly distributed by 50 L fiberglass tanks with individual faeces sedimentation columns according to Cho and Slinger (1979). Each experimental diet (containing 1% Cr_2O_3) was distributed to triplicate groups of fish that were fed approximately 2% of the biomass, twice a day (09:00 AM and 05:00 PM), during 7 days for adaptation to the diets. The collection of faeces was performed twice a day for 15 days as described by Basto et al. (2020). Faeces collected at different times from the same tank were pooled before chemical analysis.

7.2.5. Growth trial and sampling

Twelve homogeneous groups of 15 fish were individually weighted (69 ± 8 g; CV = 8%) and measured (19 ± 1 cm; CV = 3%) and randomly distributed by 160 L fiberglass tanks. Each diet was distributed to quadruplicate groups of fish, by automatic feeders, three times a day (09:00 AM, 13:00 PM and 17:00 PM). The amount of feed supplied to each tank was daily adjusted by a 5% increase when no feed losses were observed and by a 5% reduction when detected. Ten fish from the initial fish stock and four fish from each tank by the end of the experiment were collected after a 48-h fasting period, sacrificed by anaesthetic overdose (2-Phenoxyethanol, 500 µL L^{-1}), pooled and kept at −80 °C until whole-body composition was analysed. After 16 weeks of feeding the experimental diets, and after a 48-h fasting period, all fish were individually weighed (g) and measured (total length, cm). Five fish/tank were euthanized by immersion in an ice bath, and kept at 4 °C in polystyrene boxes filled with ice until sensory analysis (24 h after death). Three fish/tank were further sacrificed by a sharp blow on the head, and liver and viscera weights were registered for viscerosomatic and hepatosomatic indexes determination (VSI and HSI, respectively). A cross-sectional slab with skin (5 mm thick) was taken immediately before the dorsal fin of each fish and photographed (with scale reference) for determination of the cross-sectional area (CSA). A portion from the left part of the slab (1 × 1 cm) was frozen in isopentane, cooled by dry ice, and stored at −80 °C for further muscle histological analysis. Two other muscle samples, also from the left part of the fillet, were immediately frozen in liquid nitrogen and stored at −80 °C for DM, total lipid content and FA profile analysis. Additionally, the right dorsal fillet was collected with skin for instrumental colour and texture analysis.

7.2.6. Chemical analysis

The *d*TM, diets and pooled fish collected from the initial stock and from each tank by the end of the trial were ground, and homogenized to determine moisture content (105 °C for 24 h). Fish and faeces where then freeze-dried prior further chemical analysis. Ash was analysed by combustion in a muffle furnace at 500 °C for 5 h (Nabertherm L9/11/B170, Germany); crude protein using a Leco nitrogen analyser (Model FP 528; Leco Corporation, USA) and conversion (N x 6.25) to equivalent protein; gross energy was determined in an adiabatic bomb calorimeter (Model Werke C2000, IKA, Germany). Total phosphorus content was determined from ash according to AOAC method 965.17 (2006) with the following adaptation: phosphates were quantified after using a 2 mM ammonium heptamolybdate reagent solution with 7 mM ascorbic acid and 0.5 M sulfuric acid at 75 °C in a water bath and later determination of absorbance at 820 nm. Acid detergent fibre (ADF) was analysed according to ISO 13906:2008. Chitin was estimated using the indirect method of Finke (2007) modified by Marono et al. (2015): ADF (%) – ADIP (%), where ADIP is the amount of protein linked to ADF. The Cr_2O_3 content of diets and faeces was determined according to Bolin et al. (1952). Total lipids of *d*TM, diets, whole-body and muscle samples were extracted and quantified gravimetrically according to Folch et al. (1957), using dichloromethane:methanol 2:1 *v*/v with 0.01% butylated hydroxytoluene, BHT. The FA methyl esters (FAME) were obtained by acidic transesterification according to Lepage and Roy (1986), method modified by Campos et al. (2017), using 0.5 mg mL^{-1} of an internal standard solution of tricosaenoic acid (C23:0; Capillary GC, purity ≥99%, Sigma – Aldrich, USA) dissolved in n-hexane (instead of methanol-benzene) for lipid extracts of *d*TM and diets, and 1 mg $mL^{-1}$for lipid extracts of whole-body and muscle samples. After transesterification, FAME were recovered in 1 mL of n-hexane and analysed in a gas chromatograph (Shimadzu GC-2010, Japan) as described by Marques et al. (2022). The amount of FAs was calculated using an internal standard (C23:0) as a reference, according to Joseph and Ackman (1992).

7.2.7. Histological analysis

Muscle cross-sections (12 µm) were obtained with a cryostat, and muscle fibres membranes were marked using immunohistochemical procedures; the sections were incubated overnight at 4 °C with the dystrophin antibody MANDRA1 (Santa Cruz Biotechnology, EUA) diluted 1:200 in Phosphate Buffered Saline (PBS), and containing 1% of Tween™ 20, using the Novolink™ Polymer Detection System kit (RE7140-K, Leica Biosystems, Germany). The marked samples were then photographed and examined

under a microscope (Olympus BX51, GmbH, Germany) coupled with a camera (Olympus DP50, GmbH, Germany). An imaging software (Olympus cellSens Dimension Desktop) was used to measure the following parameters: muscle cross-sectional area, CSA (mm^2); total number of fibres per CSA; fibre number per unit of area (density; n° mm^{-2}) and frequency of fibres classes according to fibre diameter (≤ 25 µm and > 125 µm). One cross-section was analysed per fish (*n* = 12 fish per dietary treatment) and a minimum of 900 fibres were measured per cross-section to ensure results representativeness.

7.2.8. Instrumental texture and colour measurement

Skin and muscle colour measurements were done with a CR-400 chromameter (Konica Minolta Inc., Japan) with an aperture of 8 mm, with respect to CIE standard illuminant D65. The chromameter was applied onto each sample and three replicates of lightness, redness, and yellowness (L*, a* and b*, respectively) values were recorded. From a* and b* values the chroma ($C^* = (a^{*2} + b^{*2})^{1/2}$) and the hue angle ($H° = \tan^{-1}(b^* / a^*)$) were calculated (Choubert et al., 1997). Muscle texture was analysed using a TA.XT. plus Texture Analyser with a 5 kg load cell and a 2.0 mm diameter probe (Stable Micro systems Inc., UK). Texture profile parameters (hardness (N), adhesiveness (J), springiness (−), cohesiveness (−), chewiness (J), and resilience (−)) were obtained by double penetration (probe speed of 1 mm s^{-1}; probe penetration depth of 4 mm; wait time between penetrations of 5 s) on maximum thickness part of each raw fillet.

7.2.9. Consumer-liking and sensory attributes assessment

A sensory panel of 60 untrained panellists was recruited from the sensory evaluation company Sense Test's (Portugal) consumer database. All participants were regular consumers of fish (at least twice a week) and received a small financial compensation for their participation. Besides the implementation of an informed consent, the company ensures the protection and confidentiality of data through the authorization 2063/2009 of the National Data Protection Commission, and following EU Regulation (EU 2016/679), as well as a longstanding internal code of conduct. Sensory evaluation was carried in individual tasting booths at a special room equipped in accordance with ISO 8589:2007. Fish were cut into three slabs (~ 4 cm thick) that were wrapped in micro-perforated aluminium foil and steamed for 12 min at 100 °C in a preheated industrial forced convector oven with steam (Rational Combi-Master CM61, Rational AG, Germany). Each untrained panellist analysed the 3 slabs, monadically and sequentially, with their order previously balanced. The slabs were served always in the same position over a

white pre-heated (50 °C) porcelain plate blind-labelled with random three-digit codes. The consumers were provided with a porcelain spittoon, a glass of bottled natural water and unsalted crackers, and were asked to chew a piece of cracker and to rinse the mouth with water before testing each sample. For each sample, the global acceptance was evaluated using a 9-point scale and each consumer wrote a free comment to each sample, considering the main positive and negative aspects.

7.2.10. Calculations

The ADC of the experimental diets was calculated according to Maynard et al. (1979): dry matter ADC (%) = 100 × [1 - (dietary Cr_2O_3 level / faeces Cr_2O_3 level)] and nutrients or energy ADC (%) = 100 × [1 - (dietary Cr_2O_3 level / faeces Cr_2O_3level) × (faeces nutrient or energy level / dietary nutrient or energy)]; Average body weight (ABW) = (final body weight + initial body weight) / 2; Digestible nitrogen (N), lipids (L), phosphorus (P) or energy (E) intake = (dry feed consumption × N, L, P (%) or E (kJ g^{-1}) in the diet × ADC N, L, P or E) / ABW / days of experiment; N, L, P, E or FA gain = (final carcass N, L, P, E or FA content - initial carcass N, L, P, E or FA content) / ABW / days of experiment; N, L, P or E, retention efficiency (NRE, LRE, PRE, or ERE) = (N, L, P or E gain / digestible N, L, P or E intake) × 100; FA retention / consumption = 100 × (FBW × final carcass FA content - IBW × initial carcass FA content) / feed consumption per fish × FA content of diet); Fecal N, L, P or E losses = crude N, L, P or E intake × [1 - (ADC N, L, P or E / 100)]. Metabolic N, L or P losses = digestible N, L or P intake − N, L or P gain; Branchial and urinary E losses = non-fecal N losses × 25 kJ/N; Total N, L, P or L losses = crude N, L or P intake − N, L or P gain; Metabolizable energy (ME) = digestible E intake - branchial and urinary E losses; Total heat loss = E intake – E gain; Total heat production = ME - energy gain. Condition factor (K) = [final body weight / (final body length)^3] × 100; DGI = 100 × [(final body weight)$^{1/3}$ - (initial body weight)$^{1/3}$] / days; Voluntary feed intake (VFI) = 100 × dry feed intake / average body weight / day; Feed conversion ratio (FCR) = dry feed intake / weight gain; Protein efficiency ratio (PER) = weight gain / crude protein intake; Viscerosomatic index (VSI) = 100 × weight of viscera / body weight; Hepatosomatic index (HSI) = 100 × liver weight / body weight. Fish quality lipids/flesh quality lipids (FQL) = 100 × (C22:6 n-3 + C20:5 n-3) / Σ FA; Atherogenic index = [C12:0 + (4 × C14:0) + C16:0] / (Σ PUFA n-3 + Σ PUFA n-6 + Σ MUFA). Thrombogenic index = [C14:0 + C16:0 + C18:0] / [0.5 × Σ monounsaturated FA (MUFA)) + (0.5 × Σ PUFA n-6) + (3 × Σ PUFA n-3) + (Σ PUFA n-3/ Σ PUFA n-6)]. Hypocholesterolemic to hypercholesterolemic FA ratio = (C18:1n-9 + C18:2n-6 + C20:4n-6 + C18:3n-3 + C20:5n-3+ C22:5n-3 + C22:6n-3) / (C14:0 + C16:0).

7.2.11. Statistical analysis

All data are expresses as mean ± standard deviation (SD). Normality and homogeneity of variances by Kolmogorov-Smirnov and Levene's tests, respectively, and log-transformed whenever required. Differences among dietary treatments were tested by a one-way ANOVA with a significance level of 95% ($p \leq 0.05$) followed by a *post-hoc* Tukey HSD test, using SPSS Statistics 26.0 (IBM corporation, USA). A principal component analysis (PCA) was performed on the muscle cellularity and instrumental texture parameters and a correspondence analysis was applied to the free comments of untrained panellists to evaluate the linear combinations of the original variables that best separate the groups (discriminant functions), using XLSTAT 2018 ® system software (Addinsoft, USA).

7.3. Results

7.3.1. Chemical composition of experimental diets

All diets were isoproteic (47% protein on DM basis), isolipidic (20% lipids on DM basis) and isoenergetic (23 kJ g^{-1} DM) (Table 7.1). The concentration of saturated FA (SFA) increased concomitantly with *d*TM dietary inclusion (4.1% DM in CTRL vs 4.4 and 4.9% DM in TM50 and TM100, respectively), due to its high content of palmitic acid (C16:0). Despite the high concentration of oleic acid (OA; C18:1n-9) in *d*TM, all dietary treatments had very similar monounsaturated FA (MUFA; 4.4–4.5% DM) levels. The lack of EPA and DHA in *d*TM resulted in reduced n-3 PUFA content in TM50 and TM100 (2.3 and 2.0% DM, respectively) compared to the CTRL (2.7%). The *d*TM-based diets had the highest concentrations of n-6 PUFA, due to the high linoleic acid (LA; C18:2n-6) content of *d*TM, resulting in slightly lower n-3/n-6 PUFA ratio compared to the CTRL diet (2.6–2.7 in *d*TM diets vs 2.9 in CTRL).

7.3.2. Growth performance and nutrient utilization

At the end of 16-week feeding, all fish had similar growth performance (SGR = 1.2) and almost quadrupled their initial body weight (from 69 to 265–268 g final body weight). No mortality was registered, and despite fish fed TM50 had the lowest VFI, these fish had the best FCR, resulting in similar final body sizes and condition factor, irrespectively of the dietary treatment (Table 7.2). All diets were very well digested by the European sea bass (DM ADC > 78%), but the dietary inclusion *d*TM decreased protein (from 94% in the CTRL to 92 and 90% in TM50 and TM100, respectively) and lipid digestibility's (from 97% in the

CTRL to 92–97% in diets with dTM). The retention efficiency of both digested N and L increased significantly with the inclusion of *d*TM, whereas their metabolic losses decreased significantly (Table 7.3). The balance between nutrients' retention efficiency and metabolic losses resulted in similar N gain among fish (31 mg 100 g ABW day^{-1}). But whole-body L deposition and gain increased in fish fed *d*TM diets (Table 7.3 and Supplementary Table S10; Appendix). Increased P digestibility in fish fed *d*TM diets was coupled with decreased fecal and total P losses (Table 7.3).

Table 7.2. Growth performance, somatic indexes and whole-body composition of European sea bass fed experimental diets.

	CTRL	TM50	TM100	*p*-value
Growth performance				
Initial body weight (g)	68.6 ± 5.2	68.6 ± 5.2	68.6 ± 5.0	0.908
Final body weight (g)	265.4 ± 9.4	267.5 ± 12.3	268.2 ± 11.7	0.937
Initial body length (cm)	18.9 ± 0.6	18.9 ± 0.6	18.8 ± 0.5	0.309
Final body length (cm)	27.9 ± 1.1	27.9 ± 1.1	27.9 ± 1.2	0.912
Final condition factor (K)	1.2 ± 0.01	1.2 ± 0.02	1.2 ± 0.02	0.100
Specific growth rate (SGR)	1.2 ± 0.03	1.2 ± 0.04	1.2 ± 0.04	0.940
Voluntary feed intake (VFI)	1.15 ± 0.02 ^{a}	1.05 ± 0.05 ^{b}	1.13 ± 0.06 ^{ab}	0.031
Feed conversion ratio (FCR)	1.1 ± 0.03 ^{a}	1.0 ± 0.03 ^{b}	1.1 ± 0.05 ^{a}	0.012
Protein efficiency ratio (PER)	1.9 ± 0.1 ^{b}	2.1 ± 0.1 ^{a}	2.0 ± 0.1 ^{b}	0.014
Viscerosomatic index (VSI)	6.4 ± 0.2	6.8 ± 0.2	6.3 ± 0.2	0.528
Hepatosomatic index (HSI)	2.1 ± 0.5	2.2 ± 0.8	2.0 ± 0.4	0.696

Values are presented as mean ± standard deviation; n = 60 for growth-related parameters and n = 12 for somatic indexes. Different superscript letters represent significant differences among diets ($p < 0.05$; one-way ANOVA followed by Tukey's post-hoc test). The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of the fishmeal protein replaced by defatted *Tenebrio molitor* larvae meal.

Table 7.3. Apparent digestibility coefficients (ADC) and nutrient utilization of European sea bass fed experimental diets.

	CTRL	TM50	TM100	*p*-value
ADC (%)				
Dry matter	79.8 ± 0.2	78.7 ± 1.1	77.6 ± 2.1	0.211
Protein	94.2 ± 0.1 ^{a}	92.1 ± 0.5 ^{b}	90.1 ± 0.8 ^{c}	<0.001
Lipids	97.3 ± 0.1 ^{a}	96.8 ± 0.1 ^{b}	95.7 ± 0.3 ^{b}	<0.001
Energy	89.4 ± 0.4 ^{a}	88.5 ± 0.2 ^{ab}	87.6 ± 0.9 ^{b}	0.019
Phosphorus	53.9 ± 1.6 ^{c}	63.2 ± 1.6 ^{b}	74.3 ± 0.5 ^{a}	<0.001
Nitrogen (N) balance (mg 100 g^{-1} ABW day $^{-1}$)				
Digestible N (DN) intake	81.6 ± 1.1 ^{a}	73.4 ± 3.3 ^{b}	77.1 ± 4.3 ^{b}	0.018
N gain	31.5 ± 0.8	31.3 ± 1.2	31.1 ± 0.7	0.803
N retention efficiency (% DN)	38.6 ± 1.2 ^{b}	42.7 ± 2.6 ^{a}	40.3 ± 1.5 ^{a}	0.038
Fecal N losses	5.0 ± 0.1 ^{c}	6.3 ± 0.3 ^{b}	8.5 ± 0.5 ^{a}	<0.001
Metabolic N losses	50.1 ± 1.5 ^{a}	42.1 ± 3.6 ^{b}	46.1 ± 3.7 ^{b}	0.017
Total N losses	55.1 ± 1.5	48.4 ± 3.8	54.6 ± 4.2	0.051
Lipids (L) balance (g kg^{-1} ABW day^{-1})				
Digestible L (DL) intake	2.2 ± 0.03 ^{a}	2.0 ± 0.1 ^{b}	2.0 ± 0.1 ^{b}	0.018
L gain	1.9 ± 0.1 ^{b}	2.0 ± 0.1 ^{a}	2.0 ± 0.1 ^{a}	0.048
L retention efficiency (% DL)	84 ± 6.6 ^{b}	100.0 ± 3.3 ^{a}	100.0 ± 4.6 ^{a}	0.002
Fecal L losses	0.06 ± 0.001 ^{b}	0.07 ± 0.003 ^{b}	0.09 ± 0.004 ^{a}	0.002
Metabolic L losses	0.4 ± 0.1 ^{a}	0.1 ± 0.02 ^{b}	0.1 ± 0.01 ^{b}	<0.001
Total L losses	0.5 ± 0.2 ^{a}	0.2 ± 0.1 ^{b}	0.2 ± 0.05 ^{b}	0.001
Energy (E) balance (kJ kg^{-1} ABW day^{-1})				
Digestible E (DE) intake	238.6 ± 3.2 ^{a}	219.1 ± 10.0 ^{b}	237.9 ± 13.2 ^{ab}	0.032
E gain	113.4 ± 6.5	115.6 ± 3.4	117.0 ± 1.9	0.517
E retention efficiency (% DE)	47.5 ± 3.2 ^{b}	52.8 ± 1.2 ^{a}	49.3 ± 2.8 ^{ab}	0.048
Metabolizable Energy	226.1 ± 2.9 ^{ab}	208.6 ± 9.2 ^{b}	226.4 ± 12.3 ^{a}	0.033
Fecal E losses	23.3 ± 0.4 ^{b}	28.4 ± 1.3 ^{b}	33.8 ± 1.9 ^{a}	<0.001
Branchial + urinary E losses	12.5 ± 0.4 ^{a}	10.5 ± 0.9 ^{b}	11.5 ± 0.9 ^{ab}	0.017
Total E losses	153.6 ± 9.5 ^{ab}	132.0 ± 8.3 ^{b}	154.7 ± 15.4 ^{a}	0.035
Total heat production	112.8 ± 8.80 ^{a}	93.0 ± 6.2 ^{b}	109.4 ± 12.6 ^{ab}	0.037
Phosphorus (P) balance (mg 100 g^{-1} ABW day^{-1})				
Digestible P (DP) intake	7.6 ± 0.1 ^{b}	7.8 ± 0.4 ^{b}	8.7 ± 0.5 ^{a}	0.003
P gain	6.1 ± 0.3	6.2 ± 0.3	6.6 ± 0.5	0.266
P retention efficiency (% DP)	80.8 ± 4.1	80.4 ± 5.8	75.6 ± 6.1	0.366
Fecal P losses	6.5 ± 0.1 ^{a}	4.5 ± 0.2 ^{b}	3.0 ± 0.2 ^{c}	<0.001
Metabolic P losses	1.5 ± 0.3	1.5 ± 0.5	2.1 ± 0.6	0.155
Total P losses	8.0 ± 0.3 ^{a}	6.1 ± 0.7 ^{b}	5.1 ± 0.4 ^{b}	<0.001

Values are presented as mean ± standard deviation; n = 3 for ADC calculations and n = 4 for nutrient utilization calculations. Different superscript letters represent significant differences among diets ($p < 0.05$; one-way ANOVA followed by Tukey's post-hoc test). The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of the fishmeal protein replaced by defatted *Tenebrio molitor* larvae meal.

7.3.3. Whole-body and muscle composition, fatty acids profile and utilization

Whole-body moisture, protein, energy, ash and phosphorus levels were not affected by dietary treatments (Supplementary Table S10; Appendix). The whole-body total lipids significantly increased in fish fed *d*TM dietary treatments (from 12.6 g 100 g^{-1} WW in the CTRL to 13.1–13.4 g 100 g^{-1} WW in diets with *d*TM), but this pattern was not observed in muscle. Although dietary EPA and DHA decreased with dTM inclusion, due to the lack of these n-3 LC-PUFA in insects, fish whole-body retention and gain tended to increase (Figure 7.1A and Figure 7.1B; Supplementary Tables S11 and S12; Appendix), resulting in similar whole-body EPA and DHA among fish (Figure 7.1C). In *d*TM-based diets, muscle SFA increased, whilst EPA, DHA and n-3 PUFA decreased significantly. However, this reduction can only be perceived when these fatty acids are expressed in relation to total FA (% total fatty acids). The muscle content of SFA, MUFA, EPA, DHA or PUFA, expressed in percentage of wet weight (WW), showed no significant differences among dietary treatments (Supplementary Table S13; Appendix). Muscle quality indexes are shown in Figure 7.2. Muscle EPA + DHA, fish lipid quality (FQL) and hypocholesterolaemic FA/hypercholesterolaemic FA (h/H) remained similar among dietary treatments. Muscle PUFA/SFA and n-3 PUFA/n-6 PUFA ratios were significantly lower in fish fed TM100 compared to those fed CTRL. Trombogenic index (TI) increased concomitantly with *d*TM inclusion level, being highest in fish fed TM00.

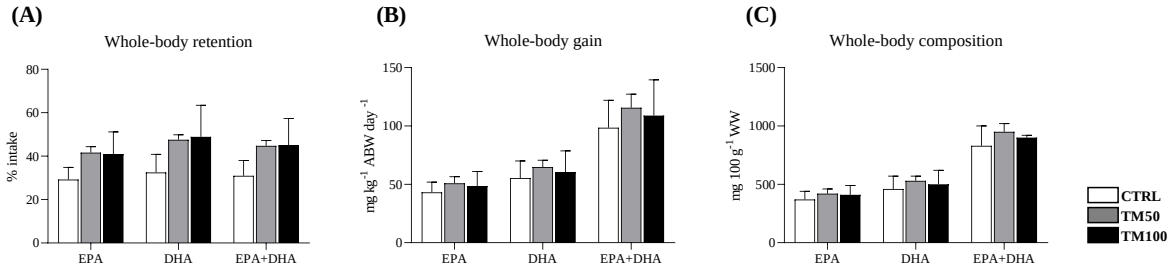


Figure 7.1. Whole-body eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and EPA+DHA retention (A), gain (B) and composition (C) of European sea bass fed experimental diets. Columns represent mean and error bars represent the standard deviation; n = 4 No significant differences were observed among diets (p > 0.05; one-way ANOVA followed by Tukey's post-hoc test). The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of the fishmeal protein replaced by defatted *Tenebrio molitor* larvae meal.

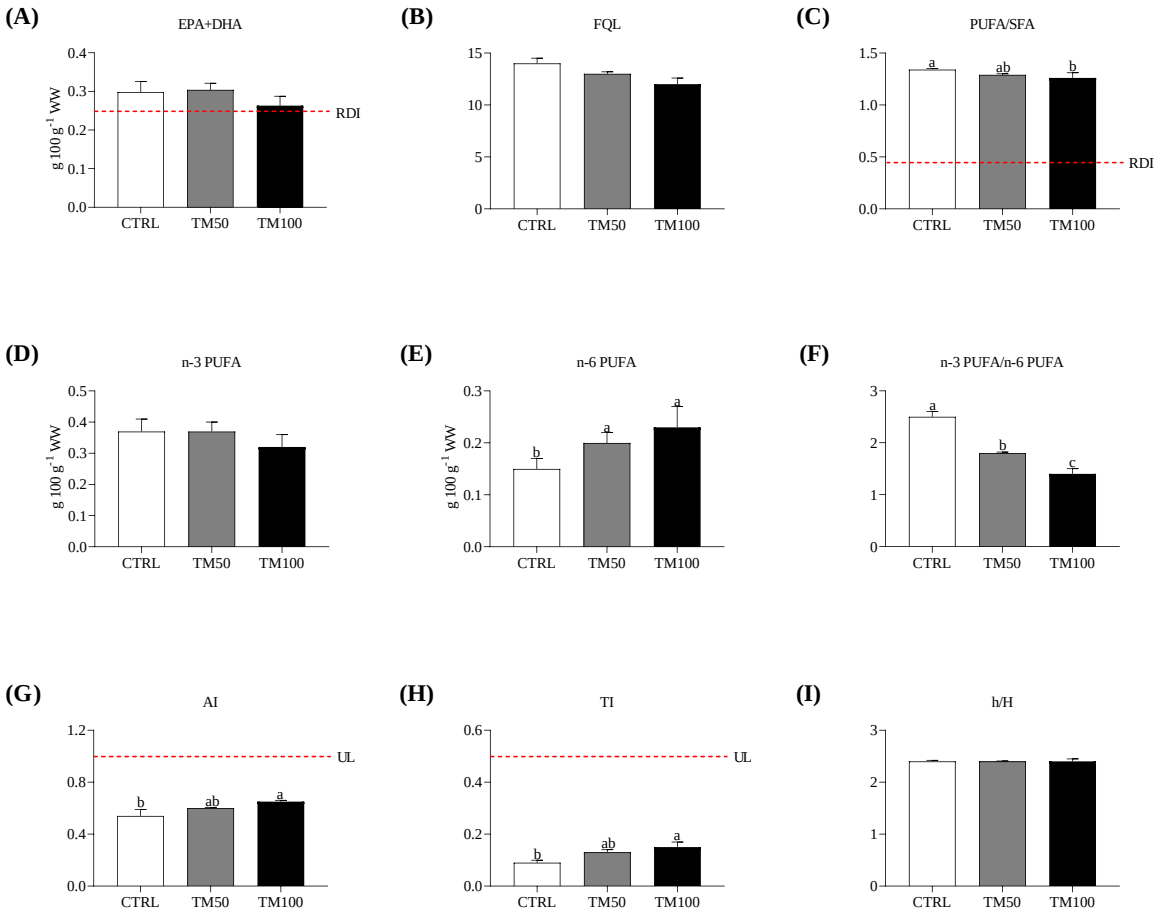


Figure 7.2. Muscle quality indexes of European sea bass fed experimental diets. Columns represent mean and error bars represent the standard deviation; n = 4. Different superscript letters represent significant differences among diets ($p < 0.05$; one-way ANOVA followed by Tukey's post-hoc test). The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of the fishmeal protein replaced by defatted *Tenebrio molitor* larvae meal. AI, atherogenic index; FQL, fish lipid quality; h/H, hypocholesterolaemic fatty acids/hypercholesterolaemic fatty acids ratio; PUFA, polyunsaturated fatty acids; RDI, recommended daily intake for human consumption; SFA, saturated fatty acids; TI, thrombogenic index; UL, upper intake level for human consumption.

7.3.4. Cellularity and instrumental texture and colour

Cellularity and instrumental texture and colour parameters are available in Supplementary Table S14 (Appendix). White muscle CSA varied between 815 and 863 mm^2, being similar among groups. The total number of fibres and their density were significantly higher in fish fed TM100 compared to those fed TM50 and were both similar to the CTRL. The muscle of fish fed TM100 had the highest percentage of small fibres (< 25 µm) and the lowest percentage of large-sized fibres (> 125 µm) (Figure 7.3). Hardness, adhesiveness, springiness, and chewiness parameters remained similar among dietary

treatments. Cohesiveness and resilience were significantly higher in fish fed TM50 compared to those fed CTRL (0.44 vs 0.39 and 0.30 vs 0.23, respectively). A PCA was applied to muscle cellularity parameters and instrumental texture attributes of fish fed the experimental diets (Figure 7.4). Two main clusters were clearly depicted along F1 (Figure 7.4), explaining 69.6% of the variance: TM50 (left side) was separated from TM100 and CTRL (right side). Muscle of fish fed TM50 was strongly associated with hardness, springiness, chewiness and with large-sized fibres (> 125 μm), whereas muscle of fish fed TM100 seems to be associated to the smallest fibre class (< 25 μm) (Figure 7.4). European sea bass skin and muscle L*, a*, b* and C* values were similar among groups (Supplementary Table S14; Appendix). Muscle H° also remained unaffected by dietary treatments, but the skin of fish fed TM100 had a significantly lower H° compared to that of fish fed the CTRL diet (131 vs 142, respectively).

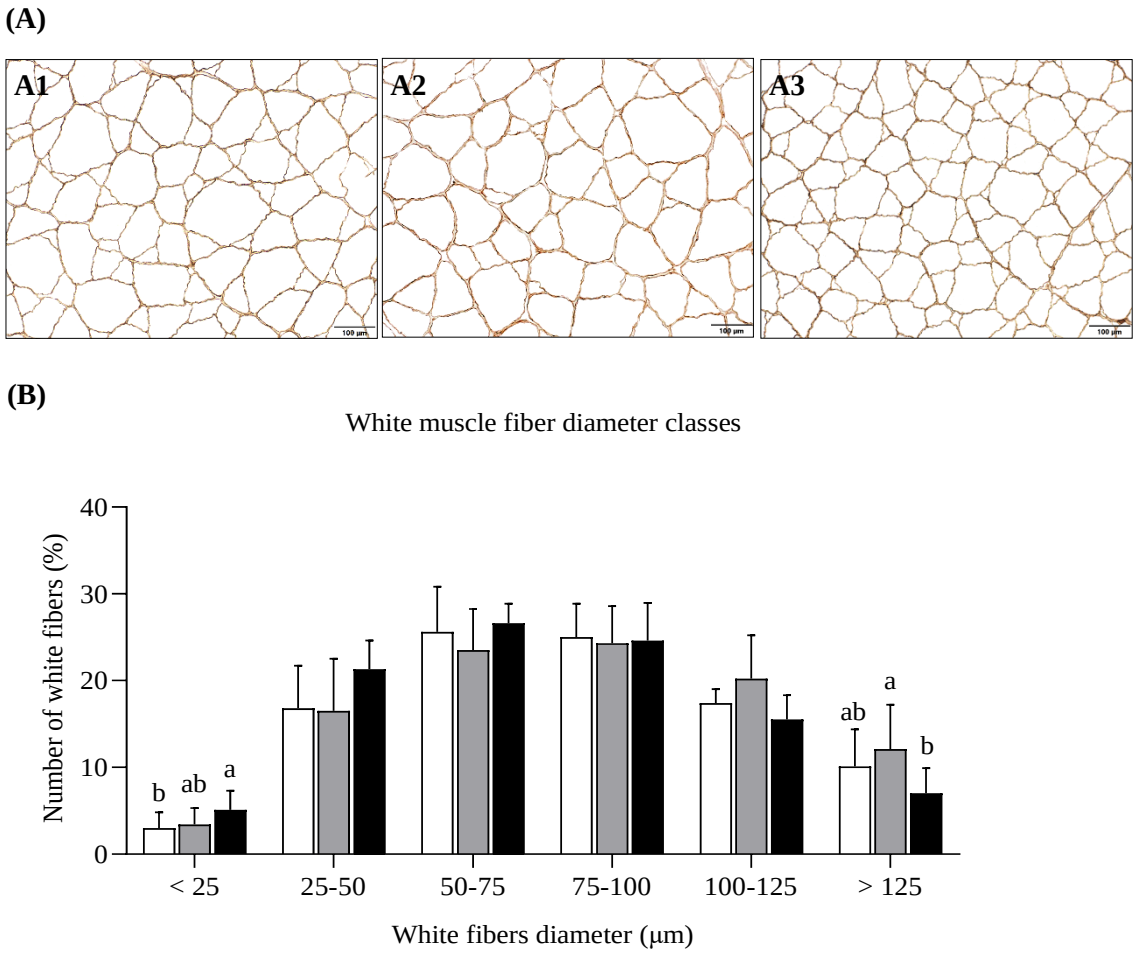


Figure 7.3. Cross section of white muscle fibres of European sea bass fed CTRL (A1), TM50 (A2) and TM100 (A3), with membranes specially marked with dystrophin antibody (100× magnification) and white muscle fibre diameter classes (B). Columns represent mean and error bars represent the standard deviation; n = 12. Different superscript letters represent significant differences among diets (p < 0.05; one-way ANOVA followed by Tukey's post-hoc test). The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of the fishmeal protein replaced by defatted *Tenebrio molitor* larvae meal.

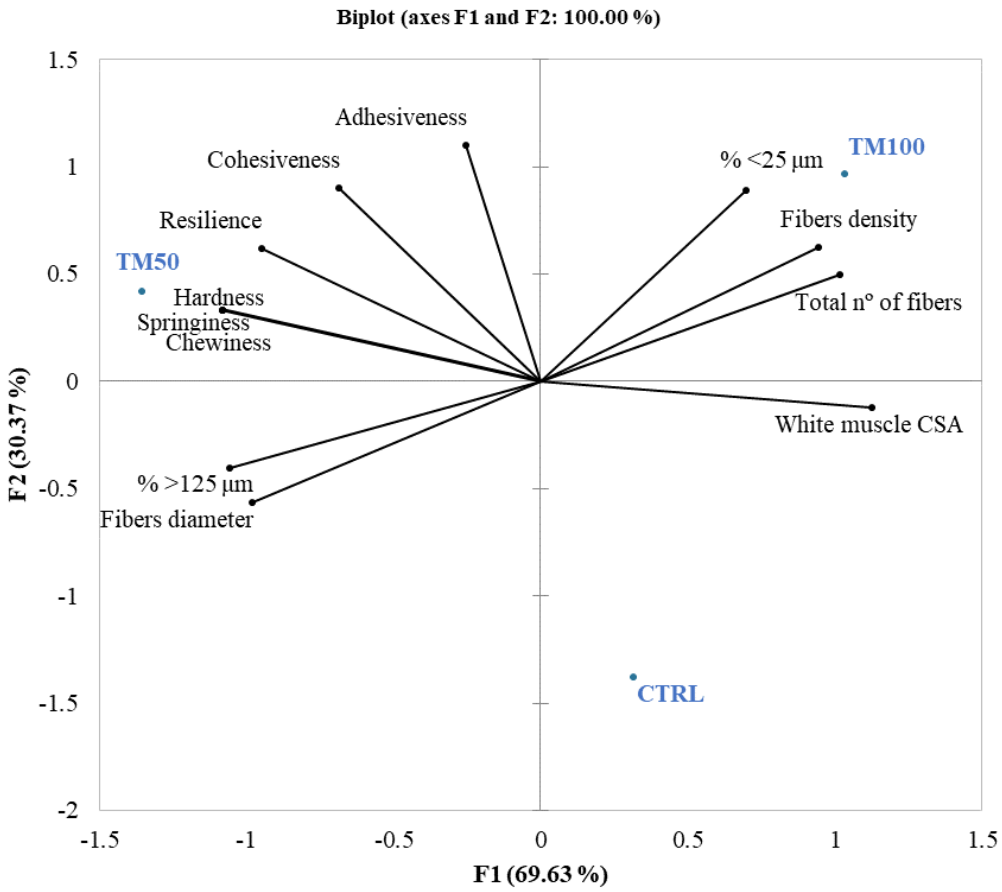
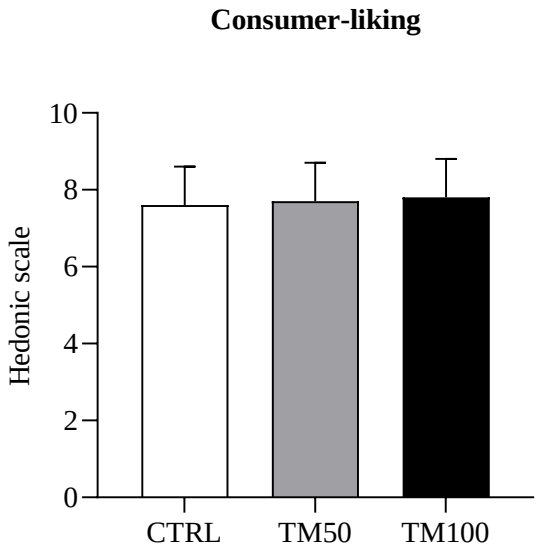


Figure 7.4. Biplot with dimensions 1 and 2 of principal components analysis of muscle cellularity and instrumental texture parameters of European sea bass fed experimental diets. The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of the fishmeal protein replaced by defatted *Tenebrio molitor* larvae meal.

7.3.5. Consumer's acceptance

The mean rates of consumer-liking were high for all fish, and the sensory panel could not perceive any significant differences in the cooked muscle slabs from fish fed the different experimental diets (Figure 7.5A). To the best of our knowledge, the impact of dietary inclusion of IM on European sea bass sensory profile was never assessed before. All samples were positively characterized by their appearance, odour, texture and taste and no negative comments of statistical relevance were recorded.

(A)



(B)

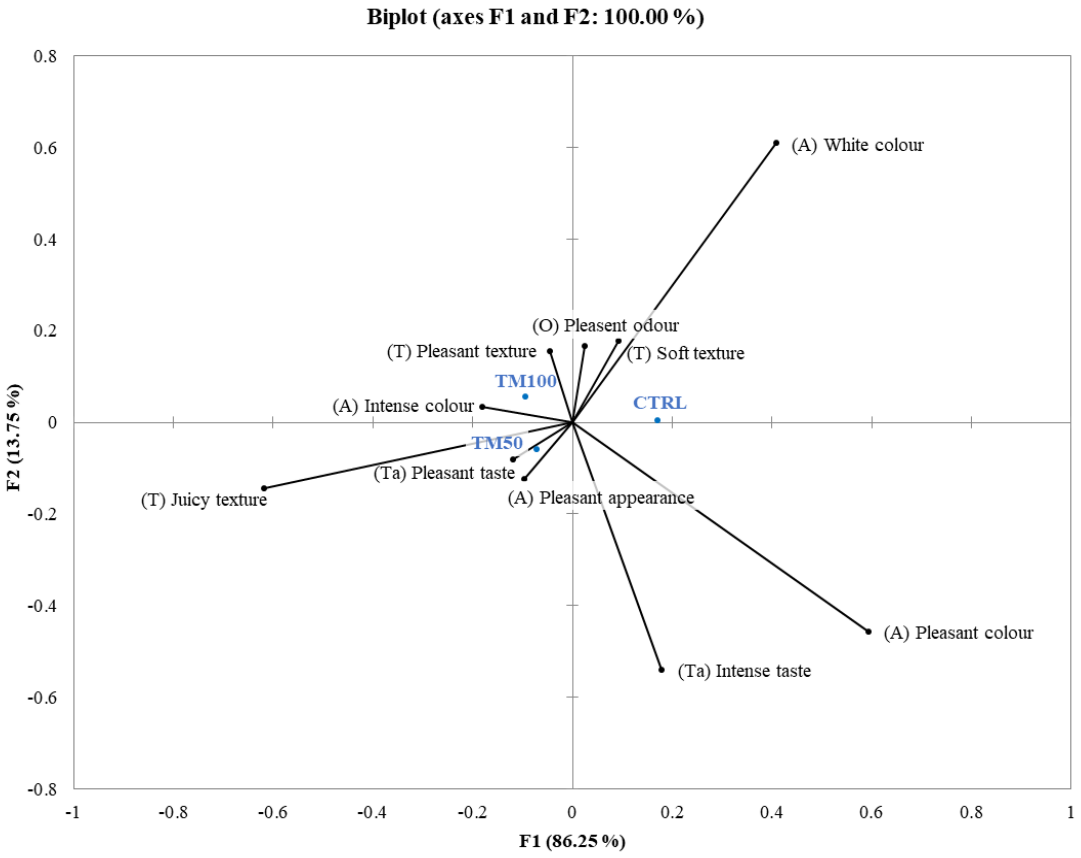


Figure 7.5. (A) Consumer-liking of European sea bass fed experimental diets. Error bars indicate the standard deviation of the mean, with n = 20. (B) Biplot with dimensions 1 and 2 of CA of free comments of consumers considering the main positive aspects of flesh of European sea bass fed experimental diets. Abbreviations stand for: A – appearance; O – odour; T – texture; Ta – taste. The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of the fishmeal protein replaced by defatted *Tenebrio molitor* larvae meal.

7.4. Discussion

Insect protein has been identified as one of the most promising ingredients to reduce the use of FM in aquafeeds, when compared with other novel protein sources (i.e., micro- and macroalgae, bacteria and yeast) (Cottrell et al., 2020). However, when included at high percentages, the insect's negligible content of EPA and DHA may compromise not only fish growth performance, but also muscle nutritional, organoleptic and sensory quality with particular relevance in market-sized marine fish species. The lack of EPA and DHA in *d*TM was reflected in decreased n-3 PUFA content in TM50 and TM100 diets. Nonetheless, due to FO inclusion, all dietary treatments still had a DHA (1.1–1.5% DM) and EPA + DHA level (2.1–2.8% DM) above minimal recommended values for European sea bass (0.5% and 0.7% DM, respectively) (NRC, 2011; Skalli and Robin, 2004). Likewise, DHA/EPA ratio (1.0–1.2) was within the recommended range of values (0.5–2.0) required by marine fish species for normal growth and development (NRC, 2011).

Fish growth performance and discharges of both phosphorous (P) and nitrogen (N) to water are highly dependent on nutrient/energy bioavailability. In the present study, P digestibility increased in fish fed *d*TM, resulting in lower fecal and total losses into the water. Such diets clearly contribute to environmentally sustainable aquaculture practices and the production of eco-friendlier products which are increasingly demanded by consumers (Carlucci et al., 2015). Despite protein digestibility decreased with *d*TM dietary inclusion, ADC values were still high (90–92%). In fish, as in other vertebrates, the impairment in protein digestibility and nitrogen utilization have often been associated to insects' chitin content, due to its capacity to bind to proteins reducing the activity of proteolytic enzymes (Piccolo et al., 2017; Belghit et al., 2018b; Cutrignelli et al., 2018; Moniello et al., 2019; Addeo et al., 2022). But in the present study, although the digestible N intake decreased in fish fed *d*TM diets, their retention efficiency increased significantly, and was associated with decreased metabolic losses, resulting in similar N gain and growth performance to fish fed the CTRL diet. These results are in line with previous findings in European sea bass juveniles, where growth performance was not affected by increasing levels of *d*TM (from 18 to 60% inclusion, replacing 40 to 100% FM protein), with a 12% FO inclusion (Basto et al., 2021). Contrarily, Gasco et al. (2016)observed that growth performance of European sea bass was compromised when fed a 50% full-fat TM dietary inclusion (71% FM replacement). However, experimental diets only contained 2% FO, as single lipid source, which could not fulfil the n-3 LC-PUFA requirements of European sea bass (Skalli and Robin, 2004), which may explain the poor fish performance.

European sea bass fed TM50 and TM100 not only had improved N retention efficiency, but also retained lipids more efficiently by reducing losses compared to fish fed the CTRL. This trade-off resulted in higher lipid gain and whole-body lipid deposition confirming previous observations in juvenile fish (Basto et al., 2021). In Atlantic salmon, Belghit et al. (2018a) also observed that 85% of FM protein replacement by HI resulted in increased whole-body lipids content, but Stadtlander et al. (2017) concluded that 50% of FM protein replacement by HI improved lipid utilization without increasing whole-body lipid content. These results suggest that high levels of IM may impact fish lipid metabolism. Indeed, in our previous study (Basto et al., 2021) the total replacement of FM by *d*TM induced alterations in juveniles' sea bass hepatic genes with key roles in lipid metabolism. More specifically, it was observed a down-regulation of the elongation of very long-chain FA protein 6 (*elovl6*) and an up-regulation of FA desaturase 2 (*fads2*), which are responsible for the elongation of SFA and $C_{12–16}$ MUFA into C_{18} MUFA and biosynthesis of LC-PUFA from LA and α-linolenic acid (C18:3n-3; ALA), respectively. Recently, Basto et al. (2022) also reported that *d*TM-based diets did not alter short- and mid-term homeostatic regulation of food intake, but induced increased levels of glucose and triglycerides in the liver of sea bass juveniles. These results suggest that the conversion of glucose into triglycerides (through glycolysis and lipogenesis) may contribute to the accumulation of body fat depots as presently observed in European sea bass fed *d*TM-based diets for 16 weeks. Such hypothesis needs to be further validated through the evaluation of the activity of enzymes involved in glycolysis and lipogenesis that may impact lipid uptake and tissues FA composition (Tocher, 2003). However, in the present study the increased whole body lipid content with *d*TM dietary inclusion was not reflected in significant alteration of n-3 PUFA or EPA + DHA levels, either at whole-body or muscle expressed on fresh basis. More importantly, all muscle samples had EPA + DHA levels above the minimum daily intake recommended by EFSA (2010) to prevent the risk of CVD (0.25 g EPA + DHA 100 g^{-1} WW). Bruni et al. (2020b) also reported that fillets of rainbow trout (*Oncorhynchus mykiss*) fed HI-based diets did not reflect the poor n-3 PUFA of dietary treatments and associated such results to an up-regulation of *fads2*. However, the nutritional value of fish fillets is not only dependent on the n-3 PUFA content, but also on their relationship with single SFA and other FA classes. The PUFA/SFA, n-3 PUFA/n-6 PUFA ratios, AI, TI, and h/H ratios have become very important parameters for evaluating the nutritional quality and healthiness of dietary lipids for human consumption (Chen and Liu, 2020; Kilar and Kasprzyk, 2021). The PUFA/SFA ratio hypothesizes that all dietary PUFA can reduce low-density lipoprotein cholesterol (LDL-C) and the serum cholesterol level, whereas SFA have the opposite effect, thus high PUFA/SFA ratios are desirable. The dietary inclusion of *d*TM resulted in a significant

decrease of muscle PUFA/SFA ratio in fish fed TM100 compared to those fed the CTRL (1.26 vs 1.34, respectively). Nevertheless, all fish still had a ratio almost 3 times higher than the minimum recommended for human consumption (PUFA/SFA ratio > 0.45) to decrease serum cholesterol levels and prevent the development of CVD (Kilar and Kasprzyk, 2021). The muscle n-3 PUFA/n-6 PUFA also decreased with increased dietary inclusion of *d*TM, probably due to the significantly increase of LA in fish fed the CTRL and TM100 diets. According to EFSA (2010), there are still no reference values for this ratio in human diets, but recent data have demonstrated that increased dietary intake of LA is associated with a reduced incidence of CVD and type 2 diabetes in humans (Marangoni et al., 2020). As the name suggests, AI and TI are also indicators of the relationship between pro-atherogenic and anti-atherogenic FA, and pro-thrombogenic and anti-thrombogenic FA, respectively (Ulbricht and Southgate, 1991). Diets with low index values (AI <1.0 and TI < 0.5) are recommended to reduce the risk of CVD (Fernandes et al., 2014). Despite of the increased indexes in the muscle of European sea bass fed *d*TM diets in comparison to those fed CTRL (AI: 0.54 vs 0.60–0.65; TI: 0.09 vs 0.13–0.15, respectively), values were largely below the tolerable upper intake level (UL) for a human healthy diet (Fernandes et al., 2014). To the best of our knowledge, there are no recommended values for h/H ratio for human consumption, but the results obtained in the present study are in line with those obtained by Testi et al. (2006) for farmed European sea bass.

Fish texture is one of the most important freshness quality attributes of fish. Among textural attributes, firmness (i.e., hardness) is one of the most appreciated attributes by consumers, but was not affected by *d*TM dietary inclusion. However, muscle of fish fed TM100 had the highest percentage of small fibres (< 25 µm), and the lowest percentage of large-sized fibres (> 125 µm). Despite all fish grew equally well, and reached similar final body size, these results suggest a particularly high potential of *d*TM diets to promote further muscle growth by fibre hypertrophy which may impact texture (Valente et al., 2013). Beyond texture, it is well known that skin and fillet colour are also important fish quality parameters, since they are directly perceived by the consumer, having a key role on the purchasing decision (Erdağ and Ayvaz, 2021). The dietary inclusion of *d*TM does not seem to have a major impact on sea bass muscle colour, and in skin, only H° value changed with dietary inclusion of *d*TM. H° correspond to the colour tone or colour name of a colour, and despite observed changes in skin, both values (142° in CTRL and 130° in TM100) belong to the discrete category of green (Sun et al., 2018).

To the best of our knowledge, the impact of dietary inclusion of IM on European sea bass sensory profile was never assessed before, but the results of the present study demonstrated that consumers accepted very well all fish samples. In Atlantic salmon

(*Salmo salar*), although trained panellists detected small changes in the sensory profile of fish fed diets with 15% HI to totally replace FM, the consumer-liking tests with untrained panellists also revealed high acceptance rates for all fish (Belghit et al., 2019; Bruni et al., 2020a). In the present study, the results of correspondence analysis applied to fish attributes identified by panellists, allowed to conclude that no differences could be perceived among experimental groups. Most attributes were close to the middle of the plot not being possible to form clusters. The sensory panel associated fish fed CTRL with a "whiter" and "pleasant colour", with 86.25% of the variance explained in F1, although no differences were detected in muscle instrumental colour. TM50 and TM100 were associated with a "juicy texture", which is defined as the moisture released in the mouth during early chewing and is considered a relevant freshness criterion (Borgogno et al., 2017). In rainbow trout (*Oncorhynchus mykiss*), untrained panellists also did not perceive differences in the sensory profile of fish fed 36% HI (50% FM protein replacement) (Sealey et al., 2011), but trained panellists detected significant changes in intensity of aroma, flavour and texture of fish fed 40% HI (Borgogno et al., 2017). These different results may be partially related to the selected consumer panel (untrained vs. trained); it is expected that trained panellists outperform untrained panellist in their ability to discriminate subtle differences among samples (Ares and Varela, 2017), but untrained ones are closer to the consumer's reality.

7.5. Conclusions

The present results indicate that the inclusion of *d*TM in diets for market-sized European sea bass does not compromise neither growth performance nor the nutritional, organoleptic and sensory quality of the muscle. Muscle EPA + DHA levels and PUFA/SFA ratio were still above the recommended daily intake of 0.25 g 100 g^{-1} WW and 0.45, respectively, to prevent the risk of CVD. Likewise, the AI and TI indexes were well below the tolerable upper intake level for a healthy diet. Despite some differences in the muscle histomorphometric and instrumental texture measurements, consumers could not perceive differences between muscle samples of fish fed the control or *d*TM diets. All samples were characterized by their soft and pleasant texture, and *d*TM samples were also associated with a "juicy texture". Altogether, these results evidence a great potential of *d*TM to fully replace FM protein in diets for market-sized European sea bass.

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CRediT authorship contribution statement

Ana Basto: Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization. **Alexandra Marques:** Investigation, Formal Analysis, Validation. **Andreia Sousa:** Investigation, Formal Analysis. **Tiago Sá:** Investigation. **Vera Sousa:** Investigation, Formal Analysis, Validation. **M. Beatriz P. P. Oliveira:** Resources, Writing – Review & Editing. **Tiago Aires:** Conceptualization, Writing – Review & Editing, Funding Acquisition. **Luisa M. P. Valente:** Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition.

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Chapter 8.

General discussion, conclusions and future directions

The present chapter aims to integrate the main findings of chapters 2 to 7, as well as build a bridge between the general and specific objectives previously delineated for this thesis. It was hypothesized that if insect meal (IM) was included in a well-balanced diet for European sea bass, fishmeal (FM) could be replaced without affecting feed intake, and digestibility, ensuring fish growth and flesh nutritional value. To test this hypothesis, three specific objectives were established. The first one focused on the characterization of the nutritional value and *in vivo* determination of the bioavailability of different IM available in the market (chapter 2). The second aimed to identify the maximum dietary FM replacement by IM without affecting feed intake regulation (chapter 3 and chapter 5), nutrient digestibility, assimilation, gut integrity (chapter 4 and chapter 7) and metabolism (chapter 6). The third objective intended to evaluate the impact of IM on the nutritional, organoleptic and sensorial quality of market-sized European sea bass (chapter 4 and chapter 7).

European sea bass is one of the most important fish species in Europe, particularly in Mediterranean aquaculture. In 2019, 219 million tonnes of this fish species were produced in Mediterranean countries valued at more than 2 billion euros (EUR) (FAO, 2022). However, despite the increasing share of FM produced from fish by-products in aquafeeds, formulations for European sea bass still rely, at least partially, on FM inclusion from whole small pelagic fish species. It is well known that fish stocks tend to be overexploited and the reliance on by-products is not enough to sustain the increasing growth of aquaculture. Thus, it is of paramount importance to find high-quality alternative ingredients to FM (Cottrell et al., 2020; Naylor et al., 2021). In 2017, the inclusion of insect protein from 7 insect species in aquafeeds was authorized in the EU (European Commission, 2017), rising interest in such protein sources. But as for all new ingredients, the feasibility of using IM as an FM substitute in aquafeed formulations, particularly for carnivorous marine fish species, like European sea bass, remains largely underexplored (Makkar et al., 2014; Henry et al., 2015).

In 2007, Glencross et al. (2007) identified five key steps for assessing the quality of new ingredients, namely, (1) characterization; (2) palatability; (3) digestibility; (4) utilization; and (5) functionality. Since then, there has been extensive development, not only related to ingredient evaluation but also final users' expectations. Consequently, two additional steps, (6) processing and (7) product quality influences, were recently proposed by Glencross (2020). In this connection, to better understand the feasibility of using IM as a protein source in diets for European sea bass, this thesis was developed considering the following steps: characterization, palatability, digestibility, utilization, and product quality.

The first key step for assessing IM quality was tackled in chapter 2. The nutritional composition of four easily accessible IM in the European market, *Hermetia illucens* (HI) and *Tenebrio molitor* larvae meal, both full fat (HI and TM, respectively) and defatted (*d*HI and *d*TM, respectively) was analysed. Additionally, an IM obtained from *Locusta migratoria* (LM) was also characterized for comparison. Although LM was less available in the European market, it aroused interest because European Commission (EC) was evaluating its potential for human consumption at the request of one of the most developed insects producing companies in Europe (Protix, The Netherlands), which was approved later in 2021 (EC, 2021). The nutritional characterization of HI, *d*HI, TM, *d*TM and LM followed standardized methods (AOAC, 2006), including mandatory variables like crude protein, crude fat, ash, moisture and gross energy, but also other key compositional parameters, such as amino acid (AA) profile, phosphorus, crude, acid and detergent fibre, and chitin.

It is well-known that the sum of total AA (true protein) is the most accurate method to determine the protein content of a food/feedstuff (Maehre et al., 2018). The determination of the total AA content of food/feedstuffs consists of two steps: (1) hydrolysis of protein or peptides for AA release, and (2) their sub-sequential analysis and quantification. The first step is the most critical since the hydrolysis degree has a direct impact on the accuracy and precision of results. Although there is no official method for protein/peptide hydrolysis, many laboratories use 6 M HCl at 110 ºC for 20-24h to hydrolyse the peptide bond between adjoining AA. However, different food/feed matrices require different hydrolysis conditions for the optimal release of different AA (Maehre et al., 2018). In this connection, before the analysis of the AA content of IM tested in this thesis, different hydrolysis periods were tested to guarantee the maximum yield, which was obtained with 6 M HCl at 110 ºC for 72h. Such methodological optimisation has not been published (Aragão, personal communication) but has been used thereafter (Basto et al., 2020; Basto et al., 2021b; Basto et al., 2021a).

Due to the high costs associated with total AA quantification, the protein content of feedstuffs has mostly been based on nitrogen determination with subsequent conversion using a nitrogen-to-protein conversion factor (Kp) of 6.25 (crude protein). However, the use of this Kp has been extensively discussed and compared to other lower factors due to the presence of non-protein nitrogen in insects, to avoid protein overestimation (Janssen et al., 2017; Belghit et al., 2019; Boulos et al., 2020; Oonincx and Finke, 2021). In chapter 2, the protein content of the tested IM was calculated not only using the Kp of 6.25 but also using two other specific Kp values previously suggested by Janssen et al. (2017) for HI and TM whole larvae protein isolates (4.76 and 5.60, respectively). The results were

hence compared with those obtained considering the sum of amino acids (AA) (true protein). The use of Kp = 6.25 resulted in a 1-4% protein overestimation for all IM tested (Figure 8.1).

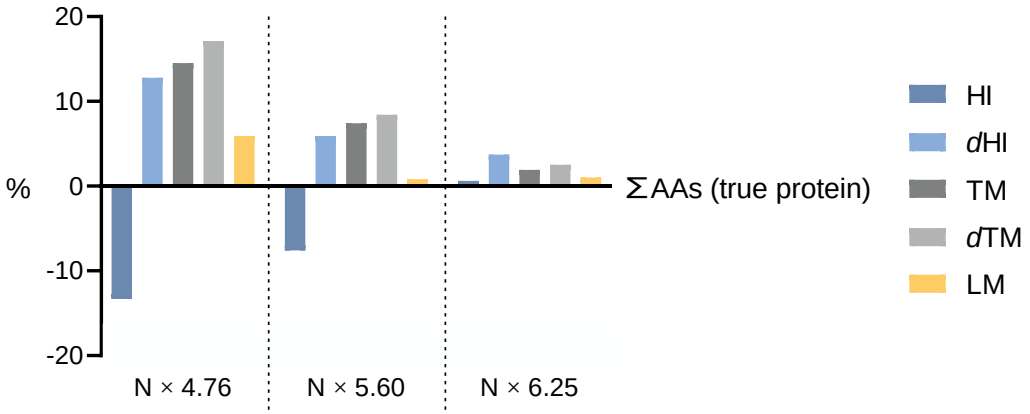

Figure 8.1. Percentage of difference between the true protein content of IM tested in chapter 2, and their protein content calculated using Kp values of 4.76, 5.60 and 6.25.

For HI, *d*HI, TM and *d*TM, the use of Kp = 4.76 and Kp = 5.60 resulted in 15-17% and 6-8% of protein underestimation, respectively (Figure 8.1). In the case of LM, differences were smaller: the use of Kp = 4.76 resulted in a protein underestimation of 6%, whereas the use of either Kp of 5.60 or 6.25 resulted in an overestimation of 1% (Figure 8.1). In fact, the calculation of the specific Kp for each IM results in values varying from 6.30 to 6.72 (Table 8.1). Although these results rely on a single sample from each species and processing treatment, the obtained data clearly demonstrate that Kp values depend on the species considered, being 6.25 the most appropriate nitrogen-to-protein conversion factor for all IM tested.

Table 8.1. Specific Kp values for IM tested in chapter 2, calculated from the ratio of the sum of AA (g 100 g^{-1}) to total nitrogen content (g 100 g^{-1}).

	HI	***d*HI**	**TM**	***d*TM**	**LM**
Kp_{HI}	6.72				
Kp_{dHI}		6.32			
Kp_{TM}			6.49		
Kp_{dTM}				6.41	
Kp_{LM}					6.30

Oonincx and Finke (2021) have recently reviewed the AA profile of 20 samples from 13 insect species (both wild harvested and produced), including different developmental stages, and recalculated the specific Kp for each one, resulting in Kp values varying from 4.56 to 6.45. These findings are in line with those obtained in chapter

2, confirming that despite a Kp of 6.25 often slightly overestimates insects' protein content, it is probably the most appropriate conversion factor when several species and/or different developmental stages are being compared.

The IM currently available on the market have a large variation in fat content, even when they are obtained from the same insect species. As observed in chapter 2, full-fat and defatted HI obtained from different producers had similar fat content values (20% on a DM basis). However, information about the rearing substrates or technological processes applied to these IM was not provided by the producers. Generally, variations in composition occur due to the lack of standardization of insect rearing methods and processing techniques. The optimization of some steps, namely those related to defatting technologies, is still warranted in many cases. Fat removal may be accomplished in many ways, such as through mechanical pressure, aqueous extraction, specific solvent extraction methods, pressurized-solvent extraction, and supercritical CO_2 extraction. The specific Soxhlet solvent extraction method has been the most used at the laboratory scale (El Hajj et al., 2022). However, even the Soxhlet method is not yet optimized since the best organic solvent or combination of solvents still is under evaluation. Ribeiro et al. (2019) reported that ethanol, followed by acetone had the highest lipid extraction yield when compared to diethyl ether, ether petroleum, and hexane. However, Laroche et al. (2019) demonstrated that ethanol was less effective at extracting non-polar fatty acids (FA). Therefore, the defatting method should be chosen based on the FA profile of the insects, and considering the combination of different solvents (e.g., hexane/ethanol or hexane/propan-2-ol) (Laroche et al., 2019). Although Soxhlet extraction has been prevalently used to extract fat from insect biomass at a laboratory scale, this process is cost-prohibitive on a large-scale since it requires a long extraction time and a large amount of solvent (López-Bascón and Luque de Castro, 2020). According to Sindermann et al. (2021), lipid extraction through mechanical screw pressure is the most used by industry. However, this method can still be optimized by: (1) the use of conveying screws with a special geometry; (2) a temperature controller to allow a good yield of the process without compromising protein quality and stability; and (3) the use of open evaporation screws or counter current heat exchangers to cool the insect press cakes immediately after pressing.

The presence of chitin has been associated with an enhancement of the immunomodulatory and antioxidant status of fish, as well as modulation of gut microbiota (Bruni et al., 2018; Weththasinghe et al., 2021a). But chitin has also been related to (1) decreased protein digestibility due to its capacity to bind proteins and immobilize or reduce the activity of proteolytic enzymes (Marono et al., 2015); (2) decreased fat

digestibility due to the suppression of bile acid level in the pylorus, which is essential for activation of lipase and efficient FA absorption (Hansen et al., 2010). Thus, more studies focused on optimal dietary insect-chitin inclusion levels merit further consideration. In chapter 2, it was possible to observe that TM and *d*TM had the lowest chitin content (5% on a DM basis) and the highest protein and fat apparent digestibility coefficient (ADC) values (89-93% and 94-95%, respectively) among all IM tested (HI, *d*HI, TM, *d*TM and LM). These results not only suggest great potential of TM and *d*TM for further incorporation into aquafeeds but also highlight the importance of selecting technologies that allow the production of IM-protein biomass with low chitin content. Decreasing the chitin content of IM can be achieved through (1) de-chitinization processes; or (2) selection of the most appropriate insects' harvesting time. According to Sindermann et al. (2021), the hard exoskeleton of insects, containing chitin, can be separated from the soft body through the use of a mechanical soft separator. This technology is already applied in the meat, poultry, fish, and shrimp industries to separate the soft fraction from harder bones or shells. Another method of de-chitinization is enzymatic hydrolysis. However, both the use of a soft separator and enzymatic hydrolysis represent additional costs to the industry. A cost-effective alternative to de-chitinization may be the identification/selection of the most appropriate time to harvest insects. According to Yu et al. (2021), the higher the number of instars of *T. molitor* larvae, the higher their chitin content, and the lower their protein content. Thus, the authors suggested that a good harvest time could be when the larvae are still young (i.e., before the 9^{th}-10^{th} instars stage). However, the number of insects' instars varies greatly with abiotic (e.g., temperature, humidity, oxygen) and biotic (e.g., population density, parental age, feed quality) factors; therefore, controlling the life cycle of each species and standardizing its cultivation conditions is crucial to being able to harvest them at the most appropriate time.

After the nutritional characterization of the five selected IM (HI, *d*HI, TM, *d*TM and LM), LM was identified as the test ingredient with the lowest potential to be incorporated into aquafeeds as an FM substitute, due to its low protein content (24% on a dry matter (DM) basis), poor essential AA (EAA) profile and high crude fibre levels. On the other hand, *d*TM was the only IM with a higher protein content (68.9% *vs* 60.2%) than a high-quality anchovy meal (5-01-985; NRC, 2011). Yet, *d*TM presented 17% less lysine (Lys) and 46% less methionine (Met), two essential amino acids (EAA), than anchovy meal (39 vs 47 mg g^{-1} DM and 10 vs 18 mg g^{-1} DM, respectively) (chapter 2). Contrarily, *d*TM presented 37% more Lys and 20% more Met than soybean meal (5-04-612; NRC, 2011) (39 vs 25 mg g^{-1} DM and 10 vs 8 mg g^{-1} DM, respectively), the most widely used alternative plant protein source to FM. Nonetheless, the nutritional characterization of an

ingredient *per si* is still not enough to assess its full potential as a nutrient source for a species. The evaluation of an ingredient's digestibility is crucial as will reflect the capability of an animal to use its various nutrients (NRC, 2011). Assessing the protein quality of a certain ingredient on a digestible protein/AA basis, rather than its crude basis, has been encouraged in literature (NRC, 2011), since nutrient ADC varies from species to species (due to physiological differences), and can be affected by a panoply of environmental factors. The classical approach consists in comparing a reference diet (containing an inert digestibility marker) with an experimental diet where 30% of the dietary mixture is replaced by the target ingredient (Belal, 2005; NRC, 2011). In chapter 2, a 20% insect inclusion level was selected instead of 30% to avoid technical difficulties during the extrusion of diets due to the high fat content of certain IM. The obtained results demonstrated that European sea bass protein digestibility was high for *d*TM (93%), intermediate for *d*HI and TM (87 and 89%, respectively), and moderate for HI and LM (76 and 74%, respectively). In fact, *d*TM was the ingredient with a protein digestibility closest to that reported for Premium Norwegian FM Lt-94 produced from whole blue whiting (94%; Davies et al., 2009). Besides, *d*TM also had the highest digestibility of EAA (93%). Since Lys and Met are the most limiting EAA in diets for European sea bass, an accurate estimation of these two AA bioavailability is of paramount importance to formulate equilibrated aquafeeds for this species. In chapter 2, ADC values of Lys (93%) were lower in European sea bass fed *d*TM than in fish either fed Premium Norwegian FM Lt- 90 or fish by-products meal (98 and 99%, respectively)(Davies et al., 2009; Campos et al., 2018). But *d*TM had the highest digestible amount of Lys (36 mg g^{-1} of DM) among all tested IM in the scope of chapter 2. Regarding Met, *d*HI displayed higher a digestible amount of this AA than *d*TM (13 and 10 mg g^{-1} of DM, respectively). However, ADC of Met in European sea bass fed *d*TM (100%) was equivalent to that observed in fish fed Premium Norwegian FM Lt- 90, and even higher than in those fed fish processing by-product meal (100 and 90%, respectively) (Davies et al., 2009; Campos et al., 2018). Finally, *d*TM had the highest amount of digestible arginine, histidine, isoleucine, leucine, valine, and phenylalanine (46, 22, 33, 52, 43 and 29 mg g^{-1} of DM, respectively).). Altogether, these results suggest that *d*TM is the most promising IM to replace FM in European sea bass diets.

In this thesis, the control diet was not the same in all experiments, and IM replaced fish meal at different levels. This information is summarised in Table 8.2. The control diets differed between studies as were formulated with different FM and PP inclusion levels. In the first control diet (CTRL.1), FM was included at 45% and PP at 23%. So, 66% of the total dietary protein was provided by FM (FM_{66}). In the second control diet (CTRL.2), a

slightly lower FM (40%) and higher PP (28.5%) level was used; in this case, 58% of the total dietary protein was provided by FM (FM_{58}), the rest being provided by PP (42%). The experimental diets were formulated to include 18, 20 and 36% *d*TM (40%, 50% and 80% FM replacement, respectively), contributing with 26, 30 and 53% to the total dietary protein (IM_{26}, IM_{30} and IM_{53}, respectively). Finally, total FM replacement was achieved using two approaches: the first diet (TM100.1) was formulated to totally replace FM by 40% *d*TM; in this case *d*TM provided 59% of the total dietary protein (IM_{59}), the rest being provided by PP sources (41%); in a second trial, a more radical diet (TM100.2) was formulated, where *d*TM was included at 60% being the single protein source (IM_{100}). All diets contained 12.5-14% fish oil (FO). To better differentiate diets and experimental trials, the contribution of either FM or IM to total protein dietary content will be used onward (FM_{66}, FM_{58}, IM_{26}, IM_{30}, IM_{53}, IM_{59} and IM_{100}).

Table 8.2. Different dietary formulations tested in each chapter of the thesis and new names based on the contribution of either FM or IM to total dietary protein content.

Diets used in each chapter	CTRL.1	CTRL.2	TM40	TM50	TM80	TM100.1	TM100.2
New designation of each diet	**FM_{66}**	**FM_{58}**	**IM_{26}**	**IM_{30}**	**IM_{53}**	**IM_{59}**	**IM_{100}**
Ingredients (%)							
Fishmeal[1]	45.0	40.0	27.0	20.0	9.0	-	-
Insect meal[2]	-	-	18.0	20.5	36.0	40.4	60.0
Soy protein concentrate[3]	8.0	10.5	8.0	10.5	8.0	10.5	-
Soybean meal[4]	10.0	13.0	10.0	13.0	10.0	13.0	-
Rapeseed meal[5]	5.0	5.0	5.0	5.0	5.0	5.0	-
Dietary protein provided by FM	**66**	**58**	40	29	13	0	0
Dietary protein provided by IM	0	0	**26**	**30**	**53**	**59**	**100**
Dietary protein provided by PP	34	42	34	41	34	41	0
Wheat meal[6]	17.0	16.2	16.8	15.2	15.5	14.3	24.0
Fish oil[7]	13.0	14.0	13.0	13.3	13.0	12.5	12.1
Vitamin and mineral premix[8]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin C	-	0.1	-	0.1	-	0.1	-
Vitamin E	-	0.1	-	0.1	-	0.1	-
Binder	1	-	1	-	1	-	1
Monocalcium phosphate	-	-	-	1.0	1.1	2.0	1.3
L-Lysine	-	-	-	-	-	0.2	-
L-Threonine	-	-	-	-	-	0.2	-
L-Tryptophan	-	-	-	-	-	0.1	-
DL-Methionine	-	0.1	0.2	0.2	0.4	0.3	0.6
Chemical composition (% DM)							
Dry matter	93.5	93.1	94.3	92.6	93.2	92.5	94.2
Protein	47.8	46.9	47.7	47.3	47.0	47.2	50.6
Lipids	18.7	19.7	19.2	19.8	20.4	19.0	22.1
Gross energy (kJ g^{-1} DM)	22.0	23.2	22.6	23.5	23.4	24.0	24.5
DP:DE (mg kJ^{-1} DM)	22.8	21.4	22.5	21.0	20.9	20.2	20.9
Ash	11.7	10.2	9.0	8.1	7.4	6.3	6.5
Phosphorus	1.4	1.2	1.1	1.2	1.0	1.0	1.0

The abbreviations for the experimental diets used in each chapter stand for CTRL, control diet; TM40, TM50, TM80 and TM100, diets with 40, 50, 80 and 100% fishmeal replacement by insect meal, respectively. The new designation for each diet stands for: FM_{66} and FM_{58}, experimental diets with 66 and 58% of the total dietary protein provided by fishmeal, respectively; IM_{26}, IM_{30}, IM_{53}, IM_{59} and IM_{100}, experimental diets with 26, 30, 53, 59 and 100% of the total dietary protein provided by insect meal. Other abbreviations stand for DP:DE, digestible protein to digestible energy ratio; DM, dry matter; FM, fishmeal; IM, insect meal obtained from defatted *Tenebrio molitor* larvae. [1] Peruvian fishmeal super prime: 71% crude protein (CP), 11% crude fat (CF), Exalmar, Peru; [2] defatted *Tenebrio molitor* larvae meal (*d*TM) obtained from different producers (*d*TM used in FM_{66}, IM_{26}, IM_{53}, IM_{100}: 65% CP, 12% CF, 4.8% chitin; dTM used in FM_{40}, IM_{30} and IM_{59}: 71% CP, 12% CF, 5.5% chitin); [3] Soy protein concentrate: 65% CP, 0.7% CF, ADM Animal Nutrition, The Netherlands; [4] Soybean meal 48: dehulled solvent extracted soybean meal: 48% CP, 2% CF, Cargill, Spain; [5] Rapeseed meal: 36% CP, 3% CF, PREMIX Lda., Portugal; [6] Wheat meal: 10% CP, 1% CF, Casa Lanchinha, Portugal; [7] Sardine oil, Sopropêche, France; [8] Vitamin and mineral premix: WISIUM, ADM Portugal S.A., Portugal

After selecting the most appropriate IM for European sea bass, the next obvious key step was the evaluation of its palatability when included in diets for this species. In chapters 3 and 5, it was evaluated for the first time the impact of increasing dietary levels of *d*TM on fish voluntary feed intake (VFI) tackling the underlying regulation mechanisms displayed at the central level. In the long-term trial (after 10 weeks of feeding), the expression of key neuropeptides involved in orexigenic or anorexigenic responses

(stimulation and inhibition of appetite, respectively) was not affected when European sea bass were fed IM_{26} or IM_{53} diets, resulting in similar VFI and weight gain among dietary treatments. The mechanisms responsible for the regulation of feed intake at the central level mainly depend on fish energy status (Soengas et al., 2018). Therefore, fish may begin to adapt to a certain diet but the ability to distinguish its effect on palatability becomes reduced in the long term, *i.e*. after weeks, months or years (Glencross, 2020). Thus, it is also important to evaluate the homeostatic response of fish to new dietary formulations in the short- and mid-term, which was evaluated in chapter 5. After the first single meal exposure of European sea bass to the experimental diets (short-term trial), or after 7 days of feeding (mid-term trial), neither IM_{30} nor IM_{59} (50 and 100% FM replacement, respectively) affected the expression of orexigenic and anorexigenic neuropeptides responsible for the homeostatic regulation of feed intake. However, in these trials VFI tended to decrease in fish fed both IM_{30} and IM_{59}; this tendency was more pronounced in IM_{30} (50% FM replacement). Such results were confirmed in a further 16 week feeding trial using the same dietary formulations, where VFI decreased in fish fed IM-based diets, but only IM_{30} significantly differed from the control (chapter 7). These results were not expected as no differences could be observed when European sea bass were fed with IM_{26} and IM_{53} diets (40 and 80% FM replacement, respectively). These differences among studies might, however, be explained by nutritional differences among diets due to distinct formulations. In fact, it has been reported that some AA and FA have a high influence on the sensing mechanisms involved in the feed intake regulation of fish (Velasco et al., 2017; Comesana et al., 2018). Although the AA profile of the diets tested in chapter 5 was not analysed, the theoretical value for each AA was calculated and is presented in Table 8.3. Overall, in IM_{30} (50% FM replacement) almost all AA were present in lower amounts (g/100 g crude protein) than in diets IM_{26} and IM_{53} (40 and 80% FM replacement, respectively). Hence, it cannot be discarded that these variations, may have influenced parameters related to the hedonic regulation of feed intake, resulting in decreased VFI in fish fed with the diet with the poorest AA profile (IM_{30}). Another aspect that deserves to be mentioned is that contrarily to the control (FM_{58}) and IM_{30} diets (0 and 50% FM replacement), the IM_{59} (100% FM replacement) was supplemented with L-Lys, L-threonine and L-tryptophane. This supplementation might have resulted in higher bioavailability of those AA in IM_{59}, resulting in activation of the hedonic pathway, enhancing the palatability of IM_{59}, and ultimately resulting in similar VFI between fish fed IM_{59} (100% FM replacement) and those fed FM_{58} and IM_{30} (0 and 100% FM replacement). However, it is extremely important to underline that these are just assumptions, and more studies are needed to better understand the impact of IM-based diets on parameters involved, not only in the homeostatic but also in the hedonic regulation of European sea

bass. Indeed, aquafeed is one of the foremost expenses of the aquaculture industry (50-70% of variable costs). Deepening knowledge about the impact of IM-based diets on hedonic regulation of feed intake would be of high interest for the proper management of feeding. This knowledge would allow: (1) to maximize production efficiency/profitability; (2) to reduce the environmental issues related to nutrients losses into the water (Glencross, 2021).

Table 8.3. Practical ([a]) and theoretical ([b]) amino acid profile (% crude protein) of experimental diets tested in this thesis.

	CTRL.1[a]	CTRL.2[b]	TM40[a]	TM50[b]	TM80[a]	TM100.1[b]	TM100.2[a]
	FM_{66}	**FM_{58}**	**IM_{26}**	**IM_{30}**	**IM_{53}**	**IM_{59}**	**IM_{100}**
Amino acids (% crude protein)							
Arginine	7.9	5.7	7.8	5.3	8.1	4.8	7.6
Histidine	2.3	2.2	2.9	2.2	3.0	2.2	3.2
Lysine	6.9	6.5	5.9	5.8	5.5	5.4	6.8
Threonine	3.6	3.7	4.2	3.4	3.8	3.4	3.8
Isoleucine	4.4	3.9	4.8	3.7	4.9	3.5	5.1
Leucine	7.9	6.4	7.3	6.4	7.7	6.3	7.8
Valine	5.0	4.5	5.5	4.4	6.2	4.3	7.4
Methionine	3.1	2.5	2.9	2.5	2.8	2.5	3.4
Phenylalanine	5.2	4.5	4.8	4.0	4.7	3.5	4.4
Cystine	0.6	0.8	0.6	1.0	0.6	1.1	0.6
Tyrosine	5.0	3.2	5.5	3.4	6.2	3.6	7.6
Aspartic acid + Asparagine	8.6	8.5	8.4	8.1	8.3	7.7	8.9
Glutamic acid + Glutamine	16.3	13.7	13.4	13.1	13.8	12.5	13.1
Alanine	5.2	4.7	5.9	4.9	6.2	5.0	7.4
Glycine	6.7	5.1	6.9	4.5	6.8	3.8	7.0
Proline	6.9	4.4	6.3	4.7	6.2	4.9	7.4
Serine	4.0	4.6	4.2	4.5	4.0	4.5	3.6
Taurine	*0.4*	*0.5*	*0.4*	*0.5*	*0.1*	*0.4*	*0.1*

The abbreviations for the experimental diets used in each chapter stand for: CTRL, control diet; TM40, TM50, TM80 and TM100, diets with 40, 50, 80 and 100% fishmeal replacement by insect meal, respectively. The new designation for each diet stands for: FM_{66} and FM_{58}, experimental diets with 66 and 58% of the total dietary protein provided by fishmeal, respectively; IM_{26}, IM_{30}, IM_{53}, IM_{59} and IM_{100}, experimental diets with 26, 30, 53, 59 and 100% of the total dietary protein provided by insect meal.

After selecting the most appropriate IM for European sea bass and evaluating its impact on the regulation of feed intake at the central level, the next key step was the evaluation of the impact of a partial or total FM replacement by *d*TM on fish nutrient utilization. For that purpose, growth performance and underlying mechanisms involved in nutrient intermediary metabolism were evaluated, both in juveniles and market-sized European sea bass (chapters 4, 6 and 7). In both chapters 4 and 7 it was clearly demonstrated that when *d*TM contributed with 59 or 100% of the dietary protein (totally replacing FM), the growth performance and feed efficiency of European sea bass were not impaired after 10-16 weeks of feeding. To the best of our knowledge, the studies conducted in the scope of this thesis are the only ones where so high percentages of dietary protein derived from IM (59 and 100%) were tested in diets for this marine species. This corresponds well with previous studies by Biasato et al. (2022) where *d*HI provided 57% of the dietary protein, also totally replacing FM, without impairing rainbow trout

growth performance or feed efficiency. Besides, Rema et al. (2019) demonstrated that total FM replacement by *d*TM even improved feed efficiency and growth performance of rainbow trout after 90 days of feeding; but in this case, IM only contributed with 35% of the dietary protein. Among all studies summarized in section 1.2.4. of chapter 1, fish growth impairment was reported by Fabrikov et al. (2021), Gasco et al. (2016), Lock et al. (2016), Reyes et al. (2020), St-Hilaire et al. (2007) and Weththasinghe et al. (2021b). But it is important to note that amongst the 23 growth trials conducted with HI (either full-fat or defatted) as FM substitute, 8 resulted in growth impairment, whereas among the 11 growth trials relying on TM (either full-fat or defatted), only 2 caused a diminished final body weight compared to fish fed the control diets (section 1.2.4. of chapter 1). Despite direct comparisons between studies being difficult due to the existence of diverse variables (e.g., fish and insect species, fish and insect stage of development, insect rearing substrate, dietary formulation, design of the experimental trial, etc.), altogether these results evidenced the great potential of TM (either full-fat or defatted) as an alternative protein source to FM.

Over the last decades, an effort has also been made towards the reduction of fish oil in aquafeeds. It would hence be important to further evaluate the dietary inclusion of *d*TM as FM replacement in more practical diets relying on a blend of oils as lipid sources. This is particularly relevant in marine fish species, like European sea bass, that have a limited capacity to biosynthesize n-3 long-chain polyunsaturated FA (n-3 LC-PUFA) due to low activity of key endogenous enzymes like the Δ5 desaturase. In freshwater fish species, such as rainbow trout, some authors already tested the FM replacement by IM in dietary formulations where a blend of FO and vegetable oils (VO) were used, and the growth performance of fish was not compromised (Cardinaletti et al., 2022; Oteri et al., 2022).

In chapters 4 and 7, it was observed that levels above 40% of FM replacement by *d*TM resulted in a reduction of protein digestibility, but ADC values of diets were still high (89-92%). These results were not expected since in chapter 2 it was observed that the protein ADC value of *d*TM was similar to that obtained for FM by Davies et al. (2009). However, since the *d*TM used in each experimental trial was obtained from different producers, the different results of protein digestibility might be related to (1) different methods of rearing and/or processing of *d*TM, emphasizing once again the need to standardize these processes; (2) chitin content of experimental diets. As abovementioned, chitin has been associated with decreased protein digestibility. On the one hand, and despite the basal mixture of the experimental diet tested in chapter 2 and experimental diet with 50% FM replacement by *d*TM (IM_{30}) having the same dietary inclusion level of

*d*TM (20%), the IM_{30} included a *d*TM with higher chitin content than that included in the basal mixture of experimental diet tested in chapter 2 (5.5% vs 4.8%, respectively). On the other hand, experimental diets with 80% (IM_{53}) and 100% (IM_{59} and IM_{100}) FM replacement by *d*TM included higher levels of *d*TM than that included in the basal mixture of experimental diet tested in chapter 2 (20% *d*TM inclusion level), which consequently results in increased chitin levels. Despite the observed impairment of protein digestibility in diets with *d*TM providing 30-100% of the dietary protein, the integrity of the anterior intestine remained well preserved, without major morphological changes, apart from submucosa thickening. Nonetheless, in the long-term, such effects cannot be overlooked and should be further explored as may impact the immunological response of fish at the local level (chapter 4). Moreover, the retention efficiency of digested nitrogen (N) increased with *d*TM dietary inclusion, whereas its metabolic losses decreased, resulting in similar N gain and final whole-body protein content between *d*TM and FM diets, not compromising fish growth.

It is well-known that fish feed formulation targets the supply of the minimum protein requirements of a species coupled with an appropriate non-protein energy source to spare the use of proteins for growth. Thus, an optimal digestible protein to digestible energy (DP:DE) ratio is essential for optimal/maximum fish growth. Any reduction of this ratio by decreasing the dietary DP level at constant or increased DE would improve protein utilization and decrease N losses. According to Kousoulaki et al. (2015), dietary DP:DE ratios reported in the literature for European sea bass juveniles smaller than 100 g varied between 20.5 and 26.5 mg protein kJ^{-1} without any pattern about growth. On the other hand, an optimal DP:DE ratio of 22-26 mg kJ^{-1} was suggested for European sea bass between 100 g and 300 g, and down to 19-21 mg kJ^{-1} for fish above 300 g (Figure 8.2). Thus, despite the previously discussed decrease in protein digestibility in diets with *d*TM providing 30-100% of the dietary protein, all tested dietary treatments provided DP:DE ratios between 20.2-22 mg kJ^{-1}, which are within the range of values recommended for optimal/maximum growth of European sea bass. Besides, all experimental diets tested in the present thesis were properly formulated to meet the EAA requirements of European sea bass. All dietary treatments containing IM were supplemented with DL-Met whilst diet IM_{59} (100% FM replacement) was also supplemented with L-Lys, L-threonine and L-tryptophane. Altogether, this might at least partially explain the similar growth among fish fed different *d*TM inclusion levels.

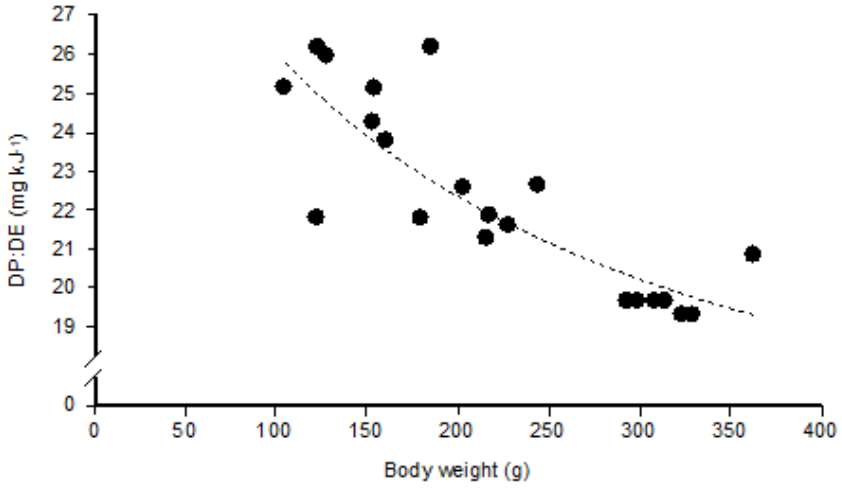

Figure 8.2. Digestible protein to digestible energy (DP:DE) ratio of high-performing European sea bass above 100 g of body weight (20-26 ºC). Adapted from Kousoulaki et al. (2015).

Fat digestibility was high (96-98%) for all diets containing IM, but some incongruent results were observed in this thesis. Fat digestibility decreased in European sea bass fed diets with *d*TM providing 30 and 59% of the dietary protein (50 and 100% FM replacement, respectively) (chapter 7), but did not vary when *d*TM provided 53% of the dietary protein (80% FM replacement); fat digestibility even increased when *d*TM was included as a single dietary protein source (chapter 4). Since fat digestibility values were alike between control diets in each trial, even though different feeding/faeces collection protocols and rearing temperatures were used, different results among IM diets probably occurred due to the different quality of *d*TM used. In fact, the *d*TM used in each trial was obtained from a different producer, emphasizing once again the need to optimize and standardize insect rearing production methods and processing technologies. Despite the discrepancies abovementioned, the present thesis it was demonstrated that total FM replacement by *d*TM, improved lipid retention efficiency and reduced losses, regardless of the level of IM contribution to dietary protein. This trade-off resulted in increased lipids gain and augmented whole-body lipid content (chapters 4 and 7). Thus, altogether the results of lipid balance in fish fed *d*TM-based diets suggest alterations in their intermediary metabolism. These alterations were further confirmed, not only through the analysis of the expression of lipid metabolism-related genes (chapters 4 and 6) but also through the analysis of key enzymes involved in this metabolic pathway (chapter 6). It was clearly demonstrated that total FM replacement by *d*TM induced *de novo* synthesis of FA and inhibits β-oxidation. This resulted in increased triacylglycerides (TAG) levels in the liver, in the mid-term (after 7 consecutive days of feeding), and increased plasma levels in the long-term (after 16 weeks of feeding), suggesting TAG mobilization for other tissues.

Altogether, these results confirm that high levels of dietary protein provided by *d*TM to totally replace FM strongly affect lipids metabolism in European sea bass.

In fish, as in mammals, the activities of aspartate aminotransferase and alanine transaminase enzymes (Ast1 and Aat1, respectively) are indicators of tissue injury or hepatotoxicity and the metabolic status of fish. Generally, the activity of these enzymes increases to meet the animals' high energy needs during pre- or stress conditions, whereas their decreased activity indicates that animals don't need to use AA to obtain energy, promoting a protein sparing effect (Hoseini et al., 2012; Gwaltney-Brant, 2016; Herrera et al., 2019). The high activity of the glutamate dehydrogenase enzyme (Gdh) has been associated with the enhancement of protein metabolism and growth (Liu et al., 2012). In chapter 6, the hepatic activity of Ast1, Aat1 and Gdh did not change in fish fed diets with *d*TM providing up to 30% of the dietary protein (50% of FM replacement), being a signal of a healthy liver and equilibrated metabolism. However, when FM was totally replaced by *d*TM (*i.e*., 59% of the dietary protein provided by *d*TM) the activity of Aat1 and Gdh was reduced. If on the one hand, the decreased activity of Aat1 may suggest a protein sparing effect, on the other hand, its reduced activity coupled with the diminished activity of Gdh, may suggest insufficient amount of some AA when high levels (> 50%) of dietary protein are provided by *d*TM. This may compromise protein deposition in fish in the long-term, and ultimately lead to growth impairment. In chapter 4, no differences were observed in signalling pathways involving the growth-hormone/insulin-growth-factors (GH/IGF) system in fish fed *d*TM diets, which is in line with the similar growth performance results among fish fed different FM replacement levels by *d*TM. However, the up-regulation of myostatin (*mstn)* coupled with a down-regulation of myoblast determination protein 2 (*myod2)* in the muscle of fish fed diets with levels above 53% of the dietary protein provided by *d*TM indicated an inhibition of muscle growth at the transcriptional level. However, the expression of key transcripts of the ubiquitin proteasome pathway remained unaltered (muscle RING-finger protein-1; *murf1*) or was even down-regulated (muscle atrophy F-box; *mafbx*/*atrogin-1*) when *d*TM was used as a single dietary protein source. Thus, this negative feature seems to have been counter-acted by a compensatory growth mechanism (i.e., inhibition of muscle atrophy) when more than half of the dietary protein was provided by *d*TM. Indeed, in chapter 7, fish fed diets with 59% of the dietary protein provided by *d*TM had the highest percentage of small fibres (< 25 µm) and the lowest percentage of large-sized fibres (> 125 µm), which may indicate a high potential for further muscular growth (Valente et al., 2013).

Finally, after selecting the most appropriate IM for European sea bass and evaluating its impact on the regulation of feed intake at the central level, and on nutrients

utilization (including digestibility, metabolism, and growth), the next key step was to evaluate its impact on nutritional, organoleptic and sensorial quality of muscle of market-sized European sea bass (chapter 7). From a consumer's point of view, fish consumption is associated with health-benefit effects. Since the fat fraction of FM is rich in healthy FA, its replacement by protein sources with low levels of such FA has a major impact on the flesh FA profile of fish. In the present thesis (chapters 4 and 7), it was demonstrated that the FA profile of muscle reflected the FA profile of experimental diets (Figure 8.3 and Figure 8.4), increasing monounsaturated FA (MUFA) and decreasing polyunsaturated FA (PUFA) contents with *d*TM dietary inclusion.

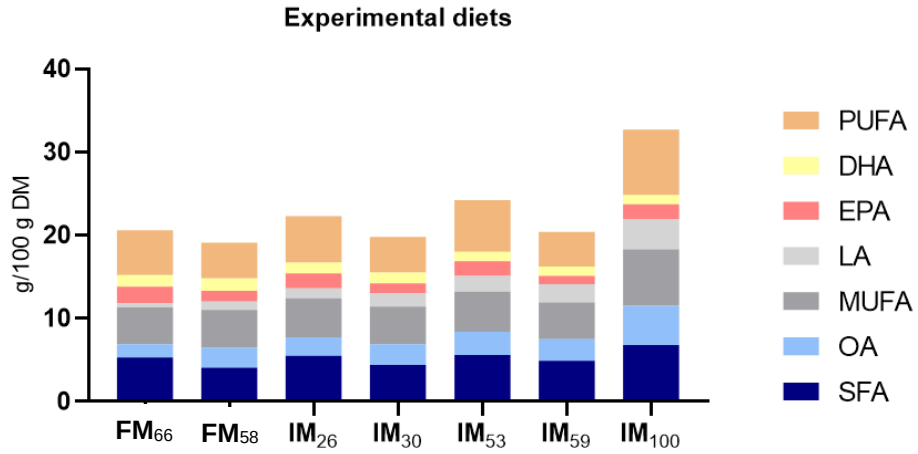

Figure 8.3. Dietary fatty acid proportions in the experimental diets used to test fishmeal replacement.

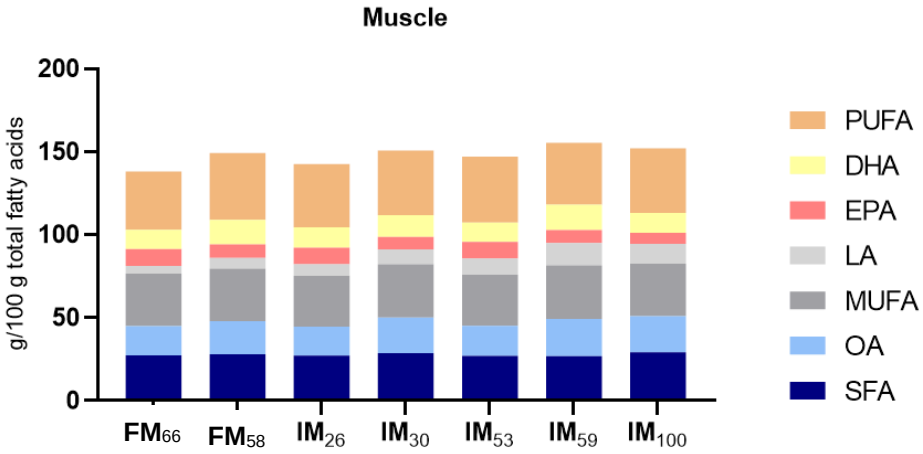

Figure 8.4. Dietary fatty acid proportions in the experimental diets and muscle of fish fed the experimental diets including increasing levels of defatted *Tenebrio molitor* larvae meal as to replace fishmeal.

Despite the dietary *d*TM inclusion resulted in decreased levels of n-3 LC-PUFA, particularly eicosapentaenoic and docosahexaenoic acids (EPA and DHA, respectively) in European sea bass, the sum of EPA and DHA was above the minimum daily intake recommended by EFSA (2010) to prevent the risk of cardiovascular diseases (i.e., 250 mg

100 g^{-1} on a wet weight basis), even in fish fed diets totally devoid of FM. The nutritional value of fish fillets doesn't only depend on the n-3 LC-PUFA content, but also on their relationship with single saturated FA (SFA) and other classes of FA. FA ratios such as PUFA/SFA, n-3 PUFA/n-6 PUFA, atherogenic, and thrombogenic indexes (AI and TI, respectively) have hence gained increasing importance in the evaluation of the nutritional quality and healthiness of dietary lipids for human consumption (Chen and Liu, 2020; Kilar and Kasprzyk, 2021). In chapter 7, it was observed that high levels of dietary inclusion of *d*TM providing more than half of the dietary protein resulted in a significant decrease of muscle PUFA/SFA ratio, even so, fish still had a ratio almost 3 times higher than the minimum recommended for human consumption (PUFA/SFA ratio > 0.45) to decrease serum cholesterol levels and prevent the development of cardiovascular diseases (Kilar & Kasprzyk, 2021). The muscle n-3 PUFA/n-6 PUFA also decreased with increased dietary inclusion of *d*TM. However, this decrease was mainly due to the high levels of linoleic acid (C18:2n-6), which has been associated with a reduced incidence of cardiovascular diseases and type 2 diabetes in humans (Marangoni et al., 2020). Altogether, these results evidence that high levels of dietary protein provided by *d*TM do not compromise the nutritional quality of European sea bass muscle, although some FA quality indexes are reduced. However, all diets contained high levels of premium quality sardine oil (12.5-14% DM), rich in n-3 LC-PUFA (e.g., EPA and DHA). So, caution is advisable when other lipid sources are concomitantly used as flesh nutritional value might be altered. Moreover, when *d*TM provided 59% of the total dietary protein to totally substitute FM, the activity of glycolytic and lipogenic genes and enzymes increased. This pattern resulted in high levels of plasmatic non-esterified fatty acids (NEFA) and TAG, which in the long-term may compromise fish health, mainly at high FM replacement levels. Further studies are imperative to evaluate the impact of such high levels of dietary protein provided by *d*TM in long-term pilot scale trials, under real farm conditions of density, temperature, oxygen and salinity, to guarantee the feasibility of using this protein source in aquafeeds in a real scenario.

Main conclusions and future directions

In this thesis, the feasibility of using IM as a protein source in diets for European sea bass was evaluated considering the 5 key steps proposed by Glencross (2020): characterization, palatability, digestibility, utilization, and product quality. From the results obtained, the following conclusions can be formulated:

1. Among HI, *d*HI, TM, *d*TM and LM, the *d*TM was the most promising alternative to FM in diets for European sea bass, not only due to its high-quality protein content with an AA profile close to that of a high-quality anchovy meal but also due to its highest protein, energy and phosphorus ADC values and highest amount of digestible EAA content.

2. Partial and total FM replacement by *d*TM did not affect underlying mechanisms responsible for homeostatic regulation of feed intake in European sea bass at the central level in the short-, mid- and long-term. However, VFI decreased after 16 weeks of feeding when *d*TM provided 30% of the dietary protein (50% FM replacement), but without compromising growth.

3. Protein digestibility decreased in European sea bass fed diets with *d*TM providing 30-100% of the dietary protein (50-100% FM replacement). Even so, ADC values were high (89-92%) and compensatory nitrogen retention together with reduced losses were observed, resulting in similar nitrogen gain and whole-body protein content. It was also clearly demonstrated that even when *d*TM contributed with high levels of dietary protein (59-100%), totally replacing FM for 10-16 weeks, fish growth performance and feed efficiency were not impaired.

4. When FM was totally replaced, and *d*TM contributed with 59% of the dietary protein, the European sea bass hepatic health was not affected, but a clear increase in liver capacity to use glucose and decreased capacity of using AA was induced. These changes resulted in enhanced lipogenic capacity reflected in high levels of plasmatic NEFA and TAG, which in the long-term may compromise fish health and flesh quality for human consumption.

5. High levels of dietary protein provided by *d*TM (up to 100%; total FM replacement) resulted in muscle EPA+DHA levels above the minimum recommended by EFSA (250 mg 100 g^{-1} WW) to prevent the risk of cardiovascular diseases in humans. However, when more than 30% of the dietary protein (50% FM replacement) was provided by *d*TM, some FA quality indexes were impaired.

6. Consumers accepted very well the cooked slabs of European sea bass fed *d*TM and even associated them with a juicier texture, which is considered one of the most important quality attributes of fish for consumers.

Overall, with the present thesis, it was clearly demonstrated that European sea bass can efficiently use dietary formulations with 53 to 59% of the total protein provided by *d*TM (i.e., totally devoid of FM) combined with 12.5-14% inclusion of high-quality FO and proper supplementation with synthetic AA, for 16 weeks under controlled conditions. However, despite some compensatory mechanisms have been observed, results suggest that such high levels of dietary protein derived from *d*TM may compromise fish health and their muscle nutritional quality for human consumption in the long-term: i.e., decreased protein digestibility and increased thickness of intestine submucosa; diminished capacity of hepatic metabolization of AA and increased circulating levels of NEFA and TAG; impairment of some FA quality indexes in muscle.

The insect producing industry is still not able to supply IM at (1) an economically viable scale; (2) in industrial quantities; (3) with standardized quality. Therefore, at present, dietary formulations with low levels of protein derived from *d*TM (<30%) are deemed more appropriate for the aquafeed/aquaculture industry, not only from the nutritional/physiological point of view but also from an economic perspective. Although no economic feasibility study has been carried out, the use of a maximal 30% of protein provided by *d*TM (i.e., 20% *d*TM dietary inclusion as 50% FM substitution) seems the most reliable if FM replacement is targeted. However, it is still imperative to evaluate the impact of such levels of dietary protein provided by *d*TM in the long-term, under real densities, temperatures, and salinities to guarantee the feasibility of using this protein source in aquafeeds in a real scenario.

Finally, and since in the present thesis were used high-quality control diets with very conservative inclusion levels of premium FM and FO (i.e., 40-45% and 13-14%, respectively), providing 58-66% and 100% of the total dietary protein and fat, respectively; it would be of extreme relevance for the aquaculture industry to evaluate the use of protein derived from *d*TM in more practical dietary formulations. This means evaluating the feasibility of using *d*TM in dietary formulations with blends of vegetable proteins and oils as the main dietary protein and lipid sources. FM and FO should also be mainly obtained from fish by-products, allowing the use of strategic levels of premium quality FM and FO.

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Appendix

Supplementary Table S1. Amino acid profile of the experimental diets.

	CTRL	TM40	TM80	TM100
Amino acids, g/100 g DM				
Arginine	3.8	3.7	3.8	3.6
Histidine	1.1	1.4	1.4	1.5
Lysine	3.3	2.8	2.6	3.2
Threonine	1.7	2.0	1.8	1.8
Isoleucine	2.1	2.3	2.3	2.4
Leucine	3.8	3.5	3.6	7.0
Valine	2.4	2.6	2.9	3.5
Methionine	1.5	1.4	1.3	1.6
Phenylalanine	2.5	2.3	2.2	2.1
Cystine	0.3	0.3	0.3	0.3
Tyrosine	2.4	2.6	2.9	3.6
Aspartic acid + Asparagine	4.1	4.0	3.9	4.2
Glutamic acid + Glutamine	7.8	6.4	6.5	6.2
Alanine	2.5	2.8	2.9	3.5
Glycine	3.2	3.3	3.2	3.3
Proline	3.3	3.0	2.9	3.5
Serine	1.9	2.0	1.9	1.7
Taurine	0.2	0.2	0.06	0.04

DM, dry matter.

Supplementary Table S2. Forward and reverse primers for real-time qPCR.

Gene	Symbol	Accession number[1]	Primers
Growth hormone receptor-type I	*ghr-i*	AF438177	F: GGT GGA TGC TGA GGA TGC R: GGT GTC TGA GCC CTG GTT
Growth hormone receptor-type II	*ghr-ii*	AY642116	F: TCC AGT CCA GAG CCC TAC R: ACG ACC TCA CCT CAC TCA
Insulin-like growth factor I	*igf-i*	AY800248	F: TAG CCA CAC CCT CTC ACT ACT G R: CCT GTT GCC GTC GGA GTC
Insulin-like growth factor II	*igf-ii*	AY839105	F: AGA CAC GGA CAC CAC ACA CTT TG R: CTC TTG ACC TTC ATT CTG CTG CTC TC
Insulin-like binding-protein 1b	*igfbp1a*	(LG10:13787250-13788417)	F: AGT GTG AAT CAT CTC TGG TTG GA R: CCC ATT CCA GGA AGA GAC ACA
Insulin-like binding-protein 2b	*igfbp2b*	EU526670	F: GCA CGG AGG CTG ACT TAC C R: CTT GGT CCA GAG TTG TTG TGA GAT
Insulin-like binding-protein 3a	*igfbp3a*	(LG4:1920612-1938180)	F: GCG TGG CAA CCG TGA AGG R: GCC TGG TGT CCA CAG ATC C
Insulin-like binding-protein 4	*igfbp4*	MN045298	F: ACT CAG CGA TGG ACA GGC AGG AT R: CGG ATG TTG CTG TTG TTG GGA TGC T
Insulin-like binding-protein 5b	*igfbp5b*	(LG15:3836279-3847001)	F: GTG CCA CTC CTT CCC AAA GAC AT R: CTG CTT GCC CAG CTT CCT CT
Insulin-like binding-protein 6b	*igfbp6b*	(LG22-25:348158-350835)	F: CCA GGG ACC ATA ATG TTG CC R: TAC ACA CCA CAG GGC TCT C
Elongation of very long chain fatty acids 1	*elovl1*	KF857295	F: TAC ACA TCT TCC ACC ACT CCT TCA T R: CCA TTC CAC CAG GAG CAT AGG
Elongation of very long chain fatty acids 4	*elovl4*	KF857296	F: ACC ATG CTT ACC GAC GCA AAC CTT R: CGA CGT GCT TGC CTC CCT TCT G
Elongation of very long chain fatty acids 5	*elovl5*	FR717358	F: CAG TCA TGT ACC TTC TGA TCG TGT GGA TGG R: GGA GTA CGG CTG CCT GTG TTT CAT

Supplementary Table S2. (*Continued*).

Gene	Symbol	Accession number[1]	Primers
Elongation of very long chain fatty acids 6	*elovl6*	KF857297	F: ACA TCA CCG TGC TGC TCT ACT CCT G R: CCG CCA CCT GGT CCT TGT AGC A
Stearoyl-CoA desaturase 1b	*scd1b*	FN868643	F: GCT TGT GGC ATA CTT CAT CCC TGG ACT C R: GGT GGC GTT GAG CAT CAC GGT GTA
Fatty acid desaturase 2	*fads2*	EU647692	F: CCG CCG TGA CTG GGT GGA T R: GCA CAG GTA GCG AAG GTA GTA AGA CAT AGA
Lipoprotein lipase	*lpl*	AM411614	F: CAA TGT GAT CGT GGT GGA CTG R: CGT CGG GTA GTG CTG GTT
Hepatic lipase	*hl*	KF857289	F: CGC AGT GGC ACC AGC AAG A R: CGG CAT CCG AGA CCG TGT T
Adipose triglyceride lipase	*atgl*	KF857294	F: GGA GCC CTC ACT GCC ACT R: ATT CGC ACC AGT CTC TCC AAG A
Hormone sensitive lipase	*hsl*	KF857293	F: GCC CTG TCT CCA GAC TAT TGC TAT C R: GCT GCT ACA CCT ATT CCT GAC TGA T
Peroxisome proliferator-activated receptor α	*pparα*	AY590300	F: CAG GAC ACG CAC AAC TCA ATC A R: GGA GAA CAC GGG ACA GTC AGA A
Peroxisome proliferator-activated receptor γ	*pparγ*	AY590303	F: CAG GAC ACG CAC AAC TCA ATC A R: GGA GAA CAC GGG ACA GTC AGA A
Carnitine palmitoyltransferase 1a	*cpt1a*	KF857302	F: TGC CAA GAG GTC ATC CAG AGT TCT R: AGT CCA CAT CAT CCG CCA GAG A
Citrate synthase	*cs*	KF857304	F: GTG TAT GAG ACC TCC GTG TTG G R: AGC AAC TTC TGA CAC TCT GGA ATG
NADH dehydrogenase subunit 5	*nd5*	KF857307	F: CCC GAT TTC TGT GCC CTA CTA R: AGG AAA GGA GTG CCT GTG A
Succinate dehydrogenase cytochrome b560 subunit	*sdhc*	KF857305	F: ACA TGG GCA AGG GCT TCA AA R: CGA TGA TGG ACA GAC CGA TAA CG

Supplementary Table S2. (*Continued*).

Gene	Symbol	Accession number[1]	Primers
Cytochrome b	*cyb*	EF427553	F: TGC CTA CGC TAT CCT TCG CTC GAT CC R: TAA CGC CAA CAC CCC GCC CAA T
Cytochrome c oxidase subunit I	*coxi*	KF857308	F: ATA CTT CAC ATC CGC AAC CAT AA R: AAG CCT CCG ACT GTA AAT AAG AAA
Cholesterol 7-alpha-monooxygenase	*cyp7a1*	KF857306	F: TGC CAT CAA AGT CCC ACC TCT T R: CAC ATC ATA GGT AGG CTG GAG GAT TC
Sirtuin 1	*sirt1*	MH138004	F: GGT GGA CCT CTT GAT TGT CAT TGG CTC TTC R: GGG ATG AGG GCA ACT GGT CGG ACT TTA
Sirtuin 2	*sirt2*	MK983171	F: TCT AAT TGA GGC TCA CGG AAC R: GAC GGG TAG ATT CTC TCC AAA G
Mitochondrial respiratory uncoupling protein 1	*ucp1*	MH138003	F: CGA TTC CAA GCC CAG ACG AAC CT R: TGC CAG TGT AGC GAC GAG CC
Mitochondrial respiratory uncoupling protein 3	*ucp3*	(LG14:12134586-12136013)	F: CCA TGC TGA GAC AGG AAG GAC CCA CAT R: CCA GTC GCA GGA AAG AAG GCA TGA ACC
Myoblast determination protein 1	*myod1*	(LG6:934633-937237)	F: GAC CGA CCT GTC AGT CCA ACC G R: TGG AGT CTC GGA GAA ATA AGA GCT GTT GT
Myoblast determination protein 2	*myod2*	(LG5:26406310-26408511)	F: CTG CTG ATG ACC TCT ACG ATG AC R: GGC GTC CAG GTC GTC AAA
Myogenic regulatory factor 4	*mrf4*	(LGx:14305213-14306264)	F: GTC TCC TCT ATA CAA CGG CAA T R: CTG TCT CGG ACG GAA CAT TAT C
Myogenic factor 5	*myf5*	(LGx:14298644-14300040)	F: CGC AAC GCC ATC CAG TAC ATC G R: GCC GTA GTA GTT TTC CAC CTG CTC AT
Myogenin	*myog*	(LG1A:13290583-13292182)	F: GAC CAA CCC TTA TTT CTT R: CAT CAT GGA GTT CCT ATC
Myostatin	*mstn*	AY839106	F: GCA GCA GCT TCT CGA CCA GTA R: ATC GTC GTC CTC CAT AAC CAC ATC

Supplementary Table S2. (*Continued*).

Gene	Symbol	Accession number[1]	Primers
Follistatin	*fst*	MK983166	F: GTG CCA GTG ACA ACA CCA CAT ATC C R: ATC CCG AGT GCT TGA CTT CCA
Fibroblast growth factor 4	*fgf4*	(LG5:29962695-29967359)	F: GGC TTT GTG ACC GGA ATG G R: GTC CGC TGT CCC GTT CAG
Fibroblast growth factor 6	*fgf6*	AY831723	F: CAA CGC CTA CGA GTC TCT GGT CTA C R: GCC ATG CTT GCT GAG TGC TAT GT
Muscle RING-finger protein 1	*murf1*	(UN:85299200-85300236)	F: TGG TGC GTC CTG TCA GTG R: CGG CTT GGT GAA CAT CTC AA
Muscle atrophy F-box	*mafbx/atrogin-1*	MK983167	F: ACT GAG GAC CGA CTG CTG TGG AAG A R: TGT CTG TCT GTG AAG TGG TAC TGG CAA AGT
Myomaker	*mymk*	(LG20:21064893-21067975)	F: ATC TGT CTC TGG CTG TGT CCT TCA T R: CAG CAT TTC GTC CCG TCC CT
Calpain 1	*capn1*	FJ821591	F: CTA CAG AGG AAA TCC GAC TAA GC R: CGG TCC ATT CCA CTT CCC
Calpain 2	*capn2*	MK983168	F: AAC GAA CTG ACA TCC GAA CTG A R: ATT GCC GCT GTC ATC CAT CA
Calpain 3	*capn3*	MK983169	F: ATA CCG ACG GGA CAG GGA AG R: GCT GCC ACG CCT TGA TCT T
Calpastatin	*cpst*	MK983170	F: AGA CGA CAC GCT GCC TCC A R: CTC AGT GGT TTA GGG ACA TCC TTG GGT TT

[1]Obtained from GenBank database or the European seabass genome project (http://seabass.mpipz.mpg.de). Accession number of seabass genome is shown in parentheses.

Supplementary Table S3. Correlations between nutrients digestibility and balance, whole body composition, muscle FA composition and plasma metabolites with VSI, HSI, or *d*TM dose.

	VSI	**HSI**	***d*TM dose**
Nutrients digestibility and balance			
ADC of protein	NS	NS	-0.80 (< 0.01)
Digestible N intake	NS	NS	-0.78 (< 0.01)
N retention efficiency	NS	NS	0.67 (0.02)
Fecal N losses	NS	NS	0.93 (< 0.01)
Metabolic N losses	NS	NS	-0.84 (< 0.01)
Total N losses	NS	NS	-0.6 (< 0.01)
ADC of phosphorus	NS	NS	0.92 (< 0.01)
P gain	NS	-0.59 (0.04)	NS
Digestible P intake	-0.62 (0.03)	NS	-0.84 (< 0.01)
Fecal P losses	NS	NS	-0.97 (< 0.01)
Total P losses	NS	NS	-0.6 (0.02)
L gain	0.59 (0.04)	0.59 (0.04)	0.78 (< 0.01)
Lipids retention efficiency	0.65 (0.02)	NS	NS
Fecal L losses	-0.64 (0.03)	NS	NS
Metabolic L losses	-0.64 (0.03)	-0.63 (0.03)	NS
Total L losses	-0.64 (0.03)	NS	NS
Digestible E intake	NS	NS	NS
E gain	NS	NS	0.78 (< 0.01)
Branchial + urinary E losses	NS	NS	-0.84 (< 0.01)
Whole body composition			
Moisture	NS	NS	-0.61 (0.04)
Lipids	NS	0.59 (0.04)	0.73 (< 0.01)
Energy	NS	NS	0.73 (< 0.01)
Phosphorus	NS	-0.60 (0.04)	NS
Muscle FA composition			
Σ MUFA	0.78 (< 0.01)	0.72 (< 0.01)	NS
n-3 LC-PUFA	NS	NS	-0.65 (0.02)
n-6 LC-PUFA	NS	0.61 (< 0.01)	0.97 (< 0.01)
Plasma metabolites			
Triglycerides	0.73 (< 0.01)	0.85 (< 0.01)	0.82 (< 0.01)
Cholesterol	0.61 (0.04)	NS	NS
NEFA	NS	NS	0.84 (< 0.01)

Values are *rs*-value (*p*-value of correlation). Differences were considered significant when $p < 0.05$. ADC, apparent digestibility coefficients; *d*TM, defatted *Tenebrio molitor* larvae meal; E, energy; FA, fatty acids; HSI, hepatosomatic index; L, lipids; LC-PUFA, long-chain polyunsaturated fatty acids; N, nitrogen; NEFA, non-esterified fatty acids; NS, not significant; P, phosphorus; VSI, viscerosomatic index.

Supplementary Table S4. Intestinal morphological measurements of European seabass fed experimental diets.

	CTRL	TM40	TM80	TM100	*p*-value
Cross-sectional area, mm^2	14.5 ± 1.6	11.1 ± 0.7	12.6 ± 0.9	12.7 ± 0.6	0.34
Muscularis externa, µm	97.9 ± 4.3	110.6 ± 3.7	96.4 ± 1.3	89.4 ± 2.5	0.61
Outer longitudinal layer, µm	34.3 ± 8.8	41.4 ± 9.3	35.2 ± 9.4	31.6 ± 3.6	0.12
Inner circular layer, µm	63.6 ± 2.9	69.3 ± 3.1	61.2 ± 3.1	57.8 ± 1.2	0.12
Submucosa, µm	26.5 ± 0.7^{c}	29.2 ± 1.0^{b}	30.2 ± 1.0^{ab}	32.8 ± 1.0^{a}	< 0.01
Lamina propria, µm	27.3 ± 1.4	28.4 ± 1.6	29.3 ± 0.4	30.3 ± 2.3	0.62
Villus length, µm	2000.6 ± 83.6	1749.1 ± 68.8	1833.6 ± 83.2	1821.0 ± 53.5	0.12
Villus width, µm	179.9 ± 11.4	160.5 ± 9.5	176.3 ± 11.0	162.6 ± 5.6	0.44
Goblet cells, nº per *villus*	304.3 ± 36.0	227.8 ± 20.8	284.3 ± 19.0	228.7 ± 22.4	0.21
Acid goblet cells, nº per *villus*	191.8 ± 25.8	157.4 ± 6.6	178.4 ± 9.6	147.1 ± 10.3	0.07
Neutral goblet cells, nº per *villus*	112.5 ± 12.5	70.4 ± 9.8	105.9 ± 12.3	81.6 ± 1.6	0.09

Values are means ± SEM; n = 9 (3 fish/replicate). Labeled means without a common superscript letter differ significantly, $p < 0.05$.

Supplementary Table S5. Muscular cellularity parameters of European seabass fed experimental diets.

	CTRL	TM40	TM80	TM100	*p*-value
White muscle cross-sectional area, mm^2	489.7 ± 12.8	495.7 ± 12.43	458.5 ± 13.8	458.74 ± 19.27	0.17
Total n° of fibers × 10^3	107.4 ± 42.0	107.5 ± 50.8	104.0 ± 54.0	105.0 ± 68.1	0.96
Fibers density (n°/mm^2)	219.6 ± 7.86	216.8 ± 8.3	227.2 ± 10.6	228.1 ± 9.8	0.78
Fibers diameter, µm	66.7 ± 3.9	66.7 ± 1.1	65.5 ± 1.10	65.41 ± 1.1	0.82
Fibers ≤ 20 µm, %	6.3 ± 0.01	5.0 ± 0.004	5.4 ± 0.01	6.25 ± 0.004	0.39
Fibers > 140 µm, %	2.2 ± 0.01	3.2 ± 0.01	2.2 ± 0.004	2.64 ± 0.01	0.54

Values are means ± SEM; n = 9 (3 fish/replicate). CSA, cross-sectional area.

Supplementary Table S6. Muscle texture profile and color of European seabass fed experimental diets.

	CTRL	TM40	TM80	TM100	*p*-value
Texture profile					
Hardness, N	0.9 ± 0.04	1.0 ± 0.06	1.0 ± 0.05	0.9 ± 0.05	0.34
Adhesiveness, J	-0.01 ± 0.002	-0.004 ± 0.001	-0.004 ± 0.002	-0.005 ± 0.001	0.40
Springiness	1.2 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.3 ± 0.1	0.29
Cohesiveness	0.4 ± 0.01	0.5 ± 0.02	0.5 ± 0.02	0.5 ± 0.02	0.83
Chewiness, J	0.5 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.16
Resilience	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.55
Color					
L*	40.7 ± 0.5	40.8 ± 0.4	40.8 ± 0.5	41.7 ± 0.5	0.41
a*	-0.2 ± 0.1	-0.5 ± 0.1	-0.3 ± 0.1	-0.3 ± 0.1	0.21
b*	-0.7 ± 0.3	-1.0 ± 0.2	-1.0 ± 0.1	0.1 ± 0.4	0.25
C*[1]	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	0.81
H°[2]	222.7 ± 22.5	238.8 ± 8.1	250.2 ± 4.2	182.8 ± 22.9	0.40

Values are means ± SEM; n = 9 (3 fish/replicate). a*, redness; b*, yellowness; C*, chroma; H°, hue; L*, lightness.
Chroma = (a* 2 + b* 2)1/2; Hue = tan−1 (b* / a*).

Supplementary Table S7. Relative expression of hepatic genes of European seabass fed experimental diets.

	CTRL	TM40	TM80	TM100	*p*-value
ghr-i	6.6 ± 0.3	7.0 ± 0.7	6.2 ± 0.6	7.5 ± 0.5	0.43
ghr-ii	2.3 ± 0.3	2.5 ± 0.1	2.2 ± 0.3	2.1 ± 0.4	0.77
igf-i	20.7 ± 1.3	21.9 ± 1.9	18.7 ± 1.6	18.5 ± 1.7	0.40
igf-ii	4.1 ± 0.6	4.4 ± 0.5	3.3 ± 0.6	3.5 ± 0.9	0.66
igfbp1b	1.5 ± 0.3	1.8 ± 0.3	1.2 ± 0.3	1.5 ± 0.5	0.70
igfbp2b	26.3 ± 2.3	30.0 ± 3.4	28.2 ± 2.6	28.1 ± 3.1	0.80
igfbp4	4.1 ± 0.5	4.1 ± 0.2	4.3 ± 0.4	3.8 ± 0.6	0.30
elovl1	0.31 ± 0.1	0.3 ± 0.04	0.3 ± 0.02	0.4 ± 0.04	0.64
elovl4	0.01 ± 0.0001	0.001 ± 0.0002	0.0001 ± 0.0002	0.0001 ± 0.0001	0.60
elovl5	0.01 ± 0.0001	0.01 ± 0.00001	0.01 ± 0.00001	0.01 ± 0.00001	0.20
elovl6	6.2 ± 1.2^{a}	4.3 ± 0.8^{ab}	4.1 ± 1.02^{ab}	2.4 ± 0.4^{b}	0.02
scd1b	1.5 ± 0.5	0.7 ± 0.1	0.7 ± 0.2	0.8 ± 0.1	0.08
fads2	0.3 ± 0.1^{b}	0.2 ± 0.1^{b}	0.3 ± 0.08^{ab}	1.0 ± 0.3^{a}	0.02
lpl	4.3 ± 0.5	4.8 ± 0.4	3.9 ± 0.3	4.4 ± 0.6	0.57
hl	4.8 ± 0.3	5.4 ± 0.4	5.8 ± 0.4	6.2 ± 0.8	0.43
atgl	1.4 ± 0.2	1.6 ± 0.2	1.8 ± 0.2	1.7 ± 0.2	0.41
hsl	1.0 ± 0.1	1.1 ± 0.1	0.9 ± 0.04	1.0 ± 0.1	0.29
pparα	0.34 ± 0.1	0.33 ± 0.03	0.32 ± 0.1	0.2 ± 0.03	0.09
pparγ	0.4 ± 0.03	0.5 ± 0.1	0.4 ± 0.03	0.5 ± 0.1	0.26
cpt1a	0.8 ± 0.1	0.8 ± 0.04	0.7 ± 0.05	0.8 ± 0.1	0.91
cs	1.9 ± 0.1	2.0 ± 0.1	1.8 ± 0.2	1.8 ± 0.2	0.60
nd5	86.8 ± 6.2	111.1 ± 6.1	98.7 ± 7.7	105.4 ± 10.6	0.18
sdhc	2.8 ± 0.2	3.0 ± 0.1	2.9 ± 0.1	3.0 ± 0.3	0.84
cyb	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.03	0.2 ± 0.01	0.27
coxi	279.9 ± 31.5	324.9 ± 19.3	283.0 ± 25.1	355.9 ± 46.9	0.41
cyp7a1	6.9 ± 0.7^{a}	4.2 ± 0.7^{b}	3.0 ± 0.5^{b}	3.2 ± 0.5^{b}	<0.01
sirt1	0.1 ± 0.01	0.1 ± 0.01	0.2 ± 0.01	0.2 ± 0.01	0.16
sirt2	0.8 ± 0.04	0.9 ± 0.04	0.8 ± 0.1	0.9 ± 0.1	0.22
ucp1	7.2 ± 0.6	8.2 ± 1.6	5.8 ± 0.6	5.8 ± 0.6	0.49

Values are means ± SEM; n = 9 (3 fish/replicate). Labeled means without a common superscript letter differ significantly.

Supplementary Table S8. Relative expression of muscle genes of European seabass fed experimental diets.

	CTRL	TM40	TM80	TM100	*p*-value
ghr-i	1.54 ± 0.22	1.41 ± 0.09	1.28 ± 0.07	1.47 ± 0.11	0.63
ghr-ii	1.10 ± 0.18	1.29 ± 0.16	1.21 ± 0.15	1.29 ± 0.20	0.85
igf-i	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.72
igf-ii	0.87 ± 0.11	0.72 ± 0.05	0.69 ± 0.04	0.69 ± 0.04	0.24
igfbp3a	1.74 ± 0.14	1.82 ± 0.22	1.84 ± 0.12	1.72 ± 0.15	0.95
igfbp5b	2.27 ± 0.29	2.65 ± 0.27	2.88 ± 0.44	2.84 ± 0.20	0.47
igfbp6b	0.29 ± 0.02	0.30 ± 0.03	0.32 ± 0.02	0.33 ± 0.02	0.56
scd1b	0.72 ± 0.08	0.63 ± 0.08	0.70 ± 0.08	0.67 ± 0.07	0.84
cpt1a	2.83 ± 0.33	2.75 ± 0.27	2.83 ± 0.26	2.91 ± 0.34	0.98
cs	23.29 ± 2.35	23.78 ± 1.21	21.76 ± 1.40	24.83 ± 1.59	0.64
sirt1	0.28 ± 0.02	0.28 ± 0.03	0.27 ± 0.02	0.30 ± 0.02	0.87
sirt2	1.41 ± 0.11	1.35 ± 0.12	1.41 ± 0.16	1.32 ± 0.05	0.92
ucp3	3.01 ± 0.29	3.12 ± 0.30	2.79 ± 0.64	2.90 ± 0.34	0.35
myod1	8.43 ± 0.88	8.78 ± 0.64	8.63 ± 0.82	7.43 ± 0.55	0.57
myod2	3.32 ± 0.23^{a}	3.23 ± 0.17^{a}	2.63 ± 0.31^{b}	2.65 ± 0.11^{b}	0.03
mrf4	0.45 ± 0.06	0.48 ± 0.07	0.40 ± 0.04	0.41 ± 0.03	0.70
myf5	0.33 ± 0.02	0.32 ± 0.02	0.30 ± 0.02	0.32 ± 0.01	0.63
myog	2.42 ± 0.24	2.34 ± 0.19	2.30 ± 0.14	2.46 ± 0.15	0.91
mstn	0.45 ± 0.09^{a}	0.61 ± 0.15^{ab}	0.81 ± 0.16^{ab}	1.04 ± 0.23^{b}	0.01
fst	0.20 ± 0.03	0.19 ± 0.02	0.19 ± 0.02	0.20 ± 0.02	0.98
fgf4	0.28 ± 0.02	0.30 ± 0.03	0.30 ± 0.01	0.31 ± 0.02	0.83
fgf6	0.86 ± 0.06	0.73 ± 0.08	0.85 ± 0.09	0.81 ± 0.04	0.53
murf1	15.23 ± 1.51	15.84 ± 1.89	15.56 ± 1.86	15.71 ± 0.61	0.99
mafbx/atrogin 1	1.73 ± 0.27^{a}	1.38 ± 0.48^{ab}	1.47 ± 0.49^{ab}	0.92 ± 0.14^{b}	0.01
mymk	0.07 ± 0.01^{b}	0.08 ± 0.01^{ab}	0.09 ± 0.02^{ab}	0.10 ± 0.01^{a}	0.04
capn1	1.20 ± 0.09	1.04 ± 0.07	1.12 ± 0.09	1.22 ± 0.06	0.36
capn2	1.02 ± 0.09	1.08 ± 0.10	1.08 ± 0.09	1.10 ± 0.06	0.92
capn3	5.33 ± 0.41	5.35 ± 0.29	5.93 ± 0.68	5.43 ± 0.43	0.96
cpst	0.87 ± 0.04	0.87 ± 0.07	0.90 ± 0.08	0.95 ± 0.04	0.72

Values are means ± SEM; n = 9 (3 fish/replicate). Labeled means without a common superscript letter differ significantly.

Supplementary Table S9. Correlations between expression of hepatic genes and plasma metabolites.

	Glucose	Protein	Triglycerides	Cholesterol	NEFA
elovl6	NS	-0.41 (0.04)	NS	-0.64 (< 0.01)	NS
fads2	NS	NS	NS	0.38 (0.04)	NS
scd1b	NS	NS	0.49 (0.04)	NS	NS
cyp7a1	0.37 (0.02)	NS	NS	-0.46 (< 0.01)	NS
pparα	NS	NS	NS	-0.37 (0.02)	NS

Values are *rs*-value (*p*-value of correlation); Differences were considered significant when $p < 0.05$. NEFA, non-esterified fatty acids; NS, not significant.

Supplementary Table S10. Whole-body composition and fatty acids profile of European sea bass fed experimental diets.

	CTRL	TM50	TM100	
Whole-body composition (g 100 g^{-1} WW)				
Moisture	62.9 ± 0.6	62.3 ± 0.3	62.0 ± 0.6	0.083
Protein	18.1 ± 0.1	18.1 ± 0.4	17.9 ± 0.2	0.431
Lipids	12.6 ± 0.1 ^{b}	13.1 ± 0.3 ^{a}	13.4 ± 0.1 ^{a}	0.022
Gross energy (kJ g^{-1} DM)	10.0 ± 0.4	10.1 ± 0.1	10.2 ± 0.2	0.457
Ash	3.6 ± 0.1	3.7 ± 0.2	3.8 ± 0.2	0.301
Phosphorus	0.6 ± 0.02	0.6 ± 0.02	0.6 ± 0.03	0.240
Fatty acids (g 100 g^{-1} total fatty acids)				
C14:0	4.2 ± 0.03 ^{a}	4.1 ± 0.01 ^{b}	4.1 ± 0.05 ^{b}	< 0.001
C16:0	19.0 ± 0.5	19.6 ± 0.2	19.8 ± 0.5	0.064
C18:0	3.4 ± 0.1	3.4 ± 0.03	3.5 ± 0.1	0.275
C21:0	1.2 ± 0.1	1.1 ± 0.03	1.1 ± 0.1	0.122
Σ SFA [1]	28.7 ± 0.5	29.0 ± 0.2	29.2 ± 0.5	0.226
C16:1	5.8 ± 0.1	5.7 ± 0.1	5.7 ± 0.2	0.598
C18:1n-9 (OA)	22.8 ± 0.7 ^{b}	24.3 ± 0.3 ^{a}	24.7 ± 1.0 ^{a}	0.008
C18:1n-7	3.0 ± 0.3	2.9 ± 0.1	2.7 ± 0.3	0.361
C20:1n-9	3.3 ± 0.4	3.1 ± 0.04	2.9 ± 0.4	0.207
C24:1n-9	0.56 ± 0.1 ^{a}	0.50 ± 0.01 ^{ab}	0.45 ± 0.1 ^{b}	0.046
Σ MUFA [2]	36.1 ± 0.2 ^{b}	37.1 ± 0.3 ^{a}	37.1 ± 0.3 ^{a}	0.001
C18:2n-6 (LA)	9.5 ± 2.6	10.3 ± 0.2	11.6 ± 2.6	0.416
C18:3n-3 (ALA)	1.6 ± 0.2	1.5 ± 0.03	1.4 ± 0.3	0.305
C18:4n-3	2.0 ± 0.1	1.9 ± 0.02	1.8 ± 0.1	0.153
C20:3n-3	0.5 ± 0.1	0.5 ± 0.01	0.4 ± 0.1	0.076
C20:4n-6	4.7 ± 0.4 ^{a}	4.2 ± 0.03 ^{ab}	3.9 ± 0.4 ^{b}	0.042
C20:5n-3 (EPA)	6.6 ± 0.5	6.0 ± 0.1	5.7 ± 0.7	0.086
C22:5n-3	1.3 ± 0.2	1.2 ± 0.02	1.1 ± 0.2	0.187
C22:6n-3 (DHA)	8.4 ± 1.2	7.6 ± 0.2	7.1 ± 1.2	0.235
EPA + DHA	15.0 ± 1.7	13.6 ± 0.3	12.8 ± 1.8	0.170
Σ PUFA [3]	35.3 ± 0.3 ^{a}	33.9 ± 0.5 ^{b}	33.7 ± 0.5 ^{b}	0.001
Σ PUFA/ Σ SFA	1.23 ± 0.04 ^{a}	1.17 ± 0.02 ^{ab}	1.15 ± 0.04 ^{b}	0.017
Σ n-3 PUFA	20.4 ± 2.3	18.7 ± 0.3	17.6 ± 2.5	0.176
Σ n-6 PUFA	14.3 ± 2.1	14.7 ± 0.2	15.6 ± 2.2	0.576
Σ n-3 PUFA/Σ n-6 PUFA	1.5 ± 0.3	1.3 ± 0.01	1.2 ± 0.4	0.382
Fatty acids (g 100 g^{-1} WW)				
Σ SFA	1.7 ± 0.2 ^{b}	2.2 ± 0.2 ^{a}	2.2 ± 0.3 ^{a}	0.018
Σ MUFA	2.1 ± 0.2 ^{b}	2.7 ± 0.3 ^{a}	2.7 ± 0.3 ^{a}	0.020
C20:5n-3 (EPA)	0.4 ± 0.1	0.4 ± 0.04	0.4 ± 0.1	0.487
C22:6n-3 (DHA)	0.5 ± 0.1	0.5 ± 0.04	0.5 ± 0.11	0.621
EPA + DHA	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	0.591
Σ PUFA	2.0 ± 0.2	2.4 ± 0.2	2.4 ± 0.3	0.056
Σ n-3 PUFA	1.1 ± 0.2	1.3 ± 0.1	1.2 ± 0.3	0.555
Σ n-6 PUFA	0.8 ± 2.1 ^{b}	1.1 ± 0.2 ^{a}	1.1 ± 2.2 ^{a}	0.011

[1] Includes C12:0, C15:0, C20:0, C22:0 and C24:0; [2] Includes: C14:1, C17:1, C18:1n-7 and C22:1n-9; [3] Includes C18:3n-6c and C20:2. Values are presented as mean ± standard deviation; n = 4. Different superscript letters represent significant differences (p < 0.05); one-way ANOVA (post-hoc Tukey's test). The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of fishmeal protein replacement by defatted *Tenebrio molitor* larvae meal, respectively. Other abbreviations stand for: ALA, α-linolenic acid; DHA, docosahexaenoic; DM, dry matter; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acids; OA, oleic acid; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; WW, wet weight.

Supplementary Table S11. Whole-body fatty acids gain (mg kg^{-1} ABW day^{-1}) of European sea bass fed experimental diets.

	CTRL	TM50	TM100	*p*-value
C14:0	30.8 ± 3.5	39.0 ± 4.6	38.9 ± 6.4	0.072
C16:0	130.6 ± 11.4 [b]	180.2 ± 23.5 [a]	183.0 ± 28.2 [a]	0.014
C18:0	22.1 ± 2.2 [b]	29.5 ± 4.0 [ab]	30.6 ± 5.2 [a]	0.029
C21:0	8.3 ± 1.7	9.8 ± 1.2	9.6 ± 2.1	0.442
Σ SFA [1]	197.4 ± 19.4 [b]	266.1 ± 34.2 [a]	269.4 ± 42.1 [a]	0.022
C16:1	38.9 ± 5.8	51.4 ± 7.2	51.0 ± 9.6	0.080
C18:1n-9 (OA)	150.6 ± 15.0 [b]	217.4 ± 30.1 [a]	221.6 ± 31.6 [a]	0.007
C20:1n-9	24.1 ± 5.3	28.9 ± 3.3	26.8 ± 7.0	0.485
C24:1n-9	3.9 ± 0.8	4.5 ± 0.5	4.0 ± 1.1	0.602
Σ MUFA [2]	241.9 ± 31.2 [b]	333.2 ± 45.1 [a]	332.7 ± 52.9 [a]	0.025
C18:2n-6 (LA)	59.7 ± 15.0 [b]	90.0 ± 9.6 [ab]	102.3 ± 26.4 [a]	0.025
C18:3n-3 (ALA)	11.3 ± 3.1	13.4 ± 1.5	12.3 ± 3.9	0.617
C18:4n-3	15.8 ± 2.5	19.3 ± 1.9	18.3 ± 3.0	0.190
C20:3n-3	3.9 ± 0.8	4.6 ± 0.6	4.2 ± 1.0	0.541
C20:4n-6	34.5 ± 6.4	39.9 ± 4.8	37.1 ± 7.8	0.526
C20:5n-3 (EPA)	43.2 ± 8.8	51.0 ± 5.6	48.6 ± 12.5	0.511
C22:5n-3	8.7 ± 2.3	10.0 ± 1.1	9.4 ± 2.8	0.701
C22:6n-3 (DHA)	55.4 ± 14.7	64.8 ± 5.9	60.4 ± 18.3	0.653
EPA + DHA	98.6 ± 23.4	115.7 ± 11.5	109.0 ± 30.7	0.595
Σ PUFA [3]	237.0 ± 29.9	298.0 ± 31.7	297.7 ± 45.7	0.066
Σ n-3 PUFA	138.3 ± 32.1	163.0 ± 16.6	153.0 ± 41.4	0.561
Σ n-6 PUFA	95.2 ± 10.5 [b]	131.0 ± 14.6 [a]	140.3 ± 24.7 [a]	0.013

[1] Includes C12:0, C15:0, C20:0, C22:0 and C24:0; [2] Includes: C14:1, C17:1, C18:1n-7 and C22:1n-9; [3] Includes 1C8:3n-6c and C20:2. Values are presented as mean ± standard deviation; n = 4. Different superscript letters represent significant differences ($p < 0.05$); one-way ANOVA (post-hoc Tukey's test). The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of fishmeal protein replacement by defatted *Tenebrio molitor* larvae meal, respectively. Other abbreviations stand for: ABW, average body weight; ALA, α-linolenic acid; DHA, docosahexaenoic; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acids; OA, oleic acid; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Supplementary Table S12. Whole-body fatty acids retention (% intake) of European sea bass fed experimental diets.

	CTRL	TM50	TM100	*p*-value
C14:0	30.9 ± 3.1 [b]	41.8 ± 3.0 [a]	37.2 ± 5.9 [ab]	0.017
C16:0	49.8 ± 3.7 [b]	68.5 ± 5.8 [a]	57.1 ± 8.7 [ab]	0.008
C18:0	37.0 ± 3.1 [b]	49.1 ± 4.5 [a]	41.5 ± 6.9 [ab]	0.024
C21:0	27.9 ± 5.4	39.8 ± 3.0	39.5 ± 8.7	0.055
Σ SFA [1]	41.5 ± 3.5 [b]	57.3 ± 4.7 [a]	48.9 ± 7.5 [ab]	0.009
C16:1	43.5 ± 6.0 [b]	65.9 ± 6.2 [a]	65.0 ± 12.1 [a]	0.008
C18:1n-9 (OA)	54.3 ± 4.7 [b]	80.8 ± 7.5 [a]	74.4 ± 10.7 [a]	0.003
C20:1n-9	32.3 ± 6.7	46.9 ± 3.2	47.4 ± 12.2	0.048
C24:1n-9	23.5 ± 4.5	31.2 ± 2.4	28.7 ± 7.5	0.160
Σ MUFA [2]	46.1 ± 5.4 [b]	69.9 ± 6.2 [a]	66.9 ± 10.6 [a]	0.004
C18:2n-6 (LA)	54.5 ± 14.0	54.0 ± 3.4	40.8 ± 10.4	0.156
C18:3n-3 (ALA)	31.1 ± 8.3 [b]	49.2 ± 3.3 [ab]	53.2 ± 16.8 [a]	0.042
C18:4n-3	33.3 ± 5.0 [b]	45.3 ± 2.5 [a]	42.4 ± 6.7 [ab]	0.020
C20:3n-3	40.7 ± 21.3	65.0 ± 12.1	73.3 ± 20.9	0.089
C20:4n-6	22.8 ± 4.0	30.1 ± 2.2	28.7 ± 5.8	0.081
C20:5n-3 (EPA)	29.2 ± 5.6	41.6 ± 2.7	40.9 ± 10.3	0.057
C22:5n-3	34.4 ± 8.8	50.0 ± 3.0	52.5 ± 15.5	0.074
C22:6n-3 (DHA)	32.5 ± 8.3	47.6 ± 2.2	48.9 ± 14.6	0.077
EPA + DHA	31.0 ± 7.0	44.8 ± 2.4	45.0 ± 12.4	0.069
Σ PUFA [3]	33.2 ± 3.8 [b]	44.7 ± 2.7 [a]	41.1 ± 6.0 [a]	0.013
Σ n-3 PUFA	31.3 ± 7.0	45.2 ± 2.6	42.2 12	0.061
Σ n-6 PUFA	35.8 ± 3.9 [b]	53.8 ± 3.6 [a]	53.6 ± 9.1 [a]	0.003

[1] Includes C12:0, C15:0, C20:0, C22:0 and C24:0; [2] Includes: C14:1, C17:0, C18:1n-7, and C22:1n-9; [3] Includes C18:3n-6c and C20:2. Values are presented as mean ± standard deviation; n = 4. Different superscript letters represent significant differences ($p < 0.05$); one-way ANOVA (post-hoc Tukey's test). The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of fishmeal protein replacement by defatted *Tenebrio molitor* larvae meal, respectively. Other abbreviations stand for: ALA, α-linolenic acid; DHA, docosahexaenoic; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acids; OA, oleic acid; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Supplementary Table S13. Muscle total lipids and fatty acids profile of European sea bass fed experimental diets.

	CTRL	TM50	TM100	*p*-value
Total lipids (% WW)	2.5 ± 0.3	2.7 ± 0.4	2.6 ± 0.4	0.870
Fatty acids (% total fatty acids)				
C14:0	3.4 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	0.112
C16:0	18.7 ± 0.1 ^{c}	19.2 ± 0.1 ^{b}	19.8 ± 0.3 ^{a}	< 0.001
C18:0	3.9 ± 0.1 ^{b}	4.1 ± 0.1 ^{a}	4.2 ± 0.1 ^{a}	0.002
C21:0	1.1 ± 0.03 ^{a}	1.0 ± 0.02 ^{b}	0.9 ± 0.01 ^{c}	< 0.001
Σ SFA ^{1}	28.1 ± 0.1 ^{c}	28.7 ± 0.1 ^{b}	29.3 ± 0.2 ^{a}	< 0.001
C16:1	4.8 ± 0.1 ^{a}	4.6 ± 0.03 ^{ab}	4.4 ± 0.2 ^{b}	0.004
C18:1n-9 (OA)	19.8 ± 0.3 ^{b}	21.3 ± 0.5 ^{a}	21.6 ± 0.9 ^{a}	0.007
C20:1n-9	3.0 ± 0.1 ^{a}	2.7 ± 0.03 ^{b}	2.3 ± 0.03 ^{c}	< 0.001
C24:1n-9	0.6 ± 0.03 ^{a}	0.5 ± 0.02 ^{b}	0.5 ± 0.02 ^{b}	0.001
Σ MUFA ^{2}	31.7 ± 0.3	32.2 ± 0.6	31.7 ± 1.0	0.506
C18:2n-6 (LA)	6.4 ± 0.2 ^{c}	9.1 ± 0.2 ^{b}	11.9 ± 0.1 ^{a}	< 0.001
C18:3n-3 (ALA)	1.6 ± 0.04 ^{a}	1.3 ± 0.02 ^{b}	1.1 ± 0.004 ^{c}	< 0.001
C18:4n-3	1.8 ± 0.1 ^{a}	1.7 ± 0.1 ^{b}	1.6 ± 0.02 ^{b}	0.001
C20:3n-3	0.4 ± 0.03 ^{a}	0.4 ± 0.01 ^{b}	0.4 ± 0.01 ^{c}	0.001
C20:4n-6	4.3 ± 0.2 ^{a}	4.0 ± 0.3 ^{a}	3.6 ± 0.1 ^{b}	0.002
C20:5n-3 (EPA)	8.2 ± 0.1 ^{a}	7.4 ± 0.1 ^{b}	6.8 ± 0.2 ^{c}	< 0.001
C22:5n-3	1.6 ± 0.02 ^{a}	1.4 ± 0.03 ^{b}	1.2 ± 0.04 ^{c}	< 0.001
C22:6n-3 (DHA)	14.9 ± 0.5 ^{a}	13.0 ± 0.4 ^{b}	11.8 ± 1.0 ^{b}	0.001
EPA + DHA	23.2 ± 0.70 ^{a}	20.4 ± 0.40 ^{b}	18.6 ± 1.30 ^{c}	0.120
Σ PUFA ^{3}	40.2 ± 0.3	39.1 ± 0.5	39.0 ± 1.2	< 0.001
Σ PUFA n-3	28.6 ± 0.6 ^{a}	25.2 ± 0.4 ^{b}	22.8 ± 1.2 ^{c}	< 0.001
Σ PUFA n-6	11.1 ± 0.3 ^{c}	13.4 ± 0.7 ^{b}	15.7 ± 0.2 ^{a}	< 0.001
Σ PUFA n-3/Σ PUFA n-6	2.6 ± 0.10 ^{c}	1.9 ± 0.02 ^{b}	1.4 ± 0.1 ^{a}	< 0.001
Fatty acids (g 100 g^{-1} WW)				
Σ SFA	0.4 ± 0.04	0.5 ± 0.04	0.4 ± 0.08	0.267
Σ MUFA	0.4 ± 0.05	0.5 ± 0.05	0.5 ± 0.1	0.372
C20:5n-3 (EPA)	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.212
C22:6n-3 (DHA)	0.2 ± 0.02	0.2 ± 0.01	0.2 ± 0.01	0.049
EPA + DHA	0.3 ± 0.03	0.3 ± 0.02	0.3 ± 0.03	0.071
Σ PUFA	0.5 ± 0.06	0.6 ± 0.04	0.6 ± 0.08	0.371
Σ n-3 PUFA	0.4 ± 0.04	0.4 ± 0.03	0.3 ± 0.04	0.096
Σ n-6 PUFA	0.1 ± 0.02 ^{b}	0.2 ± 0.02 ^{a}	0.2 ± 0.04 ^{a}	0.007

^{1} Includes C12:0, C15:0, C20:0, C22:0 and C24:0; ^{2} Includes: C14:1, C17:1, C18:1n-7 and C22:1n-9; ^{3} Includes C18:3n-6c and C20:2. Values are presented as mean ± standard deviation; n = 4. Different superscript letters represent significant differences ($p < 0.05$); one-way ANOVA (post-hoc Tukey's test). The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of fishmeal protein replacement by defatted *Tenebrio molitor* larvae meal, respectively. Other abbreviations stand for: ALA, α-linolenic acid; DHA, docosahexaenoic; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acids; OA, oleic acid; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; WW, wet weight.

Supplementary Table S14. Cellularity, instrumental texture and colour parameters of European sea bass fed experimental diets.

	CTRL	TM50	TM100	*p*-value
Muscle cellularity				
White muscle CSA (mm^2)	848.9 ± 110.5	815.0 ± 151.4	863.1 ± 132.4	0.671
Total nº of fibers × 10^3	142.9 ± 26.2 ^{ab}	132.1 ± 30.1 ^{b}	169.3 ± 36.8 ^{a}	0.022
Fibers density (nº mm^{-2})	168.5 ± 24.6 ^{ab}	162.9 ± 33.1^{b}	194.8 ± 21.0 ^{a}	0.014
Fibers diameter (μm^2)	84.1 ± 6.9 ^{ab}	86.1 ± 7.9 ^{a}	77.5 ± 4.7 ^{b}	0.007
Muscle instrumental texture				
Hardness (N)	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	0.437
Adhesiveness (J)	-0.03 ± 0.02	-0.03 ± 0.02	-0.03 ± 0.02	0.898
Springiness	1.0 ± 0.02	1.2 ± 0.6	1.0 ± 0.02	0.146
Cohesiveness	0.39 ± 0.1 ^{b}	0.44 ± 0.1 ^{a}	0.42 ± 0.1 ^{ab}	0.042
Chewiness (J)	0.4 ± 0.1	0.5 ± 0.3	0.4 ± 0.1	0.154
Resilience	0.23 ± 0.04 ^{b}	0.30 ± 0.1 ^{a}	0.25 ± 0.1 ^{b}	< 0.001
Muscle instrumental colour				
L*	40.8 ± 1.8	39.7 ± 3.2	40.2 ± 2.1	0.594
a*	-3.1 ± 0.4	-3.1 ± 0.4	-3.1 ± 0.4	0.311
b*	2.2 ± 0.5	2.2 ± 0.7	2.3 ± 0.6	0.936
C*	4.0 ± 0.4	3.9 ± 0.3	3.9 ± 0.4	0.834
H°	146.6 ± 5.6	142.5 ± 7.5	144.1 ± 9.2	0.438
Skin instrumental colour				
L*	43.7 ± 3.5	40.3 ± 2.2	43.1 ± 3.0	0.228
a*	-4.0 ± 0.9	-3.5 ± 0.8	-3.7 ± 0.8	0.328
b*	3.3 ± 1.4	3.4 ± 1.1	4.4 ± 1.6	0.123
C*	4.6 ± 0.7	4.9 ± 1.2	5.4 ± 1.0	0.206
H°	141.6 ± 6.6 ^{a}	135.9 ± 7.7 ^{ab}	129.7 ± 5.7 ^{b}	< 0.001

Values are presented as mean ± standard deviation; n = 12. Different superscript letters represent significant differences ($p < 0.05$); one-way ANOVA (post-hoc Tukey's test). The abbreviations for the experimental diets stand for: CTRL, control diet; TM50 and TM100, diets with 50 and 100% of fishmeal protein replacement by defatted *Tenebrio molitor* larvae meal, respectively. Other abbreviations stand for: a*, redness; b*, yellowness; C*, chroma; CSA, cross-section area; H°, hue; L*, lightness.

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