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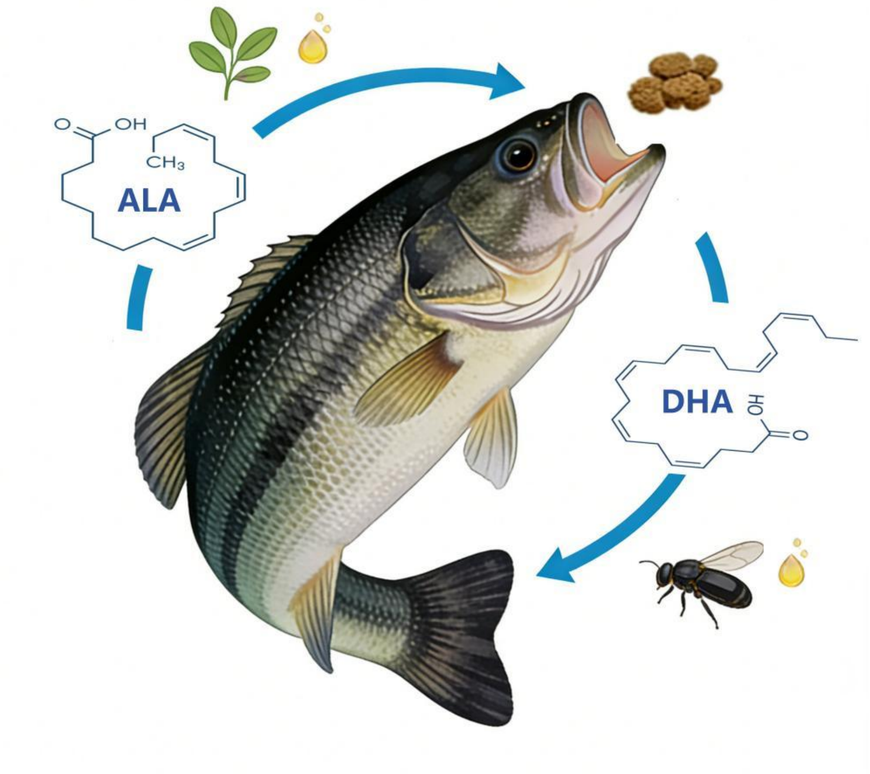
Study on the n-3 LC-PUFA Biosynthetic Capacity of Largemouth Bass *Micropterus salmoides* and the Effects of Different Lipid Sources on Its Growth and Lipid Metabolism

Yangyang Liu



Promoteur(s): Profs Rudy Megido Caparros & Min Xue

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COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

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Abstract

Yangyang Liu (2026). "Study on the n-3 LC-PUFA Biosynthetic Capacity of Largemouth Bass *Micropterus salmoides* and the Effects of Different Lipid Sources on Its Growth and Lipid Metabolism".

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Long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA), especially eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), are indispensable nutrients for human health and for optimal growth and development in fish. Although some fish species possess the capacity to biosynthesize n-3 LC-PUFA endogenously from α-linolenic acid (ALA, C18:3n-3), this biosynthesis potential varies widely among species and is strongly constrained by trophic level and feeding ecology. As a major freshwater carnivorous species in global aquaculture, largemouth bass *Micropterus salmoides* exhibits a high dependence on dietary EPA and DHA and a limited capacity for endogenous biosynthesis, which has become a critical bottleneck for fish oil (FO) replacement and the sustainable development of aquaculture. Therefore, elucidating its biosynthetic capacity, regulatory mechanisms and nutritional modulation of n-3 LC-PUFA is of fundamental importance for optimizing lipid nutrition strategies and developing sustainable alternative lipid resources.

In this thesis, we addressed the central question of "the endogenous biosynthetic capacity and nutritional regulation of n-3 LC-PUFA in largemouth bass" by integrating molecular, cellular and whole-animal approaches, including heterologous expression, primary hepatocyte culture, stable isotope tracing and multi-factor feeding trials. Through this multi-level strategy, we systematically clarified the existence, limitations and nutritional modulation of the biosynthetic pathway.

At the molecular level, heterologous expression in yeast identified two key desaturase genes: *fads2* (fatty acid desaturase 2), which exhibited Δ6 desaturase activity catalyzing the conversion of ALA to C18:4n-3, and *delta4 fads* (acyl-CoA Delta4 desaturase-like), which possessed dual Δ5 and Δ4 activities, converting C20:4n-3 to EPA and C22:5n-3 to DHA, respectively. These results provide direct enzymatic evidence that largemouth bass possesses a complete set of key desaturation steps required for n-3 LC-PUFA biosynthesis. At the cellular level, primary hepatocyte experiments showed that exogenous ALA was efficiently incorporated into cells; however, ALA incubation did not lead to a significant increase in DHA content ($P > 0.05$), suggesting that downstream elongation and terminal desaturation steps constitute major metabolic constraints. In vivo stable isotope tracing further demonstrated the physiological relevance of this pathway by detecting ^{13}C-labelled C20:4n-3 derived from ^{13}C-ALA in plasma of largemouth bass. Taken together, these molecular, cellular and in vivo results indicate that largemouth bass possesses a "functionally complete but tightly constrained" endogenous n-3 LC-PUFA biosynthesis system, in which substrate availability alone is insufficient to ensure effective DHA accumulation.

Building on this mechanistic foundation, we conducted a series of feeding trials to further clarify how nutritional factors modulate this inherently limited biosynthetic capacity. A 2 × 2 factorial feeding trial was designed with oil source (soybean oil vs. cottonseed oil) and inclusion level (50% vs. 100% vegetable oil replacement) as experimental factors, with all diets formulated to contain 30% fish meal as the basal protein source. Although the soybean oil (SO) groups exhibited higher dietary ALA and increased desaturase gene expression, neither tissue n-3 LC-PUFA levels nor hepatic desaturase abundances were significantly enhanced ($P > 0.05$), indicating that transcriptional upregulation alone is insufficient to overcome downstream metabolic bottlenecks. In contrast, cottonseed oil (CO) significantly reduced final body weight, weight gain and specific growth rate while increasing feed conversion ratio ($P < 0.05$), while inducing enhanced lipid catabolism, as indicated by elevated *atgl* (Adipose triglyceride lipase) expression and p-AMPK (Phosphorylated AMP-activated protein kinase) levels ($P < 0.05$). Collectively, these results suggest that the combination of ALA deficiency and accelerated lipid breakdown represents a key mechanism underlying CO-induced growth depression. Moreover, the findings demonstrate that when exogenous n-3 LC-PUFA supply is sufficient to support growth, additional ALA supplementation does not further stimulate endogenous n-3 LC-PUFA biosynthesis.

In the 2 × 2 factorial trial based on purified diets completely free of fish meal and FO, the complementary roles of ALA and DHA were clearly demonstrated. Fish fed linseed oil (LO) exhibited significantly higher growth indicators and tissue n-3 PUFA levels than those fed CO ($P < 0.05$), whereas *delta4 fads* and *elovl5* expression were lower in the LO-fed groups than in the CO-fed groups ($P < 0.05$). Dietary DHA inclusion significantly improved growth performance and tissue DHA levels in CO-fed fish ($P < 0.05$), while reducing liver crude lipid and triglyceride contents ($P < 0.05$). At the same time, DHA significantly downregulated hepatic *elovl 8a* expression and reduced $\Delta 5$ and $\Delta 4$ Fads protein abundances ($P < 0.05$), indicating a strong feedback inhibition of endogenous n-3 LC-PUFA biosynthesis. These results indicate that while adequate dietary ALA supported growth and DHA biosynthesis, severe ALA deficiency upregulated partial n-3 LC-PUFA biosynthesis-related genes but still reduced tissue DHA, with dietary DHA inclusion alleviating growth constraints under ALA-deficient conditions.

At the level of feed resource innovation, this study further utilized black soldier fly *Hermetia illucens* (BSF) larvae as a functional lipid carrier. Dietary fish processing by-products significantly increased larval ALA and enabled the accumulation of EPA and DHA, which were undetectable in control larvae ($P < 0.05$). In the feeding trial, largemouth bass fed the DHA-enriched BSF oil (BSFO) diet exhibited significantly higher final body weight and weight gain and a lower feed conversion ratio than those fed the control BSFO diet ($P < 0.05$). DHA inclusion markedly increased DHA, total n-3 PUFA and the n-3/n-6 PUFA ratio in muscle and liver, while reducing n-6 PUFA accumulation ($P < 0.05$). Plasma lipid profiles were significantly improved, with higher HDL-c (High-density lipoprotein cholesterol) and lower LDL-c (Low-density lipoprotein cholesterol) levels ($P < 0.05$). Meanwhile, the protein abundances of $\Delta 6$, $\Delta 5$ and $\Delta 4$ Fads were significantly decreased by dietary DHA ($P < 0.05$), further confirming feedback inhibition of hepatic n-3 LC-PUFA biosynthesis.

In conclusion, largemouth bass possesses a low-efficiency but conditionally sufficient pathway for endogenous n-3 LC-PUFA biosynthesis. Under practical aquaculture conditions, ALA functions as a key essential fatty acid, supporting tissue EPA and DHA accretion and growth when supplied at adequate levels, whereas dietary DHA compensates for insufficient precursor availability. Furthermore, DHA deposition is regulated by both dietary supply and endogenous metabolism. Overall, SO, LO, moderately supplemented CO, and n-3 LC-PUFA-enriched BSFO represent promising sustainable alternatives to fish oil in largemouth bass diets.

Keywords: largemouth bass, n-3 LC-PUFA biosynthetic capacity, vegetable oils, black soldier fly oil, DHA inclusion, lipid metabolism

Yangyang Liu (2026). "Étude de la capacité biosynthétique des n-3 LC-PUFA chez le black-bass à grande bouche *Micropterus salmoides* et des effets de différentes sources lipidiques sur sa croissance et son métabolisme lipidique".

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206 pages, 22 figures, 38 tables.

Résumé

Les acides gras polyinsaturés à longue chaîne de la série n-3 (n-3 LC-PUFA), en particulier l'acide eicosapentaénoïque (EPA, C20:5n-3) et l'acide docosahexaénoïque (DHA, C22:6n-3), constituent des nutriments indispensables à la santé humaine ainsi qu'à la croissance et au développement optimal des poissons. Bien que certaines espèces de poissons soient capables de biosynthétiser de manière endogène des n-3 LC-PUFA à partir de l'acide α-linolénique (ALA, C18:3n-3), cette capacité varie fortement selon les espèces et demeure étroitement liée au niveau trophique et au comportement alimentaire. En tant qu'espèce carnivore d'eau douce majeure en aquaculture mondiale, le black-bass à grande bouche *Micropterus salmoides* présente une forte dépendance aux apports alimentaires exogènes en EPA et en DHA, ainsi qu'une capacité limitée de biosynthèse endogène. Cette contrainte constitue un obstacle majeur à la substitution de l'huile de poisson dans les aliments formulés et compromet le développement durable de l'aquaculture. Il est donc essentiel d'élucider sa capacité biosynthétique, ses mécanismes de régulation et leur modulation nutritionnelle afin d'optimiser les stratégies de nutrition lipidique et de développer des ressources lipidiques alternatives durables.

Dans cette thèse, nous avons abordé la question centrale de la « capacité de biosynthèse endogène et de la régulation nutritionnelle des n-3 LC-PUFA chez le bar à grande bouche » en intégrant des approches moléculaires, cellulaires et in vivo, comprenant l'expression hétérologue, la culture primaire d'hépatocytes, le traçage isotopique stable et des essais alimentaires multifactoriels. Cette stratégie multi-niveaux a permis de clarifier de manière systématique l'existence, les limites et la modulation nutritionnelle de la voie de biosynthèse.

Au niveau moléculaire, l'expression hétérologue dans la levure a permis d'identifier deux gènes clés de désaturases : *fads2* (fatty acid desaturase 2), présentant une activité Δ6 désaturase catalysant la conversion de l'ALA en C18:4n-3, et *delta4 fads* (acyl-CoA Delta4 desaturase-like), possédant des activités Δ5 et Δ4, responsables respectivement de la conversion du C20:4n-3 en EPA et du C22:5n-3 en DHA. Ces résultats fournissent des preuves enzymatiques directes attestant que le bar à grande bouche possède l'ensemble des étapes de désaturation nécessaires à la biosynthèse des n-3 LC-PUFA. Au niveau cellulaire, les expériences menées sur des hépatocytes primaires ont montré que l'ALA exogène était efficacement incorporé dans les cellules ; toutefois, son incubation n'a pas entraîné d'augmentation significative de la teneur en DHA ($P > 0,05$), ce qui suggère que les étapes d'élongation et de désaturation terminales constituent des contraintes métaboliques majeures. Le traçage isotopique in vivo a confirmé la pertinence physiologique de cette voie en mettant en évidence la présence de C20:4n-3 marqué au ^{13}C dérivé du ^{13}C-ALA dans le plasma. L'ensemble de ces résultats indique que le bar à grande bouche dispose d'un système de biosynthèse endogène des n-3 LC-PUFA « fonctionnellement complet mais fortement

contraint », dans lequel la disponibilité du substrat ne suffit pas à garantir une accumulation efficace de DHA.

Sur la base de ces mécanismes, une série d'essais alimentaires a été menée afin d'évaluer l'influence des facteurs nutritionnels sur cette capacité biosynthétique limitée. Un dispositif factoriel 2 × 2 a été mis en place, combinant la source lipidique (huile de soja et huile de coton) et le niveau d'incorporation (50 % et 100 % de substitution de l'huile de poisson par des huiles végétales), avec des régimes contenant 30 % de farine de poisson comme source protéique de base. Bien que les groupes recevant l'huile de soja aient présenté une teneur alimentaire plus élevée en ALA et une augmentation de l'expression des gènes de désaturases, ni les niveaux tissulaires en n-3 LC-PUFA ni les abondances protéiques hépatiques correspondantes n'ont été significativement améliorés ($P > 0,05$). Ces résultats indiquent que la régulation transcriptionnelle ne suffit pas à surmonter les contraintes métaboliques en aval. En revanche, l'huile de coton (CO) a significativement réduit le poids corporel final, le gain de poids et le taux de croissance spécifique, tout en augmentant l'indice de conversion alimentaire ($P < 0,05$). Elle a également stimulé le catabolisme lipidique, comme en témoignent l'augmentation de l'expression de l'atgl (lipase des triglycérides adipocytaires) et des niveaux de p-AMPK (protéine kinase activée par l'AMP phosphorylée) ($P < 0,05$). Ces observations suggèrent que la combinaison d'une carence en substrat et d'une accélération de la dégradation lipidique constitue un mécanisme clé expliquant la diminution de croissance induite par l'huile de coton. Par ailleurs, lorsque l'apport exogène en n-3 LC-PUFA est suffisant pour soutenir la croissance, une supplémentation supplémentaire en ALA ne stimule pas davantage la biosynthèse endogène.

Dans un second essai factoriel 2 × 2 reposant sur des régimes purifiés totalement dépourvus de farine et d'huile de poisson, les rôles complémentaires de l'ALA et du DHA ont été clairement démontrés. Les poissons nourris avec de l'huile de lin ont présenté des performances de croissance et des teneurs tissulaires en n-3 PUFA significativement supérieures à celles nourries avec de l'huile de coton ($P < 0,05$), tandis que l'expression de Δ4 fads et d'*elovl5* était plus faible dans les groupes recevant l'huile de lin ($P < 0,05$). L'inclusion alimentaire de DHA a significativement amélioré la croissance et la teneur tissulaire en DHA des poissons recevant l'huile de coton ($P < 0,05$), tout en réduisant les teneurs hépatiques en lipides bruts et en triglycérides ($P < 0,05$). Parallèlement, le DHA a fortement réprimé l'expression hépatique de *elovl8a* et diminué l'abondance protéique des Δ5 et Δ4 Fads ($P < 0,05$), traduisant une inhibition rétrocontrôle marquée de la biosynthèse endogène des n-3 LC-PUFA. Ces résultats montrent que si un apport adéquat en ALA peut soutenir partiellement la croissance et la biosynthèse du DHA, une carence sévère en ALA entraîne une surexpression partielle des gènes impliqués dans la biosynthèse des n-3 LC-PUFA sans pour autant restaurer les niveaux tissulaires en DHA. L'inclusion de DHA alimentaire permet en revanche d'atténuer les contraintes de croissance en conditions de carence en ALA.

Au niveau de l'innovation des ressources alimentaires, cette étude a en outre utilisé les larves de mouche soldat noire *Hermetia illucens* (BSF) comme vecteur fonctionnel de lipides. Les sous-produits de transformation du poisson incorporés dans l'alimentation ont significativement augmenté la teneur en ALA des larves et permis l'accumulation d'EPA et de DHA, qui étaient indétectables chez les larves témoins ($P < 0,05$). Dans l′essai d′

alimentation, les poissons recevant une BSFO enrichie en DHA ont présenté de meilleures performances de croissance, avec un poids final et un gain de poids plus élevés, ainsi qu'un indice de conversion alimentaire réduit ($P < 0,05$). L'inclusion de DHA a permis d'augmenter de manière significative les teneurs en DHA, en n-3 PUFA totaux et le ratio n-3/n-6 dans les muscles et le foie, tout en réduisant l'accumulation de n-6 PUFA ($P < 0,05$). Les profils lipidiques plasmatiques ont également été améliorés, avec une augmentation du HDL-c (cholestérol des lipoprotéines de haute densité) et une diminution du LDL-c (cholestérol des lipoprotéines de basse densité) ($P < 0,05$). Par ailleurs, les abondances protéiques des $\Delta6$, $\Delta5$ et $\Delta4$ Fads ont été significativement réduites par le DHA alimentaire ($P < 0,05$), confirmant l'existence d'une inhibition rétrocontrôle de la biosynthèse hépatique des n-3 LC-PUFA.

En conclusion, le black-bass possède une voie endogène de biosynthèse des LC-PUFA n-3 à faible efficacité mais suffisante dans certaines conditions. Dans des conditions d'élevage pratiques, l'ALA agit comme un acide gras essentiel clé, capable de soutenir l'accumulation tissulaire d'EPA et de DHA ainsi que la croissance lorsqu'il est apporté en quantité suffisante, tandis que le DHA alimentaire compense une disponibilité insuffisante de précurseurs. Par ailleurs, le dépôt tissulaire de DHA est régulé à la fois par l'apport alimentaire et par les mécanismes métaboliques endogènes. Dans l'ensemble, SO, LO, CO modérément supplémentée et BSFO enrichie en LC-PUFA n-3 présentent un potentiel considérable comme alternatives à la FO dans l'alimentation du black-bass.

Mots-clés : *black-bass à grande bouche*, capacité de biosynthèse des n-3 LC-PUFA, huiles végétales, incorporation de DHA, huile de mouche soldat noire, métabolisme des lipidique.

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I am profoundly grateful to my supervisors, Prof. Rudy megido Caparros, Prof. Min Xue and Prof. Xiaofang Liang for their continuous guidance, critical insights, and unwavering support throughout my doctoral study. Their expertise, intellectual rigor, and constructive feedback were instrumental in shaping the scientific framework of this work, from experimental design and methodological development to data interpretation and manuscript preparation.

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Yangyang Liu
12/05/2026 in Beijing, China

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List of Abbreviations

ALA	α-Linolenic acid (C18:3n-3)
AMPK	AMP-activated protein kinase
ATGL	Adipose triglyceride lipase
ARA	Arachidonic acid (C20:4n-6)
BSFO	Black soldier fly (BSF) oil
cAMP	Cyclic Adenosine Monophosphate
CF	Condition factor
CO	Cottonseed oil
CR	Carcass ratio
DHA	Docosahexaenoic acid (C22:6n-3)
EPA	Eicosapentaenoic acid (C20:5n-3)
Elovl	Elongation of very long-chain fatty acids
FA	Fatty acid
FADS	Fatty acyl desaturase
FBW	Final body weight
FCR	Feed conversion ratio
FIFO	Fish In: Fish Out
FM	Fishmeal
FO	Fish oil
FPBs	Fish processing by-products
FR	Feed rate
HDL-c	High-density lipoprotein cholesterol
H&E	hematoxylin–eosin
HSI	Hepatopancreas somatic index
LA	Linoleic acid (C18:2n-6)
LC-PUFA	Long-chain polyunsaturated fatty acids
LDL-c	Low-density lipoprotein cholesterol
LO	Linseed oil
LPV	Lipid productive value
MUFA	Monounsaturated fatty acids
OA	oleic acid (C18:1n-9)

PER	Protein efficiency ratio
PPV	Protein productive value
PUFA	polyunsaturated fatty acids
p-AMPK	Phosphorylated AMP-activated protein kinase
SCM	*Schizochytrium sp.* meal
SEM	Standard error of the mean
SFA	Saturated fatty acids
SGR	Specific growth rate
SO	Soybean oil
SR	Survival rate
TBA	Total bile acid
TC	Total cholesterol
TG	Triglycerides
VAI	Visceral adipose index
VO	vegetable oils
VSI	Viscera somatic index
WG	Weight gain
WHC	Water-holding capacity
Δ6 Fads	Delta-6 Fatty acyl desaturase
Δ5 Fads	Delta-5 Fatty acyl desaturase
Δ4 Fads	Delta-4 Fatty acyl desaturase
^{13}C-ALA	^{13}C-labeled α-linolenic acid

1

Chapter I General introduction

Abstract

Long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA), particularly eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), are essential nutrients for human health as well as for optimal growth and development in fish. Aquatic organisms, including fish, constitute the primary dietary source of these fatty acids worldwide, yet some fish species are also capable of synthesizing them *de novo* through the endogenous conversion of α-linolenic acid (ALA, C18:3n-3). This conversion proceeds via sequential desaturation and elongation reactions catalyzed by fatty acyl desaturases (Fads) and elongases (Elovl). Notably, most teleosts lack Fads1 and instead rely on a functionally diversified Fads2 for these reactions.

Species variation in n-3 LC-PUFA biosynthetic capacity has long been interpreted through a freshwater–marine dichotomy. However, accumulating evidence indicates that trophic level and feeding ecology more accurately explain differences in *fads* and *elovl* gene complement, enzymatic function, and regulatory responses. Recent methodological advances, such as heterologous expression systems, primary hepatocyte cultures, isotope tracing, and in vivo feeding trials, have improved our understanding of LC-PUFA biosynthesis in fish. As a major freshwater carnivorous, however, the molecular regulation and species-specific limitations of largemouth bass *Micropterus salmoides* remain insufficiently resolved.

Accordingly, this chapter reviews LC-PUFA biosynthetic pathways, regulatory mechanisms, species variation and methodological approaches, identifies key knowledge gaps associated with the restricted biosynthesis potential of largemouth bass. Based on this background, the research objectives and experimental strategies of the thesis were formulated, aiming to clarify conversion capacity, regulatory mechanisms and nutritional modulation of n-3 LC-PUFA biosynthesis in this species.

Keywords: n-3 LC-PUFA biosynthesis, largemouth bass, fatty acyl desaturase and elongase, nutritional modulation

1. Background

As wild-capture fisheries have reached a plateau, the rapid expansion of aquaculture has become the dominant source of dietary long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFAs, omega-3 fatty acids with ≥20 carbon atoms and ≥2 double bonds) for human consumption (FAO, 2026) (Figure 1-1A). Global per capita apparent consumption of aquatic animal foods has more than doubled, increasing from 9.2 kg in 1961 to 21.1 kg in 2023, with preliminary estimates reaching 21.3 kg in 2024 (FAO, 2026) (Figure 1-1B). Beyond delivering high-quality protein, aquaculture is now increasingly evaluated through its ability to supply essential bioactive lipids, including eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22: 6n-3), which are linked to neurological development and cardiometabolic health in human consumers (Beyer et al., 2023; Tabilo et al., 2025).

In recent decades, the composition of aquafeeds has shifted from marine-derived lipids toward vegetable oils (VO) due to sustainability concerns and fluctuating fish oil (FO) availability. This shift has introduced challenges for LC-PUFA deposition, especially in carnivorous species that rely heavily on EPA and DHA (Tocher, 2010; Xie et al., 2021). As FO and fishmeal (FM) usage continues to decline due to sustainability and supply constraints, marked species-dependent differences in endogenous LC-PUFA biosynthesis have become more apparent (Monroig & Kabeya, 2018). Understanding how different species meet their metabolic requirements under reduced marine inputs therefore has significant implications for feed formulation, fish health, and product quality.

Moreover, recent findings suggest that LC-PUFA biosynthesis potential in fish cannot be explained solely by ecological origin. Although freshwater species are often assumed to retain higher conversion capacity, newly characterized teleosts demonstrate considerable functional diversity in fatty acid metabolism, independent of habitat classification (Castro et al., 2016). Trophic position, feeding strategy, and evolutionary history all appear to contribute to distinct lipid metabolic phenotypes across taxa, highlighting the need for functional characterization at the species level rather than relying on traditional ecology-based assumptions (Li et al., 2010a; Trushenski & Rombenso, 2020).

Even though research has expanded rapidly, our understanding of LC-PUFA biosynthesis is still incomplete for many major aquaculture species, and for several commercially important carnivorous fishes we still lack clear molecular explanations for their poor conversion capacity. These knowledge gaps constrain the development of targeted nutritional strategies, including precursor optimization, dietary lipid substitution, and regulation of lipid metabolic pathways, with the aim of improving LC-PUFA deposition under fish-oil-reduced diets (Gregory et al., 2016; Li et al., 2024; Liu et al., 2025). Therefore, a deeper mechanistic understanding of biosynthesis potential represents not only a fundamental biological question but also a prerequisite for improving the sustainability and nutritional value of modern aquaculture.

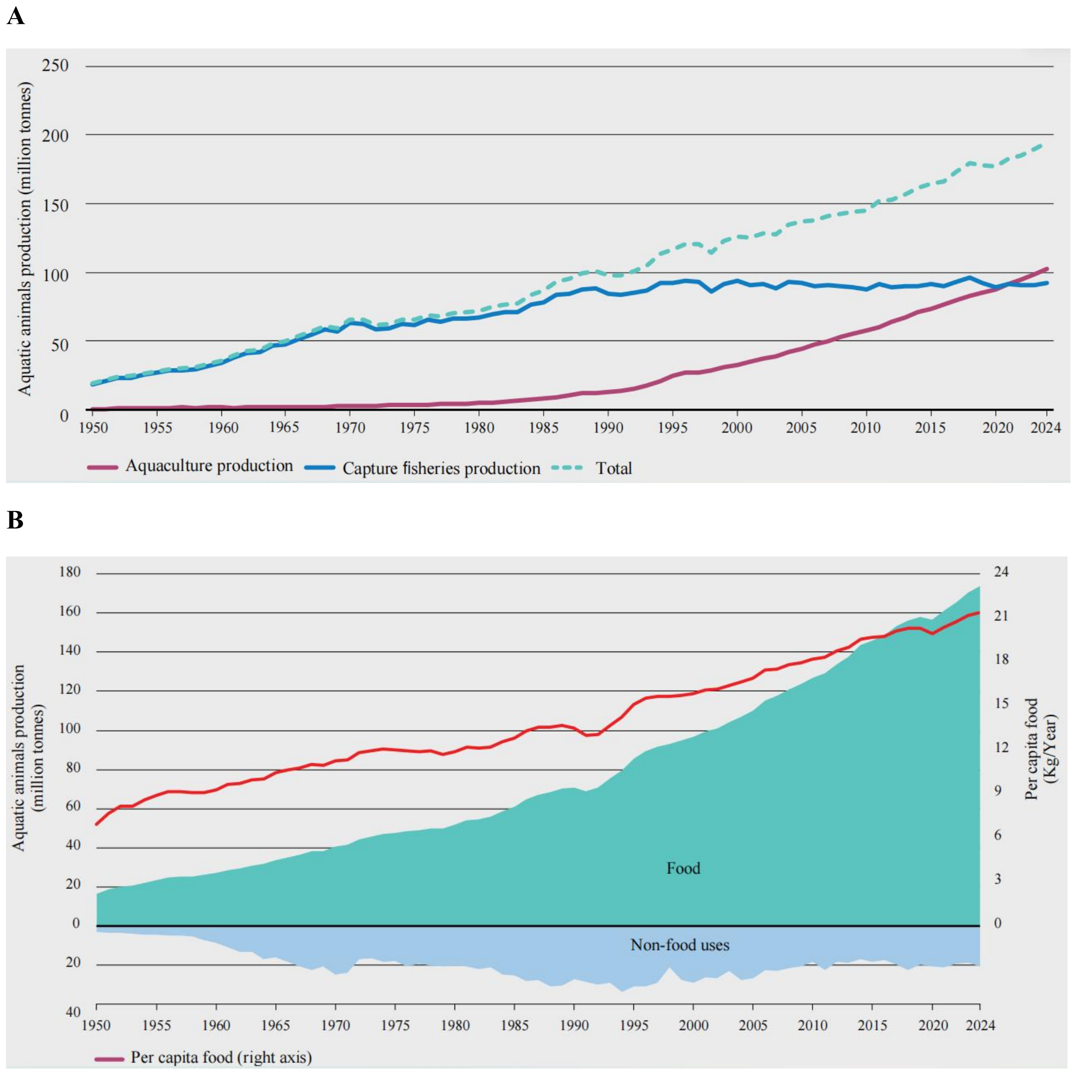


Figure 1-1 World fisheries and aquaculture production and utilization of aquatic animals (adapted from FAO, 2026). Aquatic animals exclude aquatic mammals, crocodiles, alligators, caimans, sponges, corals, pearls and algae. Data are expressed in live weight equivalent. (A) World fisheries and aquaculture production from 1950 to 2024. The red solid line represents annual global aquaculture production of aquatic animals. The blue solid line represents global capture fisheries production, while the blue dashed line indicates the combined production of global aquaculture and capture fisheries. (B) Utilization of world fisheries and aquaculture production of aquatic animals from 1950 to 2024. The green and blue areas represent food and non-food uses, respectively (left axis), while the red line represents global per capita consumption of aquatic animal foods (right axis).

2. Literature review

2.1 Global aquaculture development and the resource constraints of fish oil

Aquaculture has become the fastest-growing sector of animal food production, now accounting for more than half of the aquatic products consumed worldwide (FAO, 2026). Aquatic products, such as fish and seafood is not only a rich source of high-quality protein but also a major dietary supplier of valuable n-3 LC-PUFA, particularly EPA and DHA (Asfaw et al., 2021; Glencross, 2009). These fatty acids play crucial roles in numerous physiological and biochemical processes, including growth, reproduction, neural and visual development, immune regulation, anti-inflammatory responses, and cardiovascular health in humans (Firat et al., 2017; Li et al., 2020; Tabilo et al., 2025).

FO has long been the primary lipid source in aquafeeds due to its high content of n-3 LC-PUFA, which are essential for maintaining normal physiological functions and ensuring the nutritional quality of farmed fish (Péron et al., 2025; Tocher, 2010) (Figure 1-2). However, the supply of FO is inherently constrained by the stagnation of global capture fisheries, which have remained relatively stable over the past decade (FAO, 2026; Sahin, 2025) (Figure 1-3A). Because FO is mainly derived from wild-caught small pelagic fish, its production is tightly coupled to capture fisheries and thus characterized by limited scalability and pronounced interannual variability.

Moreover, increasing competition from the nutraceutical and pharmaceutical sectors has further diverted FO toward direct human consumption, exacerbating supply limitations (Figure 1-3B). As a result, the global availability of FO is insufficient to meet the rapidly growing demand from aquaculture, posing a significant challenge to the sustainable development of the industry. This persistent imbalance between supply and demand has accelerated the search for alternative lipid sources, particularly VO and other novel substitutes, to partially or fully replace FO in aquafeeds.

While FO replacement can reduce growth performance and fillet LC-PUFA content, the effects depend largely on the type of alternative lipid source and its interaction with endogenous LC-PUFA biosynthesis. Notably, freshwater and anadromous/diadromous species generally possess higher bioconversion capacity, enabling them to synthesize EPA and DHA more efficiently from dietary precursors. This metabolic advantage reduces the need for direct dietary supplementation of these fatty acids, thereby decreasing dependence on marine-derived oils and enhancing production sustainability (Qian et al., 2020; Roy et al., 2023). Consequently, a thorough understanding of species-specific LC-PUFA biosynthesis is essential for optimizing FO replacement strategies and developing sustainable aquafeeds.

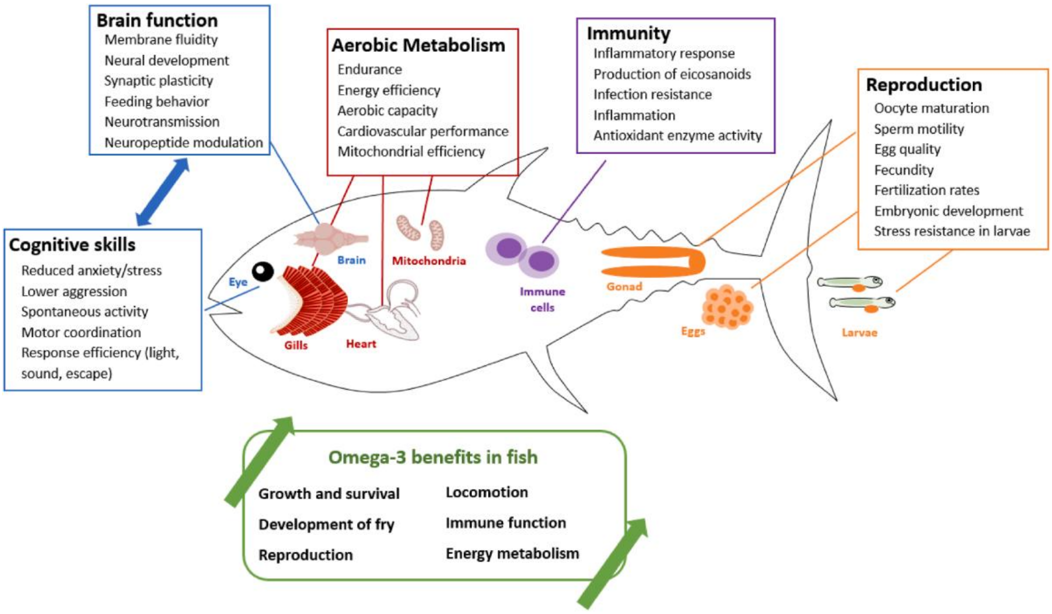


Figure 1-2 Schematic representation of the multifaceted physiological and behavioral roles of omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) in fish (Péron et al., 2025).

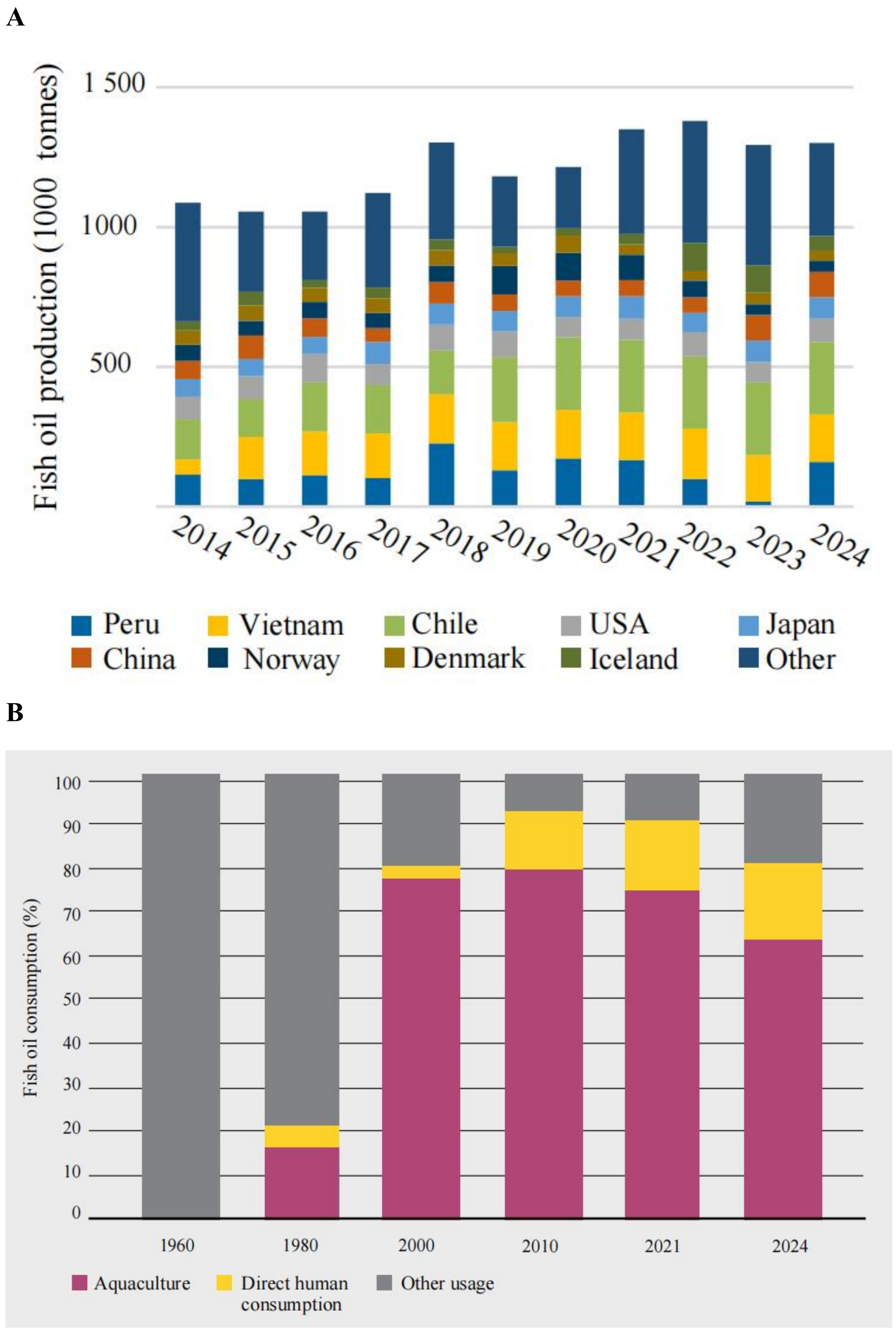


Figure 1-3 World fish oil production and utilization (adapted from IFFO, 2024 and FAO, 2026). (A) Fish oil production by country; (B) Percentage distribution of fish oil utilization by end use. Other usage includes pet food, biofuel, and cooking oil.

2.2 n-3 LC-PUFA biosynthetic pathway and key enzymes in teleosts

Essential fatty acids (EFA) refers to fatty acids that fish must obtain from external sources due to their limited ability to synthesize them (Glencross, 2009; Kaur et al., 2014; Sargent et al., 1995). Fish lack the ability to synthesize C18 PUFA—linoleic acid (LA, C18:2n-6) and α-linolenic acid (ALA, C18:3n-3) — *de novo*. Traditionally, freshwater fish have been considered capable of converting dietary C18 PUFA into LC-PUFA, rendering C18 PUFA sufficient to meet their EFA requirements; in contrast, marine fish are viewed as lacking this bioconversion capacity, making LC-PUFA their essential dietary fatty acids (Gou et al., 2020).

To be specific, the classical dichotomy posits that a species' LC-PUFA conversion capacity is shaped by its ecological background (Sargent et al., 2002; Sargent et al., 1995; Tocher, 2003). Marine fishes primarily consume zooplankton and small fish that are abundant in their natural marine environment, which supply sufficient dietary EPA and DHA, thereby reducing the evolutionary pressure to retain a fully functional endogenous biosynthetic pathway (Bell & Tocher, 2009; Castro et al., 2016). In contrast, freshwater fish have more complex diets dominated by algae, insects, and benthic invertebrates—that are typically rich in ALA but poor in n-3 LC-PUFAs, thus favoring reliance on endogenous biosynthesis (Xie et al., 2021).

However, as more species have been investigated, it has become evident that the traditional binary classification—which simply categorizes fish as either "capable" or "incapable" of synthesizing n-3 LC-PUFAs, or as "freshwater" versus "marine" species—fails to accurately capture interspecific differences (Castro et al., 2016; Tocher, 2010).

2.2.1 Biosynthetic pathways of n-3 LC-PUFA

In teleosts, n-3 long-chain polyunsaturated fatty acids (LC-PUFAs), including EPA, and DHA, can be synthesized from the essential precursor α-linolenic acid (ALA, C18:3n-3) through a series of alternating desaturation and elongation reactions catalyzed by fatty acyl desaturases (Fads) and elongases of very long-chain fatty acids (Elovl) (Monroig et al., 2018; Xie et al., 2021) (Figure 1-4). Parallel biosynthetic pathways also exist for n-6 LC-PUFAs, in which LA, serves as the precursor for the production of arachidonic acid (ARA, C20:4n-6) and other n-6 LC-PUFAs. The generally accepted pathway begins with Δ6 desaturation of ALA to produce C18:4n-3, followed by elongation to C20:4n-3 and subsequent Δ5 desaturation to generate EPA. This sequence of reactions constitutes the core pathway for the endogenous biosynthesis of n-3 LC-PUFAs in teleost fish.

The conversion of EPA to DHA represents the most critical and often rate-limiting stage of n-3 LC-PUFA biosynthesis. In most teleost species, DHA synthesis occurs predominantly through the "Sprecher pathway", which is widely recognized as the canonical route for DHA production. In this pathway, EPA is first elongated to docosapentaenoic acid (DPA, C22:5n-3), followed by further elongation to C24:5n-3. Subsequently, C24:5n-3 undergoes Δ6 desaturation to form C24:6n-3, which is then transported to peroxisomes and shortened through one cycle of β-oxidation to yield DHA (Xie et al., 2021). The Sprecher pathway has been identified in numerous freshwater and marine fish species and is considered the predominant mechanism for DHA biosynthesis in teleosts.

In addition to the Sprecher pathway, alternative biosynthetic routes have been reported in some fish species. One alternative is the Δ8 pathway, in which elongation of ALA to C20:3n-3 precedes Δ8 desaturation, thereby bypassing the initial Δ6 desaturation step. This pathway may provide a complementary route for LC-PUFA synthesis when Δ6 desaturase activity is limited. Another alternative is the Δ4 pathway, in which DPA is directly converted to DHA through the action of a Δ4 desaturase, eliminating the requirement for elongation to C24 intermediates and subsequent β-oxidation. Functional Δ4 desaturases have been identified in a limited number of marine teleost species, suggesting that this pathway is taxonomically restricted rather than universally distributed among fish (Li et al., 2010b; Xie et al., 2021; Xie et al., 2013).

Although several alternative routes have been described, the Sprecher pathway remains the predominant and most extensively documented mechanism for DHA biosynthesis in teleosts. The relative contribution of each pathway varies among species and is largely determined by the presence, substrate specificity, and catalytic efficiency of Fads and Elovl enzymes, which ultimately govern the capacity of fish to synthesize LC-PUFAs from C18 precursors.

2.2.2 Key enzymes and regulation of LC-PUFA biosynthesis

Fads play a central role in introducing double bonds at specific positions of the carbon chain. In most teleosts, only Fads2 orthologues are present, whereas mammals possess both Fads1 and Fads2 (with Δ5 and Δ6 activities, respectively) (Castro et al., 2016; Monroig et al., 2018). Teleost Fads2 with species-specific variations in gene copy number, and in rare basal groups such as the Japanese eel *Anguilla japonica*, both Fads1 and Fads2 are present (Lopes-Marques et al., 2018). To compensate for the absence of Fads1 in their genomes, teleost Fads2 enzymes appear to have functionally diversified during evolution (Xie et al., 2021), ranging from single Δ6 desaturase activity to bifunctional Δ6/Δ5 or additional Δ4 activities (Castro et al., 2016; Tocher, 2010). For example, some species, such as zebrafish *Danio rerio* (Hastings et al., 2001), African catfish *Clarias gariepinus* (Oboh et al., 2016), silver barb *Barbonymus gonionotus* (Janaranjani et al., 2018) and orange-spotted spinefoot *Siganus guttatus* (You et al., 2024) possess one Fads2 with bifunctional Δ6Δ5 desaturation activity. Pacific saury *Cololabis saira* (Matsushita et al., 2023), rabbitfish *Siganus canaliculatus* (Li et al., 2010a), Senegalese sole *Solea senegalensis Kaup* (Morais et al., 2012), del pescado blanco *Chirostoma estor* (Fads2b) (Fonseca-Madrigal et al., 2014), striped snakehead *Channa striata* (Kuah et al., 2015) possess one Fads2 with bifunctional Δ4Δ5 desaturation activity.

Complementing the desaturases, elongation of very long-chain fatty acid enzymes (Elovl2, Elovl5, and occasionally Elovl4) catalyze the stepwise addition of two-carbon units, extending the fatty acid chain to achieve the requisite length for biologically active LC-PUFAs (Ding et al., 2024; Monroig & Kabeya, 2018). Elovl5 typically extends C18–20 PUFA, while Elovl2 further extends C20–C22 PUFA, serving as a key step in LC-PUFA synthesis. Elovl enzymes cooperate with Fads to control the efficiency and direction of LC-PUFA biosynthesis, thereby playing a central role in regulating fatty acid composition in fish tissues. However, elongase functions, particularly those of Elovl5, are relatively conserved in many teleosts, whereas the presence and activity of Fads vary substantially among species (Xie et al., 2021; Xie et al., 2020). Consequently, desaturation reactions are considered key steps that largely determine the LC-PUFA biosynthetic capacity of fish.

The coordinated activity of Fads and Elovl determines species-specific LC-PUFA biosynthetic capacities and substrate preferences, which can be further modulated by dietary lipid sources and developmental stages (Castro et al., 2016; Monroig et al., 2009; Turkmen et al., 2019; Wang et al., 2023). For instance, Nile tilapia *Oreochromis niloticus* shows a stronger reliance on n-6 fatty acids, whereas rainbow trout *Oncorhynchus mykiss* exhibits a greater capacity for n-3 LC-PUFA biosynthesis, likely reflecting species-specific differences in the substrate preferences and activities of desaturases and elongases (Castro et al., 2016; Monroig et al., 2018). When FO is replaced with VO in fish feed (reducing the supply of

LC-PUFA), liver *elovl5* and *fads2* expression are usually upregulated to compensate for the lack of n-3 LC-PUFA (Marrero et al., 2021; Sourabié et al., 2019). Conversely, high intake of FO or feed rich in DHA and EPA will feedback inhibit the expression of these genes (Liang et al., 2022; Teoh & Ng, 2016). Such regulation is mediated by lipid-sensitive transcription factors, including the SREBP and PPAR pathways and integrates with physiological states such as growth and metabolic rate (Dong et al., 2017; Ferraz et al., 2021). Collectively, these coordinated regulatory mechanism underlies the adaptive diversity among fish species in response to dietary fatty acid variations.

Nevertheless, the biosynthetic capacity of fish is not fixed but rather condition-dependent, being influenced by multiple environmental and nutritional factors such as the availability of dietary precursors (e.g., ALA), water temperature, and habitat salinity (Colombo et al., 2023; Gregory et al., 2016; Villena-Rodríguez et al., 2025). These factors exert coordinated regulatory effects on the expression and catalytic activity of Fads and Elovl, leading to pronounced variations in the efficiency of endogenous LC-PUFA biosynthesis. Consequently, the actual capacity of fish to produce LC-PUFAs endogenously is shaped by the interplay between genetic potential and environmental or dietary modulation, rather than by phylogeny alone.

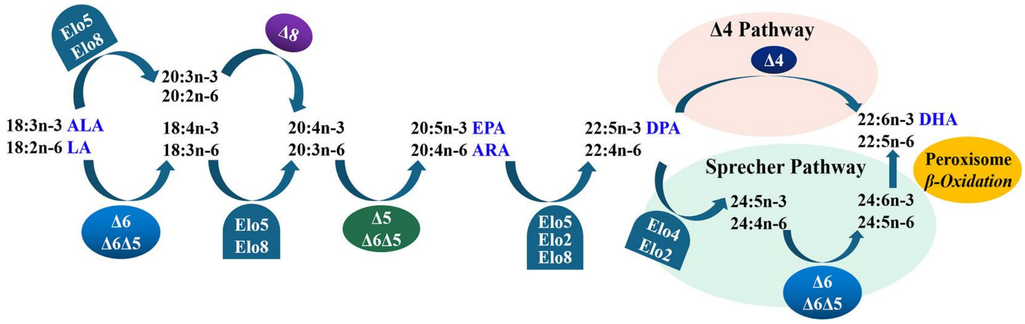


Figure 1-4. LC-PUFA biosynthetic pathway in fish (Xie et al., 2021). The biosynthesis of LC-PUFAs in fish relies on the coordinated action of desaturases and elongases. Fads2 introduce double bonds at specific positions (Δ6, Δ5, and Δ4), while elongases (Elovl2, Elovl5) extend the carbon chain by two-carbon units. Together, these enzymes convert C18 precursors such as ALA and LA into longer-chain products like EPA, DHA and ARA.

2.2.3 The LC-PUFA conversion capacities of different fish species

From an evolutionary perspective, the consistent availability of EPA and DHA in marine diets has relaxed the selective pressure to maintain a complete LC-PUFA biosynthetic pathway, leading to the loss or reduced functionality of one or more key enzymes required for their endogenous synthesis. For example, European seabass *Dicentrarchus labrax L.* and gilthead seabream *Sparus aurata* show partial or complete loss or weakening of Δ6 or Δ5 desaturase function (or rely on a bifunctional Δ4 Fads with limited activity), including sequence impairments or low transcriptional activity (González-Rovira et al., 2009; Tocher & Ghioni, 1999).

However, many freshwater species (e.g., common carp *Cyprinus carpio*) and diadromous fish species (e.g., Atlantic salmon *Salmo salar*) exhibit broad functional versatility of *fads2* genes, retaining Δ6 and Δ5 desaturation activities, and in some lineages even Δ4 activity, thereby supporting efficient conversion of dietary ALA into EPA and DHA (Bell, 2002; Castro et al., 2016; Glencross, 2009; Hastings et al., 2004; Monroig & Kabeya, 2018;

Monroig et al., 2010; Tocher, 2010; Tocher, 2003). This metabolic plasticity is considered an adaptive trait, allowing freshwater species to cope with environments where preformed DHA and EPA are limited.

While the study of rabbitfish (a marine herbivore fish with trophic level behind 2.8) confirmed that it has the function of Δ4 Fads, so it has the ability of converting DHA (Li et al., 2010a; Sargent et al., 1995; Xie et al., 2021). This finding challenges the traditional view that marine fish generally lack such biosynthetic capability. Recently, increasing evidence suggests that trophic level, an indicator of a species' position in the food chain, is a key determinant of LC-PUFA biosynthetic capacity and may represent the most reliable predictor of whether fish require C18 PUFAs or preformed LC-PUFAs (Trushenski & Rombenso, 2020). Therefore, a perspective based on trophic level and feeding habits provides a more informative framework (Monroig & Kabeya, 2018; Trushenski & Rombenso, 2020).

Freshwater fish species examined in previous studies generally occupy lower trophic levels (2.0–2.79) and primarily consume plant- or detritus-based diets (i.e., herbivores or omnivores), whereas marine species tend to occupy higher trophic levels (>2.8) and feed predominantly on animal prey (i.e., carnivores or piscivores) (Tacon et al., 2009). Consistent with this ecological differentiation, fish at lower trophic levels (<3) generally retain the capacity for de novo biosynthesis of LC-PUFAs from C18 precursors, whereas species occupying higher trophic levels (>4) typically exhibit limited biosynthetic capacity and rely largely on dietary LC-PUFAs to meet their physiological requirements (Trushenski & Rombenso, 2020). Fish at intermediate trophic levels (3-4) exhibit greater metabolic flexibility, with their LC-PUFA requirements influenced by habitat and lifestyle and life-history traits. However, the relationship between trophic level and PUFA metabolism remains complex and cannot be generalized across all fish species. Table 1-1 presents the differences in n-3 LC-PUFA biosynthesis among representative fish species, in relation to their trophic levels.

Research has shown that species at lower trophic levels or those that are predominantly herbivorous or omnivorous may retain substantial endogenous biosynthetic capacity, even across freshwater and marine environments, possess relatively strong biosynthesis abilities. Whereas carnivorous, higher-trophic-level species, depend more heavily on dietary n-3 LC-PUFAs (Trushenski & Rombenso, 2020; Wu et al., 2024). Studies have shown that several marine herbivorous or omnivorous fish, such as the rabbitfish and spotted scat *Scatophagus argus*, possess the enzymatic machinery required for n-3 LC-PUFA biosynthesis, including functional Δ6/Δ5 desaturases and elongases (Li et al., 2010b; Xie et al., 2014; Zhao et al., 2024). Conversely, freshwater carnivorous species such as Eurasian perch *Perca fluviatilis* (Geay et al., 2016), Chinese perch *Siniperca chuatsi* (He et al., 2025; Li et al., 2021), striped snakehead (Kuah et al., 2015), and largemouth bass *Micropterus salmoides* (Li et al., 2025; Yadav, 2020; Zhang et al., 2019a), as well as marine carnivorous species such as hybrid grouper *Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂ (Wu et al., 2022a) exhibited limited endogenous LC-PUFA biosynthesis capacities.

Table 1-1 Comparative LC-PUFA biosynthetic abilities of representative fish species across different trophic levels.

Category	Representative species	Trophic level[1]	EFA[2]	Fads	Notes	References
Freshwater fish	Nile tilapia	2.0	LA as the primary EFA	Δ6Δ5 Fads, Δ4 Fads	Typical fish species with "n-6 series FA dependence": higher efficiency in the LA →ARA pathway	(Oboh et al., 2017; Tocher, 2010)
	Zebrafish	3.1	ALA, LA	Δ6Δ5 Fads	Used as model for LC- PUFA biosynthesis studies	(Hastings et al., 2001; Tocher, 2010)
	Common carp	3.1	ALA, LA	Δ6Δ5 Fads	Typical freshwater omnivore with biosynthetic capability	(Marrero et al., 2022; Xie et al., 2017; Zhao et al., 2023)
	Rainbow trout	4.1	ALA as the primary EFA	Δ6 Fads, Δ5 Fads	Typical fish species with "n-3 series FA dependence": higher efficiency in the ALA→EPA, DHA pathway	(Abdul Hamid et al., 2016; Tocher, 2010; Zheng et al., 2004)
Diadromous fish	Japanese seabass *Lateolabrax japonicus*	3.1	LC- PUFA	Δ6Δ8 Fads	Lack Δ5 or Δ4 Fads required for LC-PUFA biosynthesis	(Wang et al., 2014; Xu et al., 2016)
	Atlantic salmon	4.5	ALA, LA	Δ6 Fads, Δ6Δ5 Fads	Adequate levels of LC-PUFA support growth and health during migratory and other critical physiological stages	(Hastings et al., 2004; Monroig et al., 2010; Tocher, 2010)
Marine fish	Rabbitfish	2.7	ALA, LA	Δ6Δ5 Fads, Δ4 Fads	Δ4 Fads was first found in this species	(Li et al., 2010a; Xie et al., 2018)
	European seabass	3.5	LC- PUFA	Δ6 Fads	Lack Δ5 or Δ4 Fads required for LC-PUFA biosynthesis	(Santigosa et al., 2011; Skalli & Robin, 2004)
	Gilthead seabream	3.7	LC- PUFA	Δ6Δ8 Fads		(Magalhães et al., 2020; Zheng et al., 2004)

1. Trophic level, acoording to Fishbase: https://www.fishbase.se/search.php.

2. Essential fatty acids (EFA) are fatty acids that fish cannot synthesize *de novo* in sufficient amounts and must obtain from the diet for normal growth and development. In species capable of converting C18 PUFA (LA, ALA) to LC-PUFA (ARA, EPA, DHA), LA and ALA are the EFA; whereas in species with limited or no such capacity, LC-PUFA are required as EFA(Gou et al., 2020; Kaur et al., 2014).

3. Fads: Fatty acyl desaturase.

2.3 Multi-level approaches to assess LC-PUFA biosynthesis in teleosts

To accurately evaluate interspecific variability in n-3 LC-PUFA biosynthetic capacity, it is important to adopt an integrated approach that combines evidence from multiple dimensions. At the molecular level, the presence and expression profiles of *fads* and *elovl* genes reveal the genetic basis underlying LC-PUFA synthesis (Castro et al., 2016; Monroig & Kabeya, 2018). At the metabolic level, stable isotope tracing provides quantitative insights into fatty acid conversion fluxes, thereby reflecting the actual efficiency of biosynthetic pathways (Mourente & Tocher, 1993; Ruyter et al., 2000; Tocher, 2010). At the nutritional level, feeding trials offer direct evidence of dietary dependence and physiological adaptation to different lipid sources (Liang et al., 2022; Torstensen et al., 2004; Yuan et al., 2024b). These include heterologous expression of candidate genes to determine enzymatic function, primary hepatocyte or enterocyte cultures to explore cellular-level metabolism, stable isotope tracing to quantify in vivo conversion rates, and feeding experiments to evaluate nutritional responses. Integrating these complementary lines of evidence enables a more comprehensive understanding of interspecific differences in LC-PUFA biosynthetic capacity, including insights derived from a combination of *in vitro* and in *vivo* approaches.

(1) Heterologous expression systems

Functional characterization of Fads and Elovl is often conducted using heterologous expression systems such as yeast *Saccharomyces cerevisiae* (*S. cerevisiae*). Candidate genes cloned from fish are expressed in these model systems, which lack or have minimal endogenous LC-PUFA synthesis, allowing clear assessment of enzymatic function. Substrates such as C18:3n-3 (ALA), C20:4n-3, and C22:5n-3 are supplied, and the resulting product profiles are analyzed using gas chromatography–mass spectrometry (GC–MS) to determine whether the enzyme exhibits Δ6, Δ5, or Δ4 desaturation activities.This approach provides direct evidence of enzyme specificity, activity, and substrate preference, and has been widely applied across various teleost species.

For instance, in 2001, zebrafish were demonstrated to possess both Δ5 and Δ6 desaturase activities derived from a bifunctional Fads2, as confirmed through yeast-based assays (Hastings et al., 2001). Later, in 2010, Δ4 desaturase activity was also identified in the herbivorous marine teleost rabbitfish (Li et al., 2010a), which demonstrated the two alternative pathways for DHA biosynthesis exist in teleosts. Fatty acid elongases from marine and freshwater teleosts were identified and functionally characterized, revealing interspecific differences in enzymatic competence (Agaba et al., 2005). Most elongases showed substrate preference in the order C18 > C20 > C22, with freshwater species (e.g., tilapia, catfish) exhibiting stronger PUFA elongation ability than marine species (e.g., cod, turbot), indicating a higher dietary dependence on LC-PUFA in the latter (Agaba et al., 2005).

Similarly, Δ6 Fad activity was shown to be higher in grass carp *Ctenopharyngodon idella*, rabbitfish, and Japanese eel than in mandarin fish, Atlantic salmon, and hybrid grouper, and Elovl5 activity was found to be primarily shaped by feeding habits (Xie et al., 2020). Overall, these studies indicate that teleost LC-PUFA biosynthetic capacity varies among species and is jointly influenced by ecological type and nutritional strategy. These studies also demonstrate that fish Fads and Elovl typically exhibit higher activity toward n-3 fatty acids than n-6 homologs (Agaba et al., 2005; Li et al., 2010a; Xie et al., 2020). Collectively, these studies have established heterologous expression as a widely utilized approach for elucidating the functional diversity and evolutionary adaptation of LC-PUFA biosynthesis enzymes in fish.

(2) Primary cell culture

Primary hepatocytes provide a physiologically relevant *in vitro* platform for studying fatty acid metabolism and LC-PUFA biosynthesis in fish. Unlike heterologous yeast systems, primary hepatocytes maintain the endogenous regulatory networks (e.g., nuclear receptors, binding proteins and signaling pathways) including nuclear receptors (e.g., PPARs, LXR, FXR), lipid-binding proteins, membrane transporters, and intracellular signaling cascades—thus enabling responses to nutritional and hormonal stimuli that more closely resemble the intact organism (Langan et al., 2018; Li et al., 2019; Moon et al., 1985; Pereira et al., 2022).

Accordingly, primary hepatocytes have been widely used to investigate fatty acid uptake, metabolism, and transcriptional regulation, allowing precise assessment of desaturation, elongation, and lipid incorporation in response to defined substrates or modulators, thereby complementing heterologous enzyme characterization (Jiao et al., 2020). For instance, salmonid hepatocytes have been widely used to characterize the regulation of *fads* and *elovl* genes under varying dietary lipids, temperature, and hormonal signals, revealing transcriptional patterns that parallel *in vivo* responses (Datsomor et al., 2023; Kjaer et al., 2016). Hepatocytes of Atlantic salmon and rabbitfish further demonstrated substrate-driven modulation of Δ6/Δ5 desaturase and elongase activities, providing functional validation of endogenous LC-PUFA biosynthetic pathways (Kjær et al., 2016; Liu et al., 2017).

Primary hepatocyte systems have also been instrumental in evaluating the effects of environmental stressors (e.g., hypoxia, salinity), endocrine regulation (insulin, glucagon, cortisol), or inflammatory signals on lipid metabolism, offering mechanistic insights into how physiological states shape LC-PUFA synthesis and deposition (Liu et al., 2022b; Tansutaphanit et al., 2023). More recently, hepatocytes from marine species such as large yellow croaker *Larmichthys crocea* has been used to explore cellular lipid remodeling under alternative dietary oils, demonstrating changes in SREBP1 processing, fatty acid transport, and TAG accumulation that align with feeding trials (Zhang et al., 2016). Similarly, hepatocyte models from large yellow croaker and rainbow trout reveal that substituting FO with linseed or soybean oil upregulates *fads2* expression via transcriptional control by C/EBPα and GATA3, elucidating regulatory mechanisms underlying LC-PUFA biosynthesis (Sun et al., 2022). Together, primary hepatocyte models complement heterologous expression systems by providing an intact cellular context for evaluating enzyme activity, gene regulation, and metabolic flux, and have been essential for clarifying species-specific LC-PUFA biosynthesis, dietary regulation, and lipid homeostasis.

(3) Stable isotope tracer techniques

Stable isotope labeling is a powerful *in vivo* approach for tracing fatty acid metabolic fluxes with high specificity and quantitative resolution. In this method, fish are supplied with isotopically labeled precursors, most commonly ^{13}C-ALA or ^{14}C-ALA that are either incorporated into experimental diets or delivered directly by oral gavage, to trace the novo LC-PUFA biosynthesis (Henderson et al., 1995; Mourente & Tocher, 1993), allowing the labeled atoms to be followed through successive desaturation and elongation steps. This approach offers clear advantages over conventional fatty acid profiling by directly quantifying *de novo* LC-PUFA biosynthesis and revealing tissue-specific metabolic routing, while time-course sampling further exposes conversion rates, pool turnover, and key control points, dynamic insights that static lipid profiles cannot provide (Bell & Dick, 2005; Goncalves et al., 2025; Pairohakul et al., 2021).

Moreover, isotope-labeled substrates can be applied in hepatocyte systems to resolve cell-intrinsic metabolic fluxes. Isotopic-tracing assays typically involve incubating fish hepatocytes or intestinal epithelial cells with defined fatty acid substrates, most commonly radiolabeled or stable-isotope-labeled ALA such as [^{14}C]-ALA, to precisely monitor metabolic transformations (Galindo et al., 2021; Morais et al., 2015; Mourente & Tocher, 1993; Ruyter et al., 2000). Through quantifying the production and incorporation rates of downstream metabolites including EPA and DHA, these assays provide a detailed picture of substrate utilization, pathway efficiency, and metabolic bottlenecks. At the same time, coupling isotope incorporation with transcriptional analyses allows simultaneous assessment of regulatory responses in key lipid metabolic genes such as (*fads*, *elovl*, *ppar*, *srebp*, thereby linking biochemical flux with underlying molecular control (Buzzi et al., 1996; Garrido et al., 2020; Mourente & Tocher, 1994).

(4) Feeding Trials

Nutritional intervention studies remain a cornerstone of fatty acid metabolism research, where fish are fed experimental diets differing in lipid source, such as fish, vegetable, algal, or insect oils, and tissue samples including liver, muscle, or whole body are subsequently analyzed (Glencross et al., 2016; Tocher, 2010). Fatty acid methyl esters (FAMEs) are prepared and quantified by GC–MS to characterize dietary-driven shifts in fatty acid composition and deposition (Liang et al., 2022; Liu et al., 2025; Yadav, 2020), providing a direct connection between dietary inputs and physiological outcomes.

To resolve the regulatory basis of these metabolic responses, transcriptional profiling via qPCR is widely used to quantify key LC-PUFA biosynthesis genes such as *fads2*, *elovl5*, and *elovl2* (Price et al., 2000; Ren et al., 2012). Protein-level confirmation using Western blotting or targeted enzyme quantification, together with desaturase and elongase activity assays performed on tissue extracts or cell lysates, provides complementary functional evidence (Liu et al., 2025; Valenzuela et al., 2024). Taken together, integrating compositional, transcriptional, protein, enzymatic, and flux data yields a coherent and mechanistically grounded view of how dietary lipids remodel LC-PUFA metabolism and lipid homeostasis in fish. No single approach is sufficient to fully evaluate LC-PUFA biosynthetic capacity; instead, an integrative strategy combining molecular, cellular, and *in vivo* approaches is required.

2.4 Challenges in substituting fish oil in aquafeeds

The fish in: fish out (FIFO) ratio is widely used to evaluate the dependence of aquaculture on wild marine resources (Tacon & Metian, 2008; Terpstra, 2015). To improve sustainability, reducing FIFO—particularly through the substitution of FO with alternative lipid sources—has become a major goal in aquafeed development. More recently, modified metrics such as economic FIFO (eFIFO) and nutrient FIFO (nFIFO) have been proposed, which differ in how wild fish resources are allocated, either based on economic value or nutrient contribution (Kok et al., 2020; Newton et al., 2025). However, a reduction in FIFO does not necessarily indicate successful FO substitution.

The substitution of FO with alternative lipid sources has become a central strategy to improve the sustainability of aquaculture (Tocher, 2015; Turchini et al., 2010). However, despite considerable progress, replacing FO in aquafeeds remains challenging. In practice, excessive or complete replacement of FO often leads to compromised physiological performance and reduced fillet quality (Turchini et al., 2009; Wu et al., 2024), highlighting the complexity of balancing dietary lipid composition with the metabolic requirements of fish, particularly with respect to n-3 LC-PUFA supply and utilization (Glencross, 2009; Glencross et al., 2024). Under these circumstances, the expected reduction in FIFO ratios may be limited or even negated. Therefore, a successful FO replacement strategy must achieve two objectives simultaneously: maintaining fish growth performance and product quality, while meaningfully reducing reliance on marine-derived lipid inputs. Table 1-2 summarizes the current research on alternative lipid sources for FO replacement in aquaculture.

(1) Vegetable oils

VO represent the most mature and economically viable alternative to FO, driven by supply stability and cost advantages (Nasopoulou & Zabetakis, 2012). VO are usually rich in C18 fatty acids, mainly LA, ALA, and oleic acid (OA, 18:1n-9), but lack EPA and DHA (Ruiz-Lopez et al., 2015). Decades of studies have established that VO can maintain growth in numerous freshwater species (Jin et al., 2023; Sankian et al., 2019; Yuan et al., 2024b), yet growth performance, the traditional metric of substitution success, obscures deeper metabolic perturbations.

First, the assumption that freshwater species tolerate VO better due to superior LC-PUFA biosynthesis (converting ALA→EPA→DHA) (Tocher, 2010) has been challenged by evidence that even efficient Δ6-desaturase activity fails to prevent fillet EPA+DHA decline at high VO inclusion (>75%) despite maintained growth (Liang et al., 2022; Teoh & Ng, 2016). Therefore, even species with n-3 LC-PUFA biosynthetic capacity rarely achieve tissue EPA+DHA levels comparable to FO-fed counterparts, due to β-oxidation of precursors, competitive inhibition by high dietary LA, and rate-limiting elongase/desaturase steps (Stubhaug et al., 2005; Teoh & Ng, 2016). Consequently, the decline in fillet DHA content directly reduces the health benefits of farmed seafood for human consumers, raising concerns regarding the long-term nutritional sustainability of aquaculture products (Glencross, 2009; Wang & Xu, 2024).

Second, although VO such as soybean, rapeseed, and linseed oil can partially satisfy energy requirements, they are inherently deficient in EPA and DHA and typically contain high levels of n-6 fatty acids, leading to disrupt n-6/n-3 homeostasis and triggering pro-inflammatory mediators, thereby increasing the expression of cytokines including TNF-α, IL-1β, and IL-6 (Bell & Koppe, 2010; Montero et al., 2010; Xu et al., 2022). Moreover, the deficiency of EPA and DHA compromises their anti-inflammatory and lipid-regulatory functions. At the physiological level, such imbalances have been associated with impaired lipid metabolism, including enhanced hepatic lipogenesis, reduced β-oxidation capacity, and increased lipid accumulation in tissues (Komprda, 2012; Zúñiga et al., 2011).

Cottonseed oil (CO) was selected in this study due to its regional availability and economic advantages. In China, CO is widely produced, with high output and relatively low cost, making it a competitive lipid source for aquafeeds. Although CO traditionally contains gossypol, a known anti-nutritional factor, advances in processing technologies have enabled the production of low- or even gossypol-free cottonseed oil, significantly reducing its potential toxicity. Compared with rapeseed oil, which is already well-established in aquafeeds, CO represents a cost-effective and locally sustainable alternative, particularly in regions with abundant cotton industry by-products. Therefore, evaluating its nutritional value and application potential in aquaculture is of practical importance.

Collectively, these effects may negatively influence fish health, stress resistance, and product nutritional quality when high levels of VO are used to replace FO.

(2) Animal-derived oils

Animal-derived oils, including terrestrial animal fats and marine by-product oils, have been widely explored as cost-effective alternatives to FO in aquafeeds (Monteiro et al., 2018). Terrestrial animal oils such as poultry oil, lard, and beef tallow are readily available and economically competitive, and can partially replace FO without compromising growth performance in some species (Campos et al., 2019; Li et al., 2022; Wang et al., 2024b). However, these lipids are typically rich in saturated and monounsaturated fatty acids but deficient in long-chain n-3 LC-PUFA, particularly EPA and DHA, which often results in reduced fillet nutritional quality unless supplemented or blended with n-3-rich sources (Sprague et al., 2016; Turchini et al., 2009).

In contrast, Marine by-product oils, derived from fish processing waste, offer a circular alternative by recycling trimmings into feed inputs (Alfio et al., 2021). These oils are considered nutritionally superior among animal-derived lipids because they provide preformed EPA and DHA without relying on endogenous biosynthesis, thereby supporting the maintenance of tissue LC-PUFA levels and product quality (Mamat et al., 2025).

Despite these advantages, the application of animal-derived oils remains constrained by variability in raw material composition, susceptibility to oxidation, and supply limitations linked to livestock and fisheries production (Glencross et al., 2020; Hossain, 2022). Therefore, while animal-derived oils represent practical and cost-effective alternatives to FO, their optimal use typically relies on blending strategies to balance cost, sustainability, and nutritional quality (Hasan et al., 2024; Kratky & Zamazal, 2020; Miller et al., 2008).

(3) Insect oils

Beyond established alternatives, a diverse array of emerging lipid sources—including insect oils, genetically modified crops such as camelina *Camelina sativa*, and single-cell oils—has attracted increasing attention due to their sustainability potential (Gasco et al., 2018; Napier et al., 2015).

Insects—primarily black soldier fly *Hermetia illucens* (BSF), crickets *Acheta domesticus*, and mealworms *Alphitobius diaperinus*,—are reared in industrial, climate-controlled systems. Among them, the BSF is of particular interest due to its potential to support a circular production model by converting organic waste into lipid-rich biomass. Its fatty acid profile, typically characterized by high levels of lauric acid (C12:0) and OA, has been shown to support growth performance in several fish species (Fawole et al., 2021; Hossain et al., 2023; Xu et al., 2021).

Conventional insect oils are typically low in n-3 LC-PUFAs and characterized by unfavorable n-6/n-3 ratios, restricting their applicability to fish species with limited

LC-PUFA biosynthetic capacity (Tocher, 2003). Moreover, their application is complicated by residual chitin contamination from insect exoskeletons (Pascon et al., 2025). Chitin has been reported to exert dose-dependent effects, where low inclusion may benefit gut health, whereas higher levels can bind bile acids, reduce micelle formation, and impair lipid digestion and absorption, including that of LC-PUFAs (Achinivu et al., 2022; Belghit et al., 2018; Pascon et al., 2025).

However, insect farming still faces challenges including high costs, low volumes, and limited adoption. Its proposed advantage of waste-to-feed conversion is constrained by food safety concerns, and in practice often relies on commercial feed inputs, competing with human and livestock resources (Arru et al., 2019; Brian Mboya, 2025). Nevertheless, continued technological innovation and improved regulatory frameworks may enhance its scalability and sustainability in the future (Fawole et al., 2021; Roccatello et al., 2024).

(4) Single-cell oils

Single-cell oils, derived from oleaginous microorganisms including marine microalgae and yeasts, have emerged as promising alternatives to FO in aquafeeds. Marine microalgae such as *Nannochloropsis oculata*, *Isochrysis sp.*, and *Schizochytrium sp.*, can achieve performance comparable to FO in maintaining fillet LC-PUFA levels (Guerra et al., 2023; Sarker, 2023). However, their application is constrained by fatty acid composition, potential contaminant accumulation (polychlorinated biphenyls and dioxins), and economic limitations.

First, differences in fatty acid composition limit their ability to fully replicate the balanced EPA/DHA profile of FO. For instance, *Schizochytrium sp.* oil is DHA-rich but deficient in EPA, whereas *Nannochloropsis oculata* provides EPA but little DHA, deviating from the balanced EPA/DHA profile of FO and necessitating blending strategies or partial FO inclusion (Sarker, 2023; Zahran et al., 2023). Second, variability in lipid digestibility (ranging from 50% to 98% depending on the microalgal source) and differences in cell wall structure can significantly affect nutrient bioaccessibility (Ghosh et al., 2016; Yaakob et al., 2014). In addition, concerns regarding potential contaminant accumulation (e.g., polychlorinated biphenyls and dioxins) and relatively high production costs, which are often several-fold higher than FO, further restrict their large-scale application (Chauton et al., 2015; Ma & Hu, 2024; Sarker, 2023).

In parallel, oleaginous yeasts such as *Yarrowia lipolytica* offer complementary advantages, particularly through fermentation-based systems that enable the production of tailored lipid profiles (Armenta & Valentine, 2013; Glencross et al., 2020). Compared with microalgae, yeast-based systems are less light-dependent, more scalable, and able to utilize diverse low-cost carbon sources. Research efforts have predominantly focused on n-3 LC-PUFA production, yielding strains and systems enriched in EPA and DHA (Glencross et al., 2020). However, native yeast strains typically lack the capacity to synthesize long-chain n-3 PUFA, necessitating metabolic engineering to achieve nutritionally relevant fatty acid profiles.

Therefore, the practical use of single-cell oils remains limited by uncertainties related to nutritional consistency, metabolic impacts in fish, and economic scalability. Therefore, while single-cell oils represent a highly promising and flexible class of FO alternatives, their widespread adoption will likely depend on further improvements in cost efficiency, lipid balance optimization, and processing technologies.

(5) Transgenic oilseed oils (genetic modification, GM)

Transgenic camelina and canola engineered to produce EPA and DHA represent a paradigm shift in sustainable aquafeed formulation. Field trials demonstrate that transgenic oils containing 15-25% n-3 LC-PUFA can fully replace FO in Atlantic salmon and rainbow trout diets without compromising growth performance, while significantly enhancing fillet EPA+DHA content compared to current commercial standards (Osmond et al., 2021; Tocher et al., 2024). Although no evidence supports horizontal gene transfer to fish or gut microbiota, consumer resistance, particularly in Europe and other genetically modified organisms (GMO)-averse markets, continues to constrain broader acceptance, resulting in geographically uneven adoption of these technically advanced alternatives (Hong, 2022; Saint-Eve et al., 2025).

Ultimately, the effectiveness of FO substitution should not be evaluated solely by growth performance or fillet fatty acid composition. Instead, it should be assessed based on its net impact on FIFO reduction, marine resource dependence, and long-term nutritional sustainability. In this context, improving the nutritional quality of alternative lipid sources and deepening our understanding of species-dependent LC-PUFA metabolic capacity remain critical research priorities for achieving both productive efficiency and nutritional integrity in modern aquaculture systems.

Table 1-2. Alternative lipid sources for fish oil replacement in aquaculture.

Oil source category	Specific oil source	FA characteristics	Main limitations	Typical fish species studied	References
Vegetable oils	Soybean oil	High LA (50–55%), ALA (5–10%), moderate OA, low SFA	Competes with human-edible oils; high n-6 may inhibit n-3 LC-PUFA biosynthesis and affect inflammation and immune response	Grass carp, Japanses seabass, Black seabream *Acanthopagrus schlegelii*	(Liu et al., 2022c; Peng et al., 2008; Xu et al., 2015)
	Linseed oil (Flax oil)	Very high ALA (50–60%)	Highly prone to oxidation; high ALA may increase lipid peroxidation risk	Tilapia *Oreochromis niloticus*, Atlantic salmon, black seabream	(Li et al., 2016; Menoyo et al., 2005; Zhu et al., 2022)
	Rapeseed (canola) oil	High OA (55–65%), moderate LA, low SFA	Competes with human-edible oils;Contains anti-nutritional factors glucosinolates; higher price	Atlantic salmon, rainbow trout	(Bell et al., 2001a; Gesto et al., 2021; Pettersson et al., 2009)
	Palm oil	High SFA (C16:0, 40–45%), moderate OA	Affects fish digestion and metabolism	Tilapia, rainbow trout	(Ayisi et al., 2019; Fonseca-Madrigal et al., 2005; Larbi Ayisi et al., 2018)
	Cottonseed oil	Moderate LA (~50%), notable SFA (~25%), contains cyclopropenoid FA	Contains anti-nutritional factors such as gossypol (improvements in extraction processes will reduce)	rainbow trout, black seabream, European seabass	(Güler & Yildiz, 2011) (Eroldoğan et al., 2012; Wu et al., 2022b)
Animal oils	Marine by-product oils	Rich in EPA and DHA	EPA/DHA content varies significantly depending on the source; easy oxidation	*Colossoma macropomum*	(Mamat et al., 2025; Oliveira Filho et al., 2025)
	Poultry oil	Rich in SFA and MUFA	Affects fish digestion and metabolism; impact on feed processing and stability	tiger puffer *Takifugu rubripes*; European seabass	(Campos et al., 2019; Li et al., 2022)
	Lard	High SFA and MUFA		Tiger Puffer, carp	(Liu et al., 2024; Zhou et al., 2016)
	Beef tallow	High SFA content		gilthead seabream; turbot	(Pérez et al., 2014; Zhang et al., 2023a)

Oil source category	Specific oil source	FA characteristics	Main limitations	Typical fish species studied	References
Insect oils	Black soldier fly oil	Rich in lauric acid (C12:0) (30-45%) and OA(15-25%)	Produced from organic waste, meet its commercial and sustainability expectations	mirror carp *Cyprinus carpio var. specularis*, gilthead seabream, largemouth bass	(Moutinho et al., 2024; Xu et al., 2021; Yuan et al., 2024a)
	Yellow mealworm oil	High MUFA		silver catfish *Rhamdia quelen*	(Dias et al., 2025)
Single-cell oil	Microalgae oil	Extremely rich in EPA/DHA content	Higher production cost	rainbow trout, Nile tilapia	(Bélanger et al., 2021; Sarker et al., 2016; Serrano et al., 2021)
	Yeast	Low n-3 LC-PUFA（EPA/DHA）	Fatty acid composition varies significantly depending on the source; yeast cell wall contains β-glucan and chitin, affecting fish digestion and metabolism	European sea bass, Atlantic salmon	(Berge et al., 2013; Vasilaki et al., 2023)
GM Camelina oil	GM Camelina oil; canola oil	Genetically engineered to produce EPA and DHA	Limited commercial availability	Atlantic salmon, largemouth bass, rainbow trout	(Betancor et al., 2016; Li et al., 2025; Osmond et al., 2021)

Abbreviation:SFA, Saturated fatty acids (FAs with no carbon−carbon double bonds); MUFA, Monounsaturated fatty acids (FAs with only one carbon-carbon double bond); LC-PUFA, long-chain polyunsaturated fatty acids (FAs with ⩾20 carbon atoms and ⩾2 double bonds).

2.5 Strategies to enhance n-3 LC-PUFA deposition in teleosts

Given the limited biosynthetic capacity in certain species, several nutritional strategies have been explored to enhance n-3 LC-PUFA deposition, including optimizing dietary precursor–product fatty acid ratios and supplementing feeds with DHA-rich sources, or using alternative lipid sources with favorable fatty acid profiles (Table 1-3). Nutritional strategies to enhance n-3 LC-PUFA deposition in species with limited biosynthetic capacity:

(1) Direct supplementation with n-3 LC-PUFA- rich ingredients

Directly supplying n-3 LC-PUFAs through ingredients such as FO, algal oil, krill oil, or purified DHA concentrates enables efficient incorporation into membrane phospholipids and storage lipids, thereby effectively enhancing tissue n-3 LC-PUFAs levels, fillet nutritional value, and associated physiological functions (Diken et al., 2025). This approach bypasses the endogenous biosynthesis bottleneck typical of most marine and many freshwater teleosts, which often exhibit limited ability for converting C18 precursors into long-chain products (Tocher, 2015).

Among available options, FO has always been traditional and popular ingredients for supplying lipids in the diet of aquafeeds (FAO, 2026; Torstensen & Tocher, 2010). However, on an annual basis, FO is a finite natural resource and its supply has reached its sustainable limit, and so increasing demand has resulted in prices rising year on year (Council et al., 2011; Liu et al., 2018; Turchini et al., 2010). Due to the limitation of resources, it is urgent to find ideal alternative lipid sources of FO.

Given these limitations, researchers have increasingly focused on alternative sources, with n-3 LC-PUFA-rich ingredients emerging as sustainable and effective substitutes that consistently enhance n-3 LC-PUFA deposition across species. For example, microalgae-derived oil substitution has been shown to elevate DHA levels in rainbow trout and gilthead seabream while maintaining growth performance (Flores-Moreno et al.; Kajbaf et al., 2025). In largemouth bass and other carnivorous teleosts, feeding trials consistently report greater muscle and hepatic DHA retention when diets contain preformed LC-PUFAs compared with ALA-based formulations, primarily due to the limited activity of endogenous desaturases. Besides lipid sources, *Schizochytrium sp.* meal (SCM) has been shown to enhance fillet nutritional quality and sensory attributes in several fish species, such as in rainbow trout (Bélanger et al., 2021; Santigosa et al., 2020) and Atlantic salmon (Zatti et al., 2023).

Novel insect-derived oils, such as trophically upgraded BSF oil (BSFO), offer promising avenues for enhancing the n-3 LC-PUFA value of insect lipids. These upgraded oils are typically produced by enriching larvae with n-3 LC-PUFAs or ALA through substrates such as microalgae, fish byproducts, or formulated feeds (Barroso et al., 2019; Hoc et al., 2021b; Xu et al., 2021), thereby narrowing the nutritional gap between insect-based and marine-derived lipid sources. BSF larvae themselves efficiently convert organic matter into lipid and protein for feed applications and generally exert minimal negative effects on fish growth performance and fillet fatty acid composition (Barroso et al., 2019). These traits open the possibility of deliberately engineering BSF fatty acid profiles through substrate selection and optimized harvesting time; however, the degree of modification achievable through dietary manipulation also presents inherent limitations (Ewald et al., 2020).

In addition, DHA-rich supplementation can modulate lipid metabolic pathways by downregulating *fads2* and *elovl* transcription through end-product feedback inhibition, further reinforcing the reliance on dietary long-chain n-3 PUFA inputs (Glencross et al., 2015; Gregory et al., 2016; Panserat et al., 2009; Tocher et al., 2002b). This regulatory pattern has been demonstrated in multiple fish species, indicating a progressive shift toward dependence on dietary n-3 LC-PUFAs rather than *de novo* synthesis.

(2) Optimizing precursor (ALA)

Enhancing dietary ALA represents a key strategy for stimulating endogenous synthesis of n-3 LC-PUFAs in species that retain functional desaturase and elongase pathways. As the metabolic precursor for EPA and DHA production, increasing ALA availability elevates substrate flux through the desaturation–elongation sequence catalyzed by Fads2 (Δ6/Δ5 activity) and elovl5/elovl2, thereby promoting the formation of downstream LC-PUFAs (Xie et al., 2021). In practical feed formulation, ALA supply can be optimized through the inclusion of ALA-rich ingredients such as linseed oil, perilla oil, and rapeseed oil, which have been shown to enhance n-3 LC-PUFA deposition in several freshwater and euryhaline species without compromising growth performance (Bell, 2002; Li et al., 2024; Liu et al., 2025; Liu et al., 2022c).

However, the efficacy of this approach is strongly species-dependent. Some fish species exhibit reduced Δ6/Δ5 desaturase activity and limited capacity to convert ALA to DHA, resulting in minimal improvement in LC-PUFA deposition even when precursor supply is increased (Tocher, 2010). For instance, in black seabream, dietary perilla oil has been reported to up-regulate key LC-PUFA biosynthesis genes such as *elovl5* and *fads2*. Yet, compared with other oil sources, this did not translate into higher DHA or EPA levels in muscle and liver (Jin et al., 2017b). Together, these observations underscore that molecular up-regulation of the pathway alone may be insufficient, species-specific enzyme capacity, substrate use, and other metabolic or nutritional factors can limit actual n-3 LC-PUFA deposition even when precursor intake is increased.

Moreover, LA competes with ALA for LC-PUFA biosynthesis enzymes leading to the production of ARA. Therefore, formulation needs to keep n-6:n-3 or ALA:LA ratios within a favorable range. For example, in the marine teleost rabbitfish, incorporation of n-3 LC-PUFA into liver and fillet was proportional to dietary ALA:LA ratios; diets with an ALA:LA of about 1.93 maximized hepatic Δ4 fad, Δ6/Δ5 fad, and *elovl5* mRNA levels. (Xie et al., 2018). Similarly, in Atlantic salmon raised on diets lacking preformed EPA or DHA, the endogenous production of n-3 versus n-6 LC-PUFA shifted dramatically with the ALA:LA ratio. A 3:1 ALA:LA ratio yielded roughly 27:1 n-3:n-6 LC-PUFA production, while a 1:3 ratio reduced this to about 2:1, underscoring how lower LA or higher ALA can strongly favor n-3 LC-PUFA biosynthesis (Sprague et al., 2019). A recent salmonid review underscores the broader relevance: maintaining an optimal n-3:n-6 balance is crucial for metabolic and immune functions (Carr et al., 2023).

Overall, precursor optimization serves as a valuable strategy in species with conserved LC-PUFA biosynthetic pathways. While in marine fish or other species with more limited conversion potential, ALA enrichment alone may not suffice; it is most effective when combined with dietary inclusion of n-3 LC-PUFA or other means to meet final LC-PUFA requirements.

(3) Additional or complementary strategies

While the two strategies above form the backbone of most dietary or formulation approaches, additional methods can broaden or reinforce their effectiveness. Beyond these conventional options, a wider set of tactics, including genetic modification, microbiome management, environmental control, and life-stage planning, can create a multilayered, combinatory strategy, especially when biological or environmental constraints hinder results.

Overexpress or introduce key biosynthesis enzymes. Introduce or enhance genes encoding high-activity Δ6/Δ5 desaturases, elongases, or other pathway enzymes to boost the fish's own ability to convert ALA to EPA/DHA. For example, researchers introduced a Δ6 desaturase gene from a salmon species into zebrafish (Alimuddin et al., 2005), resulting in markedly elevated tissue levels of EPA and DHA compared with non-transgenic controls (approximately 1.4-fold and 2.1-fold increases, respectively). Similar, dual expression of Atlantic salmon Δ5 and Δ6 desaturases in tilapia not only elevated n-3 PUFA content but also enhanced disease resistance and altered gut microbiota composition, thereby indicating broader physiological benefits of such genetic enhancement (Chiang et al., 2020). Collectively, these results confirms that direct enhancement of pathway enzymes can markedly improve n-3 LC-PUFA accumulation, even in species not naturally strong converters. In addition to transgenic approaches, selective breeding for enhanced endogenous LC-PUFA biosynthesis to improve the capacity of fish to convert precursor fatty acids into DHA and EPA, thereby reducing the need for direct marine lipid supplementation (Napier et al., 2020; Tocher, 2015).

Modulate gut microbiota and related metabolic pathways. Bacteria can also be a source of PUFAs, but their concentrations can vary significantly between bacterial species (Wanka et al., 2018). Vibrio species are especially rich in EPA and DHA (Estupiñán et al., 2020). Probiotics in the gastrointestinal tract support nutrition and growth through multiple pathways, including secretion of digestive enzymes, uptake of nutrients such as cofactors, vitamins, and unsaturated fatty acids, and indirect enhancements of overall nutrient absorption (Wuertz et al., 2021). In addition, a positive correlation between the level of probiotic bacteria *Shewanella spp* and fatty acids ALA and LA in fish Senegalese sole *Solea senegalensis* was demonstrated (Tapia-Paniagua et al., 2014).

Environmental or husbandry control to stimulate endogenous synthesis. Adjust environmental factors, such as salinity, temperature, or other culture conditions, to influence gene expression or regulatory pathways that govern LC-PUFA synthesis. Studies have shown that altering salinity can modulate expression of microRNAs, transcription factors, and key desaturase or elongase genes (Chen et al., 2020). In addition, tilapia LC-PUFA biosynthesis is boosted when fed VO at winter temperatures. At 22 °C, Δ4 desaturation increased with dietary ALA increase, while at 28 °C, fish fed the diet poor in 18:3n-3 had no apparent activity of this enzyme (Corrêa et al., 2023). Overall, these environmental or husbandry factors can enhance the fish's intrinsic metabolic capacity without requiring drastic changes to the diet.

Feeding stage and life-cycle management. Target specific developmental stages when the fish naturally has higher or more flexible LC-PUFA biosynthetic capacity, or when nutritional demands make precursor use most effective. LC-PUFA biosynthetic capacity in tilapia varies markedly with growth stage, sex, and dietary fatty acid composition, with males generally exhibiting higher *fads2* and *elovl5* expression than females, and LC-PUFA levels were lowest at stage II (feeding fifth-eighth week) and highest at stage III (feeding ninth-sixteenth week), demonstrating strong stage-dependent metabolic flexibility (Chen et al., 2025). Therefore, early-life or particular growth phases may respond strongly to precursor balance or other dietary manipulations, setting the stage for better later outcomes. Ontogenetic studies in bay snook *Petenia splendida* or other species have tracked biosynthetic capacity across growth stages and found stage-specific differences in expression of desaturases and elongases, indicating windows of opportunity where diet or environment manipulation can produce stronger LC-PUFA deposition (Jiménez-Martínez et al., 2025).

Table 1-3. Strategies to enhance n-3 LC-PUFA deposition

Strategy	Mechanism	Representative species	References
Nutritional strategies	Microalgae (direct n-3 LC-PUFAs supply) Low-to-moderate inclusion (0.7–5% diet; many studies 1–3%)	Rainbow trout, Nile tilapia, Atlantic salmon, Florida pompano *Trachinotus carolinus*	(Bélanger et al., 2021; Peng et al., 2024; Riche et al., 2023; Santigosa et al., 2020; Sarker et al., 2020; Zatti et al., 2023)
	Optimize precursor/product (increase ALA or adjust n-3/n-6 ratios); FO replacement with ALA-rich oils (partial to full replacement, e.g., 30–100%)	Rainbow trout (linseed oil)	(Trattner et al., 2008; Turchini et al., 2011)
	Blend alternative oils (e.g., insect oil + microalgae; fish by-product oils + algae); 50% BSFO + 1–3% *Schizochytrium* in some trials	Turbot *Scophthalmus maximus*, Black soldier fly	(Monteiro et al., 2024; Truzzi et al., 2020)
	Bioactive fatty acids (conjugated LA, 3-thia acids, petroselinic acid) PPAR activators (such as the lignin sesamin)	LA, 0.6% 3-thia fatty acids, peroxisome proliferator, 1.5% coriander oil (in canola oil diets), sesamin (0.58%), resveratrol	(Kennedy et al., 2006; Moya-Falcón et al., 2004; Ruyter et al., 1997) (Randall et al., 2013; Torno et al., 2017; Trattner et al., 2008; Vestergren et al., 2013)
	Micronutrient levels (trace Zn, vitamins at nutritionally relevant doses)	Rainbow trout	(Lewis et al., 2013)
Transgenic approach	Transgenic/molecular overexpression	*fat1* (common carp), *fat1* and *fat2* (zebrafish), Transgenic expression of *fads6* and *elovl5a* (zebrafish)	(Cheng et al., 2015; Pang et al., 2014; Zhang et al., 2019b)
	Genome editing (CRISPR)	CRISPR/Cas9 disruption of *elovl5a* (common carp)	(Zhang et al., 2024a)
Biochemical approach	Gut microbiome / probiotics modulation		(Fu et al., 2021; Mang et al., 2024)

2.6 Knowledge gap: LC-PUFA biosynthesis in largemouth bass

Largemouth bass, a typical carnivorous freshwater fish species, is now one of the most important aquaculture species worldwide, especially in China. According to the China Fisheries Statistics Yearbook (2013–2025 editions), its production increased substantially from 0.24 million tonnes in 2012 to 0.94 million tonnes in 2024 (Figure 1-5). As a high-yield, economically valuable species with growing aquafeed demands, largemouth bass currently requires FO inclusion levels of 4%−6% in commercial diets, comparable to those used in certain marine species (Song et al., 2024a; Zhang et al., 2019a). This phenomenon, characterized as “freshwater fish physiology with marine fish nutritional requirements”, indicates substantial potential for FO substitution. The dietary requirements for EPA and DHA, together with the capacity for endogenous biosynthesis of these n-3 LC-PUFAs, are critical factors influencing feed optimization, culturing efficiency, FIFO reduction, and overall sustainability. Therefore, the largemouth bass is a typical research object with both scientific value and industrial status.

Existing studies have demonstrated that largemouth bass exhibit a certain demand for diet n-3 LC-PUFA. A study using purified FO indicated that both weight gain and feed efficiency, as well as whole-body concentrations of EPA and DHA, can be improved in largemouth bass by dietary supplementation with approximately 1% EPA + DHA, whereas the ratio of EPA to DHA does not significantly affect growth (Yadav, 2020). Similarly, another study reported that the optimal requirement of dietary n-3 LC-PUFAs was 0.76 % (dry weight), and 0.80-1.10 % was beneficial for maintaining hepatic lipid metabolism balance in largemouth bass (An et al., 2023).

Although previous studies have suggested that largemouth bass may possess the capacity to maintain LC-PUFA homeostasis, this supports the notion that largemouth bass similarly have a limited ability to convert ALA into EPA and DHA (Li et al., 2025; Yadav, 2020; Zhang et al., 2019a). However, compared to well-studied species such as salmonids (Kjær et al., 2016; Xie et al., 2021; Zheng et al., 2004), the endogenous n-3 LC-PUFA biosynthetic capacity of largemouth bass has not been systematically validated across different biological levels. In particular, the functions of key Fads involved in the n-3 LC-PUFA biosynthetic pathway have not yet been fully characterized.

Accordingly, based on previous studies linking fish trophic level to LC-PUFA biosynthetic capacity (Trushenski & Rombenso, 2020), largemouth bass, as a carnivorous freshwater species with a trophic level of approximately 3.8, is predicted to possess some intrinsic ability to convert ALA into n-3 LC-PUFAs; however, this capacity is relatively limited, and dietary supplementation of n-3 LC-PUFAs is therefore required. Verifying this hypothesis is therefore of practical significance for Chinese aquaculture: it can inform optimized feed formulation to ensure adequate EPA and DHA supply, improve growth performance and feed efficiency, reduce dependence on FO, and support sustainable production. Moreover, as a representative inland carnivorous species, understanding the LC-PUFA biosynthetic capacity of largemouth bass provides a reference for similar aquaculture species, facilitating more targeted nutritional strategies.

Besides, previous studies have increasingly investigated the effects of replacing FO with different alternative oil sources on the growth performance, fatty acid composition and lipid metabolism of largemouth bass (Guo et al., 2019; Shi et al., 2019; Song et al., 2024a; Tidwell et al., 2007; Yuan et al., 2024a; Zhang et al., 2019a). Although alternative lipid sources can partially replace FO in largemouth bass diets, their effects on n-3 LC-PUFA retention and lipid metabolic regulation remain key limitations for achieving optimal growth and nutritional quality. However, studies examining the effects of different oil sources on n-3 LC-PUFA biosynthesis remain limited (Liang et al., 2022), and the species-specific conversion capacity under varying dietary conditions is still insufficiently understood, hindering the development of optimized feed formulations.

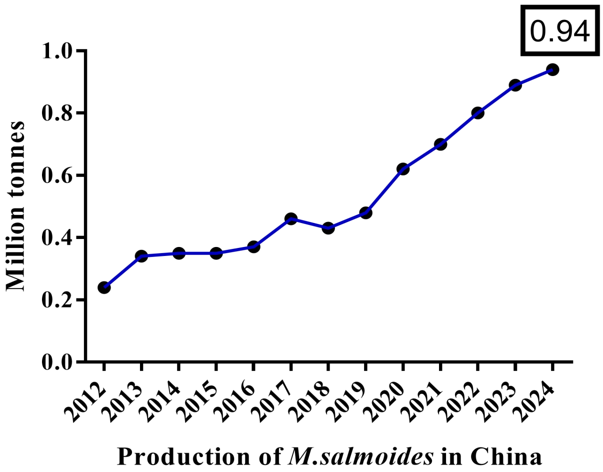


Figure 1-5. Production of largemouth bass in China from 2012 to 2024 (China Fisheries Statistics Yearbook, 2013–2025 editions).

2.7 Prospects

Reducing the reliance of aquafeeds on FO while ensuring adequate n-3 LC-PUFA deposition in teleosts is fundamental to improving both the sustainability and economic viability of aquaculture, and requires the development of species-specific strategies. In practice, no single strategy optimally balances sustainability, cost, and efficacy. Strategy selection should be guided by LC-PUFA conversion capacity and practical constraints, after which one or two complementary approaches can be added.

Species with limited endogenous conversion benefit most from direct n-3 LC-PUFA supplementation paired with precursor balancing, supported by microbiome or husbandry adjustments. In species with moderate or strong biosynthetic capacity, precursor optimization, life-stage targeting, and environmental modulation become more effective, with biochemical or genetic interventions applied where feasible. Genetic approaches may deliver long-term FIFO reductions but require substantial regulatory oversight and sustained evaluation. Environmental, microbiome-based, and life-stage strategies, by contrast, provide adaptable solutions that can be rapidly adjusted to shifting production goals and market demands.

Although multiple nutritional, biochemical, and environmental strategies have been developed to enhance n-3 LC-PUFA deposition, their practical application often constrained by several biological, economic, and regulatory challenges. Future research should prioritize elucidating lipid metabolism, optimizing precursor utilization, identifying environmental and microbiome-based enhancers of biosynthesis, and developing novel sustainable lipid sources, thereby reducing reliance on marine FO and enabling more efficient, resilient feed formulations.

3. Scientific question and objectives

Given the dual role of largemouth bass as a representative freshwater fish with both scientific value and industrial significance, accurately characterizing its n-3 LC-PUFA biosynthetic capacity is crucial for understanding its nutritional physiology and for supporting the sustainable development of its aquaculture industry. However, despite its economic significance, the capacity of this species to endogenously biosynthesize n-3 LC-PUFAs remains insufficiently characterized.

Moreover, clarifying how different dietary lipid sources regulate n-3 LC-PUFA biosynthesis and deposition in largemouth bass is essential for the development of cost-effective and sustainable aquafeeds. Although the replacement of FO has become inevitable, such substitution often leads to reduced tissue DHA levels, disrupted lipid metabolism, and potentially impaired growth performance, highlighting the urgent need for species-specific nutritional strategies.

Based on these background, this study aims to address the following question:

1.Does largemouth bass possess a complete Δ6/Δ5/Δ4 desaturase system for n-3 LC-PUFA biosynthesis, and what are its essential fatty acid requirements?

2.How do different dietary lipid sources affect growth performance, fatty acid composition, and the capacity for n-3 LC-PUFA conversion in largemouth bass?

Hypothesis: Largemouth bass possess a restricted capacity for endogenous LC-PUFA biosynthesis, and this limitation can be strategically compensated through nutritional modulation and DHA supplementation, enabling sustainable aquafeeds independent of marine-derived lipids.

To expound on the main questions above, this study addresses these knowledge gaps by integrating functional characterization of *fads* genes with controlled feeding trials to elucidate the conversion capacity of largemouth bass and the physiological impacts of different dietary lipid sources. The specific objectives were formulated:

1.To functionally verify desaturase activities and to characterize the *in vitro/in vivo* conversion of ALA into LC-PUFA and involved in LC-PUFA biosynthesis.

2.To evaluate the physiological response of largemouth bass to VO sources, focusing on growth, fatty acid composition, health, and lipid metabolism, to further extend the objectives of these feeding trials by validating its n-3 LC-PUFA biosynthetic capacity.

4. Overview of the chapters

The technology rout of our research is shown in Figure 1-6. First (Chapter II), we determined the endogenous conversion capacity of ALA in largemouth bass through *in vitro*, *in vivo*, and functional verification approaches. Second (Chapter III), we assessed how VO sources influence growth, fatty acid deposition, and LC-PUFA biosynthesis based on a commercial diet. Based on this validation (Chapter IV), we examined whether ALA is functionally indispensable and whether DHA can compensate for limited conversion under FM- and FO-free conditions. Finally (Chapter V), we evaluated n-3 PUFA-enriched BSFO as a practical nutritional strategy to replace marine-derived lipids.

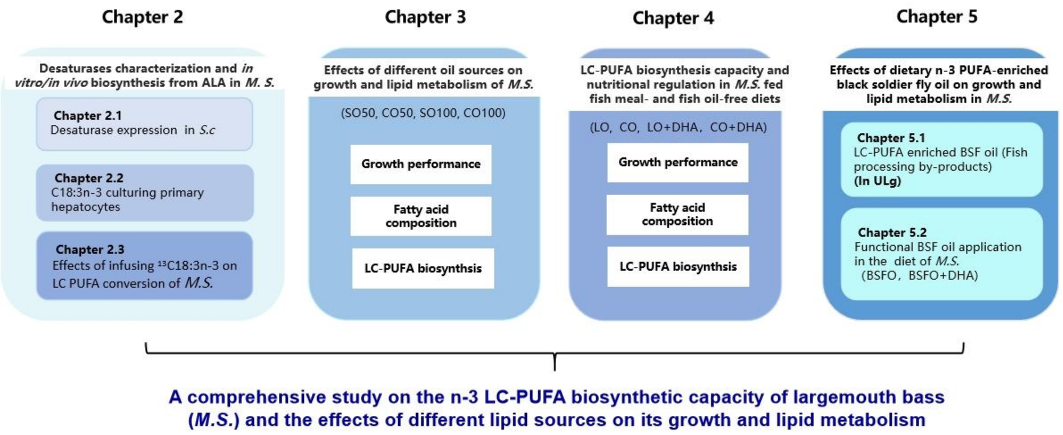


Figure 1-6. The technology route of this thesis.

Chapter I General introduction.

In this chapter, general information on the research progresses and objectives was described. The main content included reviewing the LC-PUFA biosynthetic pathways, regulatory mechanisms, species variation and methodological approaches, identifies key knowledge gaps associated with the restricted biosynthesis potential of largemouth bass. Based on this background, the research objectives and experimental strategies of the thesis were formulated, aiming to clarify conversion capacity, regulatory mechanisms and nutritional modulation of n-3 LC-PUFA biosynthesis in this species.

Chapter II Desaturase characterization and *in vitro/in vivo* LC-PUFA biosynthesis from ALA in largemouth bass.

This chapter aimed to characterize the intrinsic ability of largemouth bass to biosynthesize long-chain n-3 PUFA from dietary ALA and to verify the functional activities of key desaturase genes. To achieve this, desaturase genes were cloned and expressed in *S. cerevisiae* by inserting the amplified fragments into the pYES2 vector followed by galactose-induced expression, allowing the evaluation of substrate–product shifts and functional verification of enzymatic activities responsible for LC-PUFA biosynthesis. Furthermore, primary hepatocytes of largemouth bass were isolated and incubated with exogenous ALA to determine their cellular utilization and conversion efficiency. In parallel, an *in vivo* gavage trial using ^{13}C18:3n-3 was performed to analyze isotopic enrichment and metabolic products before and 24 h after administration, thereby quantifying the conversion of labeled substrates under physiological conditions.

Chapter III Effects of different oil sources on growth and lipid metabolism of largemouth bass.

This chapter aimed to determine how dietary replacement of marine lipids with VO in a commercial diet formulation affects growth performance, lipid metabolism, and physiological health of juvenile largemouth bass. A two-factor feeding trial was conducted to evaluate the effects of SO and CO, supplied at 50% or 100% dietary lipid inclusion, on growth performance, lipid metabolism, and fillet quality in largemouth bass. SO was used as the primary VO in commercial diet formulations, and CO, with lower ALA content and potential application in aquafeed, was included as a comparative treatment. Through this nutritional comparison, the physiological responses and metabolic plasticity of largemouth bass under alternative lipid use were examined, thereby establishing the nutritional feasibility and limitations of replacing marine lipids.

Chapter IV LC-PUFA biosynthetic capacity and nutritional regulation in largemouth bass fed FM- and FO-free diets.

This chapter aimed to verify whether ALA functions as a functional indispensable fatty acid for largemouth bass, to assess whether exogenous DHA can compensate for ALA deficiency, and to elucidate regulatory mechanisms limiting endogenous LC-PUFA biosynthesis under FM- and FO-free conditions. To address these objectives, a strictly controlled diet system was established based on a 2 × 2 factorial design in which fish were fed LO (ALA-rich), CO (LA-rich), either with or without 3% *Schizochytrium Sp.*-derived DHA supplementation. By manipulating substrate (ALA) availability and end-product (DHA) feedback simultaneously, responses in growth performance, fatty acid biosynthesis, and lipid metabolic regulation were assessed to reveal limiting steps and feedback inhibition that constrain LC-PUFA biosynthesis in this species.

Chapter V Effects of dietary n-3 PUFA-enriched black soldier fly oil on growth and lipid metabolism in largemouth bass.

This chapter aimed to evaluate whether n-3 PUFA-enriched insect oil can nutritionally substitute for marine-derived lipids in largemouth bass and thereby offer a practical solution to their limited endogenous conversion capacity. To this end, BSF larvae were reared on diets containing gradients of fish processing by-products to generate n-3-enriched insect oils without impairing larval growth, and subsequent feeding trials investigated whether BSFO enriched with ALA, EPA, and DHA—either alone or in combination with additional DHA supplementation—could effectively fulfill the n-3 LC-PUFA requirements of fish raised under FM- and FO-free conditions. Through evaluating growth, lipid metabolism, and fatty acid deposition, the feasibility of BSFO as a sustainable n-3 PUFA source was systematically validated.

Chapter VI General discussion, conclusion and perspectives.

In this chapter, the main findings of the previous experiments were integrated to clarify the actual biosynthetic capacity of largemouth bass, the nutritional limitations behind it, and the effectiveness of DHA supplementation and n-3 enriched BSFO in compensating these limitations. The answers to the central research questions were summarized and the broader implications for sustainable feed formulation were emphasized. Finally, we summarized key knowledge gaps and discussed potential directions for future research.

2

Chapter II Desaturase characterization and *in vitro*/*in vivo* LC-PUFA biosynthesis from ALA in largemouth bass

Abstract

To evaluate long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA) biosynthetic capacity in largemouth bass *Micropterus salmoide*s and to provide a mechanistic basis for subsequent dietary lipid strategies, we conducted a series of complementary experiments integrating molecular, cellular and whole-animal approaches. First, functional characterization of fatty acyl desaturase genes was performed using heterologous expression in yeast. Three desaturase genes were identified: *fads2* (fatty acid desaturase 2) catalyzing C18:3n-3 → C18:4n-3, and *delta4 fads* (acyl-CoA Delta4 desaturase-like) exhibiting dual Δ5Δ4 activity (C20:4n-3 → C20:5n-3; C22:5n-3 → C22:6n-3), and *fads6* (fatty acid desaturase 6) showed no detectable desaturase activity. To further assess fatty acid utilization at the cellular level, primary hepatocytes of largemouth bass were incubated with α-linolenic acid (ALA, 18:3n-3). Hepatocytes effectively incorporated exogenous fatty acids; however, ALA incubation did not result in a significant increase in docosahexaenoic acid (DHA, C22:6n-3) content. Finally, an *in vivo* isotope tracing experiment was conducted by oral gavage of ^{13}C-labelled ALA (^{13}C18:3n-3) to largemouth bass (initial body weight 44.13 ± 0.07 g) to directly examine endogenous conversion under physiological conditions. The detection of ^{13}C20:4n-3 in fish plasma provides direct evidence that dietary ALA can be converted into n-3 LC-PUFA *in vivo*, confirming the physiological relevance of the biosynthetic pathway. Overall, these integrated results demonstrate that largemouth bass possesses a functional but tightly regulated endogenous n-3 LC-PUFA biosynthetic pathway. While the enzymatic machinery and *in vivo* conversion capacity are clearly present, substrate availability alone does not guarantee downstream LC-PUFA accumulation.

Keywords: largemouth bass, n-3 LC-PUFA biosynthesis, fatty acyl desaturases, hepatocytes, isotope tracing

1. Introduction

Long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA) are essential components of fish physiology, and their endogenous biosynthesis relies on a series of desaturation and elongation reactions catalyzed by key enzymes (Tocher, 2010; Xie et al., 2021). Considerable species-specific variation exists in the capacity for LC-PUFA biosynthesis among teleost fish, highlighting the need for comprehensive evaluation at the species level (Castro et al., 2016).

In previous studies, assessments of LC-PUFA biosynthesis in largemouth bass and related species have mainly relied on tissue fatty acid composition or transcriptional responses of lipid metabolism–related genes (Li et al., 2025; Yadav, 2020; Zhang et al., 2019a). Although previous studies suggest a restricted ability of largemouth bass to convert ALA to EPA and DHA, these assessments were made under dietary conditions that included FM or FO as sources of n-3 LC-PUFAs. Nevertheless, the specific roles of three annotated *fads* genes in the largemouth bass genome—*fads2* (fatty acid desaturase 2), *delta4 fads* (acyl-CoA Delta4 desaturase-like), and *fads6* (fatty acid desaturase 6), in LC-PUFA biosynthesis, particularly in the key desaturation steps remain to be elucidated (Xie et al., 2021). To address these limitations, an integrated experimental strategy is required, in which enzyme functionality (Ding et al., 2024; Li et al., 2010a), cellular fatty acid utilization (Jiao et al., 2020; Liu et al., 2017) and whole-animal metabolism (Bell et al., 2001b; Tocher & Ghioni, 1999) are evaluated in a complementary manner.

Accordingly, this chapter aimed to evaluate the endogenous n-3 LC-PUFA biosynthetic capacity of largemouth bass. To achieve this, desaturase genes were cloned and expressed in *S. cerevisiae* by inserting the amplified fragments into the pYES2 vector followed by galactose-induced expression, allowing the evaluation of substrate–product shifts and functional verification of enzymatic activities responsible for LC-PUFA biosynthesis. Furthermore, primary hepatocytes of largemouth bass were isolated and incubated with exogenous ALA to determine their cellular utilization and conversion efficiency. In parallel, an *in vivo* gavage trial using ^{13}C-labelled ALA (^{13}C18:3n-3) was performed to analyze isotopic enrichment and metabolic products before administration and 24 h after administration, thereby quantifying the conversion of labeled substrates under physiological conditions.

2. Materials and methods

2.1 Functional characterization of largemouth bass fads genes by heterologous expression

2.1.1 Materials preparation

Analytical-grade fatty acid substrates utilized for functional assays were purchased from the following suppliers, with purities ≥99% as stated by the manufacturers: ALA (C18:3n-3; Solarbio, Beijing, China), eicosatetraenoic acid (C20:4n-3; ZZstandard Co., Ltd., Zhengzhou, China), and docosapentaenoic acid (C22:5n-3; Nu-Chek Prep, USA). Stock solutions were prepared in ethanol at appropriate concentrations and stored at −20 °C until use. All substrates used in the experiments were from the same manufacturer batch to ensure consistency.

2.1.2 Molecular cloning of largemouth bass *fads* genes

Three *fads* genes annotated as fatty acyl desaturase (Fads)–related sequences were identified from the genome of largemouth bass, including *fads2* (Gene ID: 119893991; coding sequence, CDS 1338bp), *delta4 fads* (Gene ID: 119884848; CDS 1326bp), and *fads6* (Gene ID:119915965; CDS 1220bp). For clarity and consistency with prior literature, gene names are presented using English form (e.g., *fads2*, *delta4 fads*, *fads6*), whereas enzyme functions are indicated with Greek symbols (e.g., Δ4 Fads activity) to denote the positions of the desaturation reaction.

Gene annotation was based on NCBI genome annotation and pairwise amino acid sequence similarity analyses. As summarized in Table 2-1, *delta4 fads* showed complete nucleotide query coverage (100%) and high amino acid identity (80%) relative to *fads2*, indicating that these two genes represent closely related *fads2*-like paralogs. In contrast, *fads6* exhibited low query coverage (11%) and low sequence identity (34%) compared with *fads2*, supporting its annotation as a distinct desaturase gene.

The CDS of *fads2*, *delta4 fads*, and *fads6* were amplified by PCR using 2×T8 high-fidelity DNA polymerase (Tsingke Biotechnology, Beijing, China). Following amplification, the DNA fragments were digested with the appropriate restriction endonucleases (Thermo Fisher Scientific, USA) and ligated into the pYES2 vector (Invitrogen, USA) to generate the recombinant plasmids named pYES2-*fads2*, pYES2-*delta4 fads*, and pYES2-*fads6*. Three verified recombinant vectors were individually transformed into *S. cerevisiae* strain INVSc1 through the pYES2-INVSc1 yeast expression system (Thermo Fisher Scientific, USA) (Kabeya et al., 2021; Li et al., 2010a). Yeast cells harboring the empty pYES2 vector were grown under identical conditions and used as the control. Following the transformation, plasmid DNA was extracted from recombinant yeast colonies, and Sanger sequencing covering the entire CDS was performed to verify the correct insertion and integrity of the target genes.

2.1.3 Heterologous expression of largemouth bass *fads* genes in yeast

Verified transformed yeast colonies carrying pYES2 (empty-vector control), pYES2-*fads2*, pYES2-*delta4 fads*, and pYES2-*fads6* (Figure 2-1, corresponding to clone numbers 3, 4, 9, and 12, respectively) were cultured overnight in 15 mL SD/-Ura medium at 30 °C and 250 rpm. After being pelleted by centrifugation (1,500 × g, 5 min, 4 °C), the cells were resuspended in 50 mL of SG/-Ura induction medium for subsequent induction. Gradual inoculation was performed to adjust to an optical density at 600 nm (OD_{600}) of 1.

Each of the four transformed yeast cultures was supplemented with one of the following three FA substrates. These substrates, initially dissolved in ethanol, were added from stock solutions to the culture medium, to achieve final concentrations of 0.3 mmol/L ALA, 0.3 mmol/L C20:4n-3, and 0.5 mmol/L C22:5n-3; the elevated C22:5n-3 level was set in accordance with previously published protocols (Fonseca-Madrigal et al., 2014; Geay et al., 2016; Li et al., 2010a). For each transformed yeast group, a vehicle control receiving an equivalent volume of ethanol was included. In total, 16 treatments (4 constructs × 4 substrate/control conditions) were prepared, each with two independent technical replicates.

After incubation at 30 °C with agitation for 48 h, cells were harvested by centrifugation (500 × g, 2 min, 4 °C), rinsed twice with 5 mL of ice-cold Hank’s Balanced Salt Solution (HBSS, Thermo Fisher Scientific, USA), and subsequently freeze-dried for fatty acid analysis (Abdul Hamid et al., 2016; Hastings et al., 2001; Li et al., 2010a; Oboh et al., 2017).

2.1.4 Fatty acid analysis of yeast

Total fatty acids were extracted from the sample by saponification with 0.5 M NaOH in 80% methanol at 80 °C for 3 h, followed by neutralization with HCl. The extracted fatty acids were spiked with DMAQ-$^{13}C/^{15}N$-derivatized fatty acids as internal standards, then derivatized using the DMAQ-$^{12}C/^{14}N$ reagent (20 mM) in the presence of EDC (750 mM) and HOAt (15 mM) at 20 °C for 30 min. The reaction was quenched with 10% formic acid, concentrated under vacuum, and reconstituted in acetonitrile. The derivatized samples were subsequently analyzed by liquid chromatography-mass spectrometry (LC-MS) on a C8 column under positive electrospray ionization in multiple reaction monitoring (MRM) mode, with detailed procedures and analytical principles described previously (Feng et al., 2022). Analyses were performed using an ACQUITY Ultra Performance Liquid Chromatography system (Waters, USA) interfaced with a QTRAP 6500 triple quadrupole mass spectrometer (USA). FA peaks were identified by comparing retention times and ion patterns with authentic standards analyzed under identical conditions. Chromatograms of standards peaks are shown in Figure 2-2. Based on LC-MS data, extracted ion chromatograms (EICs) were generated in Origin 2021 to illustrate variations in the fatty acid profile of yeast. The relative conversion rate of FA substrates to their desaturated FA products was calculated as [peak area of FA product/(peak area of FA product + peak area of FA substrate)] × 100% (Abdul Hamid et al., 2016; Hastings et al., 2001; Li et al., 2010a; Oboh et al., 2017). The relative conversions obtained from two independent technical replicates were highly consistent (variation $< 5\%$), confirming the reliability of the results. The data are presented as the mean values of the two replicates.

Table 2-1. Summary of desaturase gene characteristics and sequence similarity in largemouth bass.

Gene name	NCBI annotation[1]	Gene ID	CDS length[2] (bp)	Protein length (aa)	% Query cover (vs *fads2*)	% Per. ident (vs *fads2*)
fads2	*fatty acid desaturase 2*	119893991	1338	445	-	-
delta4 fads	*acyl-CoA Delta4 desaturase-like*	119884848	1326	441	100%	80%
fads6	*fatty acid desaturase 6*	119915965	1220	406	11%	34%

1.Annotation was based on NCBI genome records and pairwise amino acid sequence similarity analyses, including query coverage and sequence identity.

2.CDS lengths (bp) correspond to the coding sequences used for PCR amplification and functional analyses.

3. Query Cover: percentage of the query sequence length that is included in the alignment. Per. ident: percentage of identical amino acids in the aligned region between two protein sequences.

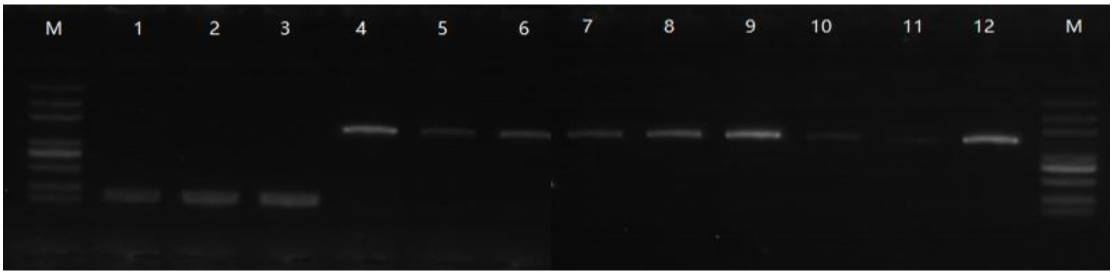
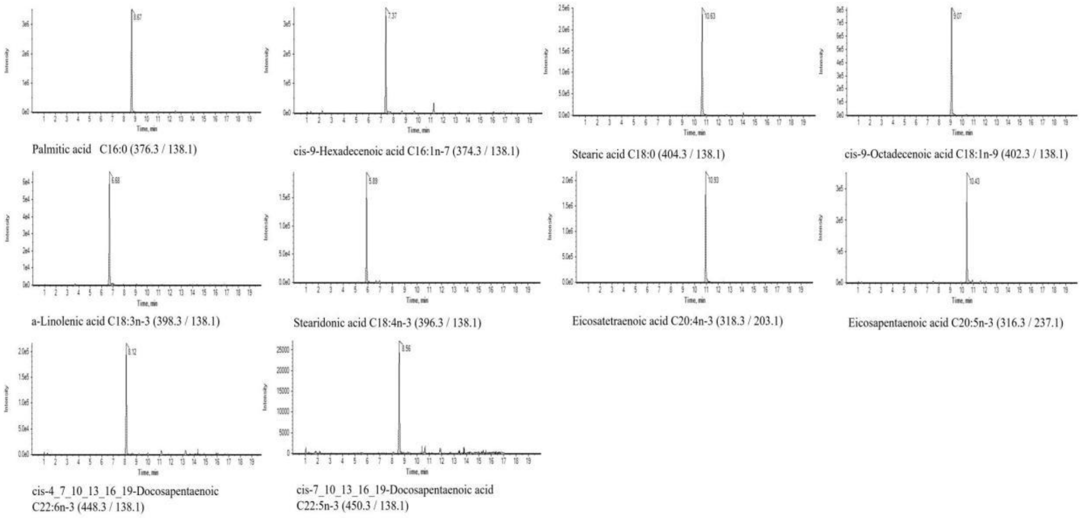


Figure 2-1. Electrophoresis pattern of amplified product from the transgenic yeasts containing recombinant vectors. Note: lanes 1–3 represent pYES2 (empty vector); lanes 4–6 represent pYES2-*fads2*; lanes 7–9 represent pYES2-*delta4 fads*; and lanes 10–12 represent pYES2-*fads6*. Lane M represents the DNA ladder.



Palmitic acid C16:0 (376.3 / 138.1)

cis-9-Hexadecenoic acid C16:1n-7 (374.3 / 138.1)

Stearic acid C18:0 (404.3 / 138.1)

cis-9-Octadecenoic acid C18:1n-9 (402.3 / 138.1)

a-Linolenic acid C18:3n-3 (398.3 / 138.1)

Stearidonic acid C18:4n-3 (396.3 / 138.1)

Eicosatetraenoic acid C20:4n-3 (318.3 / 203.1)

Eicosapentaenoic acid C20:5n-3 (316.3 / 237.1)

cis-4_7_10_13_16_19-Docosapentaenoic C22:6n-3 (448.3 / 138.1)

cis-7_10_13_16_19-Docosapentaenoic acid C22:5n-3 (450.3 / 138.1)

Figure 2-2. Chromatograms of fatty acid standards with retention times (min, x-axis) and signal intensities (cps, y-axis) analyzed under identical LC–MS conditions.

2.2 In vitro ALA incubation in primary hepatocytes of largemouth bass

2.2.1 Materials preparation

Medium 199 (M199, Thermo Fisher Scientific, USA) and Leibovitz's L-15 medium (Thermo Fisher Scientific, USA), Fetal Bovine Serum (FBS, Sigma-Aldrich, Merck KGaA, Germany), ALA (Solarbio, China), ice-cold Hanks' balanced salt solution (HBSS).

2.2.2 Hepatocyte isolation and identification

Healthy largemouth bass (mean body weight: 30.00 g; n = 40) were randomly selected from the culture tanks and anesthetized with 30 mg/L MS-222. Fish were sampled in five independent batches (biological replicates), with 8 individuals per batch. Liver tissues were aseptically excised and used for subsequent primary hepatocyte isolation. Primary hepatocytes were isolated from liver tissues following previously described procedures and analytical principles (Figueiredo et al., 2021). Cells obtained from each batch were processed independently to maintain biological replication. The isolated hepatocytes were cultured in M199/L-15 medium supplemented with 10% FBS at 28 °C. After seeding, cells were allowed to attach for 48 h before medium replacement.

Hepatocyte identification was confirmed by periodic acid–Schiff (PAS) staining for glycogen. Cell viability was then assessed using the Cell Counting Kit-8 (CCK-8, Beyotime, China) according to the manufacturer's instructions, based on the colorimetric measurement of formazan dye produced by metabolically active cells to calculate inhibition percentages.

2.2.3 Fatty acid treatment

Prior to fatty acid incubation, hepatocytes were starved in M199/L-15 medium containing 2% FBS for 12 h. A stock solution of ALA was prepared in absolute ethanol at 0.1 mol/L and subsequently diluted in M199/L-15 supplemented with 2% fatty acid–free FBS to obtain a working solution. Cells were then treated with medium containing 0.25 mmol/L ALA.

Experiments were initiated when the cells reached approximately 70% confluence, typically at 6 days post-isolation. The experimental design included a blank control group and an ALA treatment group. The control group was maintained in M199/L-15 medium containing 10% FBS, whereas the treatment group was cultured in medium supplemented with ALA. Both the control and ALA treatment groups were performed in two replicates.

2.2.4 Fatty Acid extraction and analysis

After 48 h incubation, approximately equal amounts of hepatocytes were collected, centrifuged at 500 × g for 2 min. Prior to lipid extraction, cell pellets were washed three times with ice-cold HBSS to remove residual culture medium, serum-derived lipids, and extracellular contaminants. Following the final wash, the supernatant was completely discarded and the cell pellets were immediately subjected to lipid extraction. Total lipids were extracted according to a modified Folch procedure (Folch et al., 1957). Briefly, cell pellets were homogenized in chloroform:methanol (2:1, v/v) containing butylated hydroxytoluene (BHT) as an antioxidant to minimize oxidation of unsaturated fatty acids during extraction. Following thorough mixing and incubation to ensure complete lipid solubilization, phase separation was achieved by centrifugation. The lower organic phase was carefully collected, while the aqueous phase was re-extracted with the chloroform:methanol (2:1, v/v). The combined organic extracts were evaporated to dryness under a stream of nitrogen. Lipid classes were separated by thin-layer chromatography (TLC), and the corresponding fractions were recovered and transmethylated to fatty acid methyl esters (FAME). The resulting FAME were derivatized to pyridyl esters by incubation with potassium tert-butoxide in tetrahydrofuran and 3-pyridylmethanol at 40 °C for 30 min. The pyridyl esters were then extracted with hexane, evaporated to dryness under nitrogen, and reconstituted for subsequent analysis.

Gas chromatography–mass spectrometry (GC–MS) analysis was performed in electron ionization (EI) mode using a Zebron ZB-Wax (Phenomenex) fused silica capillary column (30 m × 0.32 mm i.d.) with helium as the carrier gas. The oven temperature was programmed from 80 °C to 250 °C at a rate of 40 °C/min. Quantification was conducted using an internal standard method, and fatty acid composition was determined based on normalized peak areas, expressed as the percentage of each individual fatty acid relative to the total identified fatty acids. Data are expressed as mean ± standard error of the mean (SEM).

2.3 In vivo conversion of ALA revealed by ^{13}C-labelled isotope tracing

2.3.1 Materials preparation

^{13}C18:3n-3, C18:4n-3, C24:5n-3, C22:5n-3, C24:6n-3, C22:5n-3 were obtained from zzstandard (Industrial Co., Ltd., China).

2.3.2 Experimental design

A total of 32 healthy largemouth bass (mean body weight 44.13g) were randomly allocated into four experimental groups, with 8 fish per tank, at the National Aquafeed Safety Assessment Station (Nankou, Beijing, China). Two tanks received ^{13}C18:3n-3 solution via oral gavage and were sampled at 24 and 48 h post-gavage, respectively. The remaining two tanks were orally gavaged with an equal volume of 0.9% NaCl and sampled at the same time points.

2.3.3 Pre-Gavage preparation

Before gavage, fish were fasted or fed a diet free of EPA and DHA for 1–2 weeks to deplete endogenous LC-PUFA. The 10 mg/mL ^{13}C18:3n-3 solution was prepared by dissolving 10 mg of ^{13}C18:3n-3 in 50 µL absolute ethanol, followed by dilution with 950 µL 0.9% NaCl solution to achieve a uniform concentration. Gavage dosage was calculated as a 0.5% of body weight, following previous studies that adjust nutrient dosing relative to body weight in largemouth bass nutrition experiments (Liu et al., 2025; Mourente & Tocher, 1994; Mourente & Tocher, 1993)

2.3.4 Gavage and sampling

Fish were gavaged with equal volume of the prepared ^{13}C18:3n-3 solution (treatment) or 0.9% NaCl solution (control) using gavage needles. Plasma samples were collected at 24, and 48 h post-gavage. Fatty acid composition of plasma was analyzed by LC-MS to detect incorporation and conversion of the ^{13}C-labeled precursor.

3. Results

3.1 Functional characterization of genes related to fatty acid desaturation

Based on NCBI genome annotations, three candidate desaturase genes (*fads2*, *delta4 fads*, and *fads6*) potentially involved in fatty acid desaturation were chosen from genome of largemouth bass. Yeast transformed with the pYES2 vector alone exhibited the main fatty acids typically found in *S. cerevisiae* specifically 16:0, 16:1n-7, 18:0, and 18:1n-9, along with the exogenously added FAs (Figure 2-3A, E, I, M). Yeast expressing pYES2-*fads2* and cultured with the Δ6 substrate (ALA) exhibited new fatty acid peaks in their profiles (Figure 2-3F). Similarly, yeast expressing pYES2-*delta4 fads* displayed additional fatty acid peaks when cultured with pathway precursors (ALA, C20:4n-3, C22:5n-3), reflecting Δ6, Δ5, and Δ4 desaturation steps (Figure 2-3G, K, O).

The results in Table 2-2 showed that yeast expressing pYES2-*fads2* demonstrated Δ6 Fads activity (Conversion rate, 13.6%). In comparison, yeast transformed with pYES2-*delta4 fads* only a very small amount of conversion was detected when a Δ6-specific substrate (4.52%) was supplied, while displayed Δ4 and Δ5 Fads activities were detected with relative conversion rates of 40.52% and 20.49%, respectively. No desaturase activity was detected in yeast expressing pYES2-*fads6*.

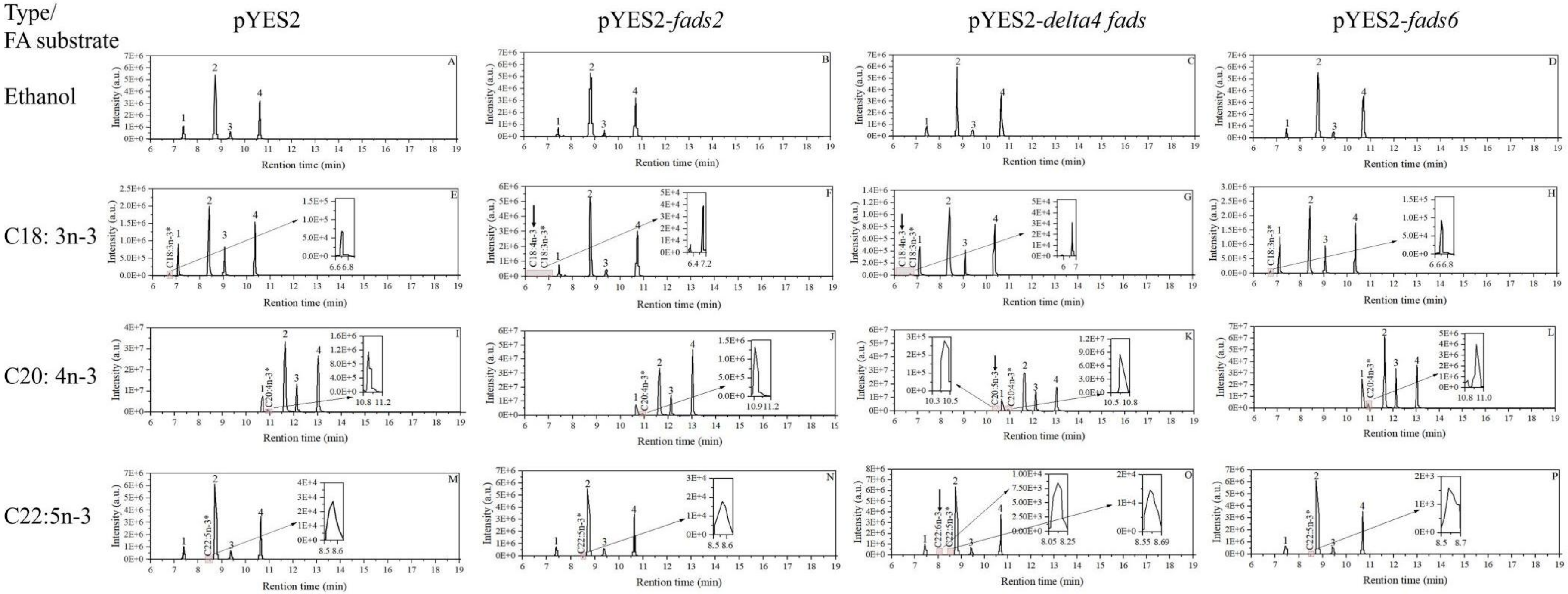


Figure 2-3. Functional characterization of fatty acyl desaturase genes from largemouth bass expressed in yeast (*S. cerevisiae*). Extracted ion chromatograms (EICs) reconstructed from LC-MS data show retention times (min, x-axis) and signal intensities (cps, y-axis). Yeast tranformed with pYES2 (empty-vector control) (A, E, I, M), pYES2-*fads2* (B, F, J, N), pYES2-*delta 4 fads* (C, G, K, O), or pYES2-*fads6* (D, H, L, P) were cultured with ethanol (vehicle control, A-D) and fatty acid substrates C18:3n-3 (E-H), C20:4n-3 (I-L), and C22:5n-3 (M-P). Peaks 1 to 4 represent the main endogenous fatty acids in *S. cerevisiae*: C16:1n-7, C16:0, C18:1n-9, and C18:0.Arrows mark desaturation products: C18:4n-3 (F, G), C20:5n-3 (K), and C22:6n-3 (O).

Table 2-2. Relative conversions catalyzed by recombinant pYES2-*fads2*, pYES2-*delta4 fads*, and pYES2-*fads6* in transformed yeast with Δ6, Δ5, and Δ4 fatty acid substrates.

Fatty acid substrate	Fatty acid product	Desaturase activity	Recombine pYES2	Conversion (%)
C18:3n-3	C18:4n-3	Δ6 Fads	pYES2-*fads2*	13.60
			pYES2-*delta4 fads*	4.52
			pYES2-*fads6*	-
C20:4n-3	C20:5n-3	Δ5 Fads	pYES2-*fads2*	-
			pYES2-*delta4 fads*	20.49
			pYES2-*fads6*	-
C22:5n-3	C22:6n-3	Δ4 Fads	pYES2-*fads2*	-
			pYES2-*delta4 fads*	40.52
			pYES2-*fads6*	-

1. Δ6, Δ5, and Δ4 fatty acyl desaturase (Fads) indicate desaturase activities at the corresponding Δ6, Δ5, and Δ4 positions, respectively.

2. Conversion (%) was calculated using the formula: [peak area of fatty acid product/(peak area of fatty acid product + peak area of fatty acid substrate)] × 100%.

3.2 In vitro ALA incubation in primary hepatocytes of largemouth bass

According to the development process of hepatocytes in largemouth bass, the cells growth reached confluence phase on the 6th day (Figure 2-4A). The cultured cells exhibited typical polygonal morphology and showed positive PAS staining, indicating abundant glycogen storage characteristic of hepatocytes (Figure 2-4B). Besides, cell viability determination showed that 0.2-0.3 mmol/L of ALA was added to the culture medium has no effect on liver cell viability (Figure 2-5A). As shown in Figure 2-5B, treatment with ALA at a concentration of 0.25mmol/L for 48 hours did not result in a significant increase in cellular DHA content in primary hepatocytes of largemouth bass.

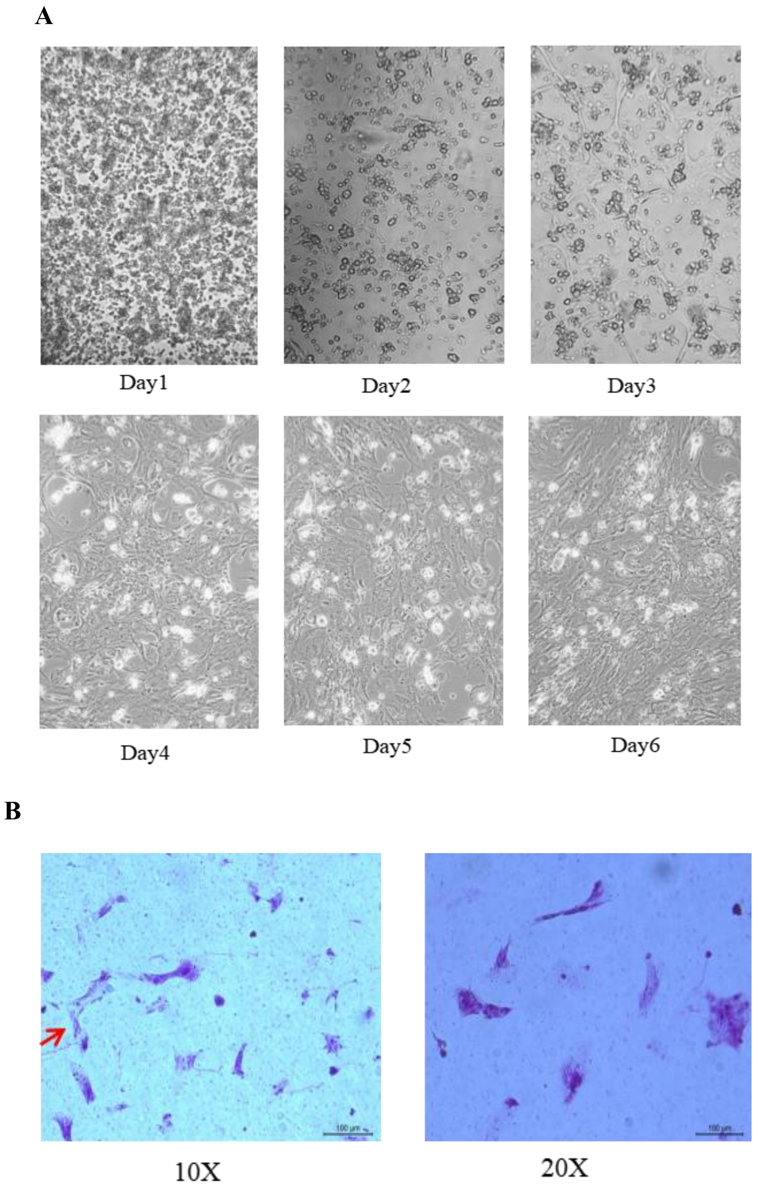
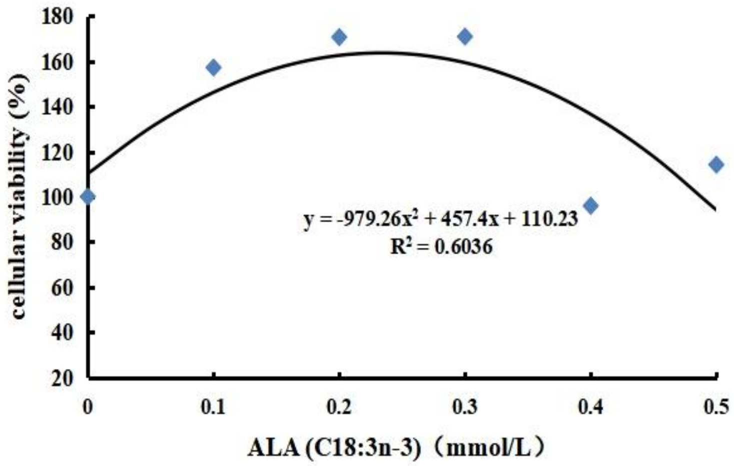


Figure 2-4. Development process of hepatocytes in largemouth bass. (A) Primary hepatocyte culture; (B) Primary hepatocytes identified by PAS glycogen staining.

A



B

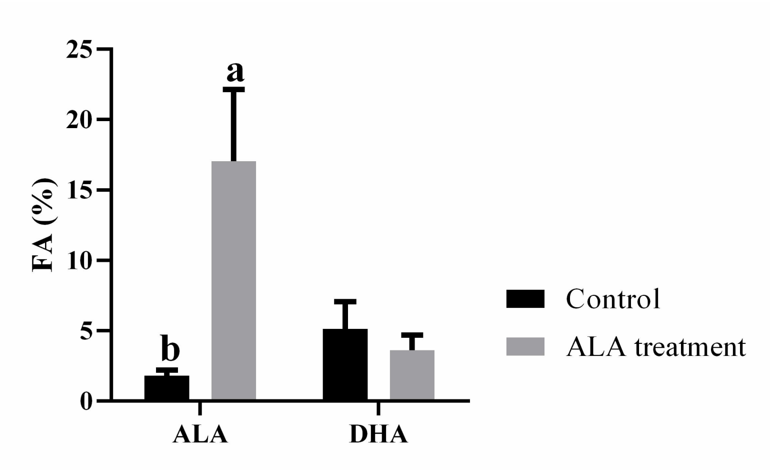


Figure 2-5. Effects of largemouth bass hepatocytes after exposure to C18:3n-3. (A) Cellular viability of hepatocytes in largemouth bass supplied with different concentration of C18:3n-3; (B) Changes in ALA and DHA levels in hepatocytes following C18:3n-3 supplementation.

3.3 Effects of ^{13}C18:3n-3 gavage on n-3 LC-PUFA biosynthesis of largemouth bass

As shown in Table 2-3, no significant differences were observed in the plasma fatty acid profile between the NaCl and ^{13}C18:3n-3 groups at 24 h post-gavage ($P > 0.05$). Absolute concentrations and relative proportions of all endogenous fatty acids did not differ significantly between the NaCl and ^{13}C18:3n-3 groups, demonstrating that the ^{13}C18:3n-3 gavage load did not disturb baseline fatty acid composition. Consequently, the endogenous background can be reliably used for subsequent ^{13}C-tracer-based metabolic flux analyses.

Based on plasma fatty acid composition of largemouth bass at 24h and 48h gavage, C18:3n-3 was converted to C20:4n-3 (Figure 2-6), indicating the presence of Δ6 or Δ8 desaturation activity in this species. However, no other downstream n-3 PUFAs were detected.

Table 2-3. Fatty acid composition (ug/mL) of plasma in largemouth bass after gavage 24h (mean ± SEM).

Item	Nacl treatment	^{13}C18:3n-3 treatment
C17:0	61.01±6.80	44.33±2.25
C18:0	1367.5±105.16	1215.18±45.12
C20:0	104.33±8.66	90.95±6.48
C21:0	23.65±5.28	10.82±0.58
C22:0	28.81±5.05	16.56±0.63
C23:0	28.43±7.10	58.79±1.24
C24:0	9328.61±1325.98	16175.51±3109.71
C16:1n-7	256.96±28.91	239.24±28.43
C17:1n-7	64.72±3.88	62.43±4.89
C18:1n-9	1287.57±29.94	1359.40±61.57
C20:1n-9	250.37±8.89	229.83±23.78
C22:1n-9	107.78±5.54	122.05±8.06
C24:1n-9	72.59±7.88	62.48±3.68
C18:3n-3	203.56±12.93	198.59±14.17
C18:4n-3	63.97±5.00	53.46±3.30
C20:3n-3	46.17±15.11	57.01±3.42
C20:4n-3	11780.84±960.08	11690.09±962.76
C20:5n-3	509.65±16.50	508.73±41.16
C22:5n-3	294.63±21.34	298.19±15.33
C22:6n-3	3959.37±167.69	4006.73±267.95
C24:5n-3	18.62±4.23	9.94±0.69
C24:6n-3	105.92±7.67	98.07±5.53
C18:2n-6	1618.18±41.79	1642.06±130.03
C18:3n-6	10471.96±1306.21	12505.50±1575.74
C20:2n-6	118.45±5.67	115.55±9.17
C20:3n-6	33.41±3.66	26.46±2.39
C20:4n-6	16.80±5.44	3.88±0.49
C22:2n-6	24.17±4.68	13.59±1.23
C22:4n-6	36.46±4.33	22.70±1.23
C22:5n-6	83.58±4.27	306.78±15.55
SFA	10942.34±1304.17	17612.13±3148.20
MUFA	2039.98±57.83	2075.42±119.37
n-3PUFA	16982.73±1017.26	16920.81±795.38
n-6PUFA	12403.01±1272.26	14636.52±1644.78
PUFA	29385.74±2055.65	31607.15±1987.20

Notes: SFA: Saturated FAs; MUFA: Monounsaturated FAs; PUFA: Polyunsaturated FAs.

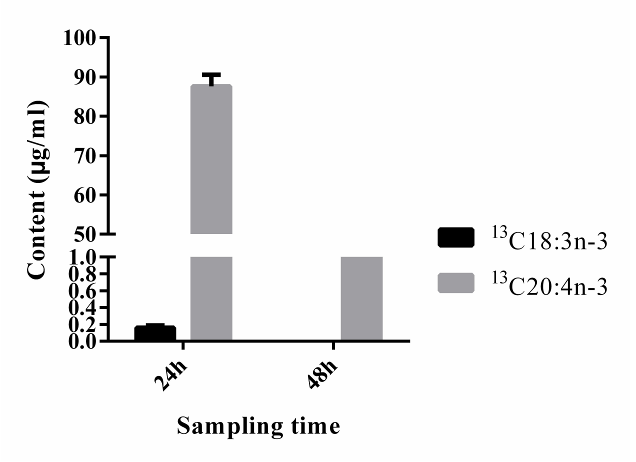


Figure 2-6. The effects of ^{13}C18:3n-3 gavage on target fatty acid of largemouth bass plasma from different sampling time.

4. Discussion

4.1 Largemouth bass fads genes exhibit distinct desaturase activities

The conversion of ALA into n-3 LC-PUFA in largemouth bass is mediated by a complex biosynthetic pathway involving multiple desaturase genes (*fads2*, *delta 4 fads*, *fads6*) and elongase genes (*elovl4a*, *elovl5*, *elovl6*, *elovl8a*) (Li et al., 2025; Liang et al., 2023; Zhang et al., 2023b). Our study provides evidence that largemouth bass possess the enzymatic basis for n-3 LC-PUFA biosynthesis by demonstrating, through heterologous expression, that *fads2*, *delta4 fads* exhibit intrinsic desaturase activity. The yeast-based heterologous expression assay was employed to characterize the intrinsic catalytic function of desaturase genes, as previously described in zebrafish (Hastings et al., 2001) and rabbitfish (Li et al., 2010b). Although these results suggest potential *in vivo* pathway capacity, actual conversion efficiency in largemouth bass may be influenced by factors such as substrate availability, tissue specificity, and competing metabolic pathway (Valenzuela et al., 2025; Valenzuela et al., 2024).

The expression of *fads2* conferred Δ6 desaturase activity, as evidenced by the conversion of ALA to its corresponding Δ6 desaturation product, with a relative conversion rate of 13.6%. This activity is consistent with the monofunctional desaturase function of Fads2 reported in most marine carnivorous species (Amara et al., 2009; Tocher et al., 2006). In contrast, *fads2* gene of largemouth bass showed neither Δ5 nor Δ4 desaturase activity, differing from the bifunctional Fads2 of the striped snakehead (Kuah et al., 2015).

Earlier research has demonstrated that *delta4 fads* encodes a broad-specificity desaturase, capable of catalyzing multiple desaturation steps (Fonseca-Madrigal et al., 2014; Oboh et al., 2017). Consistent with previous reports, the *delta4 fads* gene in largemouth bass exhibited multifunctional desaturation activity, with Δ4 (40.52%) and Δ5 (20.49%) activities. When a Δ6-specific substrate was supplied, only 4.52% of the product was converted—a value too low to be deemed functional Δ6 Fads activity according to the previous studies (Monroig et al., 2018; Shi et al., 2018b). Therefore, our results in this study support active Δ4 and Δ5 desaturase functions, whereas Δ6 activity appears negligible under experimental conditions.

Notably, *delta4 fads* exhibited their highest enzymatic activity toward the Δ4 substrate (C22:5n-3), which is consistent with a Δ4 desaturation capacity reported for this enzyme in other teleosts. This activity trend is compatible with a potential role of "Δ4 Fads2" in DHA biosynthesis via the "Δ4 pathway" (i.e., direct Δ4 desaturation of C22:5n-3 to DHA). Previous comparative studies have suggested that some more recently diverged teleosts may rely less on the "Sprecher pathway", (i.e., DHA biosynthesis via elongation of C22:5n-3 to C24:5n-3 followed by Δ6 desaturation to C24:6n-3 and subsequent β-oxidation), coinciding with the occurrence of "Δ4 pathway" (Oboh et al., 2017; Xie et al., 2021). Nevertheless, the present data do not distinguish between the "Δ4 pathway" and "Sprecher pathway".

Based on the yeast transformation results, the *fads6* gene failed to exhibit any detectable desaturation activity, its role in largemouth bass remains unknown. Although *fads6* has been reported to contribute to LC-PUFA biosynthesis in other teleost species (Wang et al., 2025; Xing et al., 2023; Zhu et al., 2021), its function in largemouth bass remains unclear and warrants further investigation.

4.2 In vitro ALA incubation in primary hepatocytes of largemouth bass

In the present study, supplementation of ALA in primary hepatocyte cultures of largemouth bass did not result in a detectable increase in cellular DHA content. This observation is consistent with the ^{13}C-labeled isotope tracing results, which demonstrated that n-3 PUFA conversion was largely restricted to early intermediates, with no detectable accumulation of downstream long-chain products.

The lack of DHA accumulation in primary hepatocytes may indicate a limited efficiency of the downstream elongation and desaturation steps involved in n-3 LC-PUFA biosynthesis, a phenomenon that has been widely reported in carnivorous teleosts with restricted endogenous LC-PUFA biosynthetic capacity (Ferosekhan et al., 2020; Geay et al., 2016). Although early desaturation activity (Δ6 and/or Δ8) may occur, as suggested by the appearance of C20:4n-3 *in vivo*, subsequent conversions toward DHA appear to be strongly constrained, likely due to rate-limiting Δ5/Δ4 desaturation or elongation steps (Castro et al., 2016; Shajahan, 2022).

In addition, primary hepatocyte culture is an *in vitro* model that cannot completely replicate the complex physiological environment of the whole organism, since it lacks hormonal regulation and tissue-level interactions present *in vivo* (Mleczko, 2017; Scown, 2009). These regulatory factors—including insulin, thyroid hormone, and LXRα-mediated signaling—are known to modulate lipid metabolism and the expression of enzymes involved in LC-PUFA biosynthesis, potentially resulting in DHA production levels below the detection threshold under *in vitro* conditions (Xie et al., 2021).

From a physiological perspective, largemouth bass is a carnivorous freshwater species that relies heavily on dietary sources of n-3 LC-PUFA. Therefore, a relatively weak endogenous DHA biosynthetic capacity may represent an adaptive nutritional strategy rather than a complete absence of the biosynthetic pathway (Carassou et al., 2025; Yadav, 2020). This interpretation is supported by the functional characterization of desaturase genes and further corroborated by *in vivo* isotope-tracing experiments presented in this study.

4.3 Physiological constraints on endogenous n-3 LC-PUFA biosynthesis in largemouth bass

The absence of significant changes in the overall plasma fatty acid profile following ^{13}C18:3n-3 gavage indicates that the tracer dose did not affect systemic fatty acid composition. This methodological feature is critical, as it ensures that subsequent detection of labeled metabolites reflects physiological metabolic flux rather than secondary responses to dietary disturbance (Felip et al., 2012).

Notably, the appearance of ^{13}C20:4n-3 in plasma provides direct *in vivo* evidence that largemouth bass is capable of converting C18:3n-3 through early desaturation and elongation steps. This finding supports the presence of functional Δ6 and/or Δ8 desaturation activity under physiological conditions and is consistent with the enzymatic activities identified through heterologous expression assays (Hastings et al., 2001; Monroig & Kabeya, 2018; Zheng et al., 2005). In contrast, no labeled downstream n-3 LC-PUFA were detected following gavage, suggesting that subsequent conversion steps toward DHA are strongly constrained *in vivo*. This limitation may arise from low catalytic efficiency of downstream desaturases or elongases, rapid dilution of labeled intermediates within large endogenous lipid pools, or insufficient tracer incorporation within the experimental time window (Bell et al., 2001a; Monroig & Kabeya, 2018; Tocher, 2010). Similar patterns of restricted downstream n-3 LC-PUFA biosynthesis have been reported in other carnivorous teleosts, which rely predominantly on dietary sources to meet their DHA requirements (Tocher, 2015). Together, these results indicate that while early steps of n-3 PUFA conversion occur *in vivo*, the overall capacity for endogenous DHA synthesis in largemouth bass remains limited.

Considered together, although metabolic conversion of ALA can occur *in vivo*, its contribution to tissue LC-PUFA pools appears to be minor under physiological conditions, suggesting that increases in ALA supply alone are unlikely to enhance tissue n-3 LC-PUFA deposition (Bell et al., 2001a; Tocher, 2010). Therefore, the subsequent chapter evaluates whether dietary lipid manipulation under aquaculture conditions can modulate tissue n-3 LC-PUFA deposition and hepatic fatty acid metabolism.

5. Conclusion

This study integrates heterologous functional characterization of desaturase genes with *in vivo* nutritional regulation analyses, providing new evidence for elucidating the n-3 LC-PUFA biosynthetic capacity of largemouth bass. Two desaturase (Δ6 Fads and Δ5Δ4 Fads) exhibited catalytic activity in heterologous expression assays, supporting the potential enzymatic capacity for DHA biosynthesis in largemouth bass. The combined evidence from heterologous expression, primary hepatocyte incubation and *in vivo* isotope tracing demonstrates that largemouth bass possesses a functional and physiologically active pathway for endogenous n-3 LC-PUFA biosynthesis. However, the efficiency and nutritional significance of this pathway under practical feeding conditions remain unclear. Therefore, further feeding trials are required to determine whether endogenous conversion of ALA can meaningfully contribute to tissue LC-PUFA deposition and growth performance, particularly in comparison with direct dietary LC-PUFA supply.

3

Chapter III Effects of different oil sources on growth and lipid metabolism of largemouth bass

From: Liu, Y., Xue, M., Francis, F., Megido, R. C., Wang, J., Wang, H., & Liang, X. (2025). Growth, fatty acid composition, and fillet quality of largemouth bass (*Micropterus salmoides*) fed soybean oil and cottonseed oil diets. Aquaculture Reports, 43, 102938.https://doi.org/10.1016/j.aqrep.2025.102938

Abstract

A 2 × 2 factorial feeding trial was conducted to evaluate the effects of lipid source (soybean oil, SO; cottonseed oil, CO) and replacement level (50% and 100% substitution of fish oil, FO) on growth performance, lipid metabolism, and fillet quality in largemouth bass *Micropterus salmoides*. A total of 480 fish (51.66 ± 0.01 g, mean ± SEM) were randomly assigned to four dietary treatments with four replicate tanks per treatment: SO50, CO50, SO100, and CO100. Fish fed with CO exhibited reduced growth performance, particularly at the 100% inclusion level ($P < 0.05$). Diets with SO or higher VO levels (50→100%) significantly reduced FIFO (Fish In : Fish Out ratio) ($P < 0.05$). Fillet quality was also altered, as indicated by reduced drip loss, increased muscle pH at 100% vegetable oil (VO) inclusion, and improved cohesiveness of fresh fillets in the CO-fed groups ($P < 0.05$). CO inclusion significantly decreased total cholesterol and low-density lipoprotein cholesterol in plasma ($P < 0.05$), which was accompanied by elevated adipose triglyceride lipase (*atgl*) expression and increased phosphorylation of AMP-activated protein kinase (p-AMPK) ($P < 0.05$); CO100 group exhibited lower triglyceride level both in plasma and liver ($P < 0.05$). Although SO-fed fish had higher dietary ALA levels and higher expression of *fads2* and *delta4 fads* ($P < 0.05$), there were no significant differences between SO- and CO-fed fish in hepatic protein abundance of fatty acyl desaturases (Fads) or in long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA) contents in muscle and liver ($P > 0.05$). Similarly, although a significant increase in *fads2* and *delta4 fads* expression, as well as in Δ5 Fads and Δ4 Fads protein expresssion, was observed at the 100% VO inclusion level ($P < 0.05$), the concentrations of n-3 LC-PUFA contents in tissues .decreased significantly when the VO inclusion level reached 100% ($P < 0.05$). In conclusion, inferior growth performance in CO-fed largemouth bass was associated with the lack of dietary ALA, limited endogenous n-3 LC-PUFA biosynthesis, and enhanced lipid metabolism, particularly in the CO100 group. These findings indicate that inclusion of SO at 50% and 100% levels maintained growth performance while reducing FIFO, whereas 100% CO inclusion negatively impacted growth performance. Moreover, endogenous ALA conversion contributes little to tissue n-3 LC-PUFA deposition under fish meal–based diets, despite elevated desaturase expression.

Keywords: largemouth bass, vegetable oils, n-3 LC-PUFA biosynthetic capacity, lipid metabolism, fillet quality

1. Introduction

Aquaculture has emerged as a critical component in food production, significantly bolstering global food security and nutritional supply (Zhang et al., 2022). In 2024, capture fisheries reached 92 million tons, a slight 1.10% increase from 2022. In contrast, aquaculture production experienced a 9.11% increase in 2024, reaching 103 million tons, surpassing 100 million tonnes for the first time (FAO, 2026). Notably, China remains the largest producer and exporter of aquaculture products, contributing over 60% to global production (FAO, 2026; Zheng et al., 2022). The rapid growth of aquaculture has led to a growing demand for fish feed, particularly for lipids. Fish oil (FO) is valued in fish feed for its unique fatty acid profile, which includes eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) (Sargent et al., 2003; Tocher et al., 2019). These FAs play a significant physiological role in aquaculture species (Cheng et al., 2018; Glencross et al., 2024). However, the rising costs and limited supply of FO necessitate alternative lipid sources to support sustainable aquaculture development (Qin et al., 2022; Tocher et al., 2019; Zorlu & Gümüş, 2022). Vegetable oils (VO), which are cost-effective and widely available, are considered promising alternatives to FO provided that essential fatty acid requirements are met (Nasopoulou & Zabetakis, 2012; Sargent et al., 1999). In recent years, global VO production has continued to increase, reaching approximately 234.5 million tons in 2025, with soybean oil (SO) representing one of the dominant vegetable oils globally, whereas cottonseed oil accounts for a comparatively minor share (USDA, 2025).

Throughout the preceding twenty years, numerous studies have assessed the impacts of substituting FO with VO or animal-derived oils, either partially or entirely, in various fish species (FAO, 2026; Zhang et al., 2019a). Despite challenges, including imbalanced fatty acid compositions (Sargent et al., 2003), reduced palatability, and difficulties in digestibility (Caballero et al., 2002; Guillou et al., 1995), increasing evidence suggests that VO, particularly those characterized by C18 polyunsaturated FAs (PUFA) could serve as viable alternative oil sources for certain species.

SO is mainly composed of linoleic acid (LA, C18:2n-6; 50 – 60% of total fatty acids) and α -linolenic acid (ALA, C18:3n-3; 5 – 8%) (USDA, 2019). The incorporation of SO into fish diets, either partially or fully, has been shown to be effective across various species. Examples include the gilthead seabream (48%), black seabream (60-80%), sharpsnout seabream *Diplodus puntazzo*, (100%), European seabass (50%), grass carp (100%), and largemouth bass (100%) (Figueiredo Silva et al., 2005; Gong et al., 2024; Liu et al., 2022c; Martínez-Llorens et al., 2007; Peng et al., 2008; Piedecausa et al., 2007; Yun et al., 2012; Zhang et al., 2019a). SO is also widely used as the primary VO in aquafeeds for largemouth bass, and blends of FO and SO (50:50, w/w) have also been commonly applied in both commercial and experimental diets to maintain fatty acid balance and support growth performance (Chen et al., 2024a; Liu et al., 2022a; Sun et al., 2025; Xie et al., 2022b; Xue et al., 2022). However, SO is also subject to significant price fluctuations and unstable supply. Consequently, it is essential to develop alternative VO sources to diversify lipid sources in aquafeed and promote sustainable aquaculture development.

Cottonseed oil (CO), a by-product of the cotton industry, is considered a non-food oil resource, represents a cost-effective vegetable oil derived from a non-food oilseed crop widely produced in China with stable yields. It is characterized by a high level of LA

(44%-55%) and low ALA content (Eroldoğan et al., 2012; Hou et al., 2024), and has gained increased attention due to its high C18 PUFA content, and potential antioxidant and anti-inflammatory properties (Park, 2019; Riaz et al., 2023). Although CO traditionally contains gossypol, a known anti-nutritional factor, industrial refining processes can effectively remove gossypol and other undesirable compounds, making the oil suitable for feed and food applications (Gutierrez & Komarnytsky, 2025). Various studies have documented successful instances that adding CO to fish diets, including gilthead seabream (60%), European seabass (100%), rainbow trout (50%), black bream *Acanthopagrus schlegelii* (60%) (Eroldoğan et al., 2012; Wassef et al., 2015; Wu et al., 2022b; Yıldız, 2018). Additionally, the growth of spotted seabass *Lateolabrax japonicus* was enhanced when provided with a diet consisting of 50% FO and 50% CO compared to a diet containing 50% FO and 50% SO (Hou et al., 2024).

Importantly, as demonstrated in the previous chapter, largemouth bass exhibits a functionally present but quantitatively limited capacity for endogenous long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA) biosynthesis. This metabolic constraint suggests that the efficacy of VO rich in C18 PUFA may depend not only on growth performance but also on their effects on fatty acid deposition and hepatic lipid metabolism. Therefore, controlled feeding trials are necessary to evaluate the practical relevance of this biosynthetic pathway under aquaculture conditions. In this context, CO was selected as a representative VO, as its LA content is comparable to that of SO, while differing in ALA levels, thereby providing a suitable model to elucidate diet-induced variations in tissue n-3 fatty acid profiles. Moreover, although extensive studies have evaluated the effects of various lipid sources on growth performance in this species (Gong et al., 2024; Liang et al., 2022; Yun et al., 2012), the application of CO in largemouth bass diets remains insufficiently investigated.

Accordingly, this study thoroughly explored the influences of SO and CO on on growth performance, fatty acid profiles, fillet quality, and lipid metabolic processes in largemouth bass, aiming to provide a theoretical basis for the utilization of CO in compound aquafeeds. Particular attention was given to whether dietary fatty acid composition could modulate n-3 LC-PUFA deposition and reveal the practical significance of endogenous fatty acid bioconversion capacity in this species.

2. Materials and methods

2.1. Diet preparation

A blend of 50 % FO and 50 % SO, commonly used in commercial largemouth bass diets, was selected as the reference lipid source in this study. Based on this, a 2 × 2 factorial feeding trial was designed to investigate the effects of lipid source (SO, CO) and inclusion level (50% and 100% FO replacement) on largemouth bass. Specifically, the experimental groups were set as follows: SO50 (50% SO and 50% FO, as dietary lipid source, w/w), CO50 (50% CO and 50% FO, w/w), SO100 (100% SO), CO100 (100% CO). SO was obtained from Yihai Kerry Group (China), and CO was sourced from Xinjiang Jinlan Plant Protein Co., Ltd. (China). Four experimental diets were formulated according to Table 3-1, with 30.2% fishmeal (FM) as the major protein source. The preparation and preservation of diets for future use following previous methods (Liu et al., 2022a; Xie et al., 2022b). Table 3-2 demonstrates the fatty acid composition of the lipid sources and experiment diets.

Table 3-1. Feed formulation and proximate composition of the experimental diets

Ingredients (%)	SO50	CO50	SO100	CO100
Fishmeal	30.20	30.20	30.20	30.20
Cottonseed protein concentrate	25.00	25.00	25.00	25.00
Soybean protein concentrate	8.00	8.00	8.00	8.00
Soybean meal	14.00	14.00	14.00	14.00
Wheat flour	4.20	4.20	4.20	4.20
Cassava tuber meal	5.00	5.00	5.00	5.00
Microcrystalline cellulose	0.10	0.10	0.10	0.10
Kelp powder	1.50	1.50	1.50	1.50
Permix[1]	1.00	1.00	1.00	1.00
DL-Met	0.04	0.04	0.04	0.04
$Ca(H_2PO_4)_2H_2O$	0.36	0.36	0.36	0.36
Choline chloride	0.40	0.40	0.40	0.40
Fish oil	5.10	5.10	-	-
Soybean oil	5.10	-	10.20	-
Cottonseed oil[2]	-	5.10	-	10.20
Proximate Composition (%, dry matter)				
Dry matter	96.50	96.41	97.36	95.22
Crude protein	52.02	51.39	51.33	52.69
Crude lipid	12.07	12.23	11.33	11.82
Ash	10.33	10.51	10.48	10.46
Energy, MJ/Kg	21.92	21.13	21.13	21.57

1. The premix provides the following per kg of diets: vitamin premix: VA 20 mg, VD3 10 mg, VE 400 mg, VB1 10 mg, VB2 15 mg, VB6 15 mg, niacin 100 mg, VC ester 1000 mg, calcium pantothenate 40 mg, VK3 20 mg, biotin 2 mg, VB12 8 mg, folic acid 10 mg, corn gluten powder 150 mg; inositol 200 mg; mineral premix: $MgSO_4$ 2040 mg, $FeSO_4 \cdot H_2O$ 300 mg, $ZnSO_4 \cdot H_2O$ 200 mg, $MnSO_4 \cdot H_2O$ 100 mg, Na_2SeO_3 10 mg, $CoCl_2 \cdot 6H_2O$ 5 mg, KI 80 mg, zeolite powder 705 mg; mould inhibitor 3400 mg.

2. According to the examining report provided by the manufacturer, the free gossypol content in cottonseed protein concentrate is 392 mg/kg (≤400 mg/kg), and in cottonseed oil is 12 mg/kg (≤200 mg/kg). After calculation, the content of gossypol in the SO50, CO50, SO100, CO100 diet is 98.00, 98.61, 98.00, 99.22 mg/kg, respectively, which are far lower than the requirement of the limit of China National Standard (≤150mg/kg) (GB13078-2017, 2017).

Table 3-2. Fatty acid composition of oil sources and diets (mg/g)

Items	Oil sources			Diets			
	FO	SO	CO	SO50	CO50	SO100	CO100
C12:0	0.89	-	-	-	-	-	-
C13:0	0.65	-	-	-	-	-	-
C14:0	56.30	0.72	6.09	4.71	4.83	2.31	2.60
C15:0	6.46	-	-	0.40	0.39	-	-
C16:0	148.00	94.40	181.00	17.56	20.88	15.56	24.53
C17:0	5.03	0.61	0.48	0.53	0.51	0.34	0.30
C18:0	32.80	35.00	17.60	4.51	3.71	4.92	3.30
C20:0	7.62	3.68	2.10	0.49	0.45	0.33	0.27
C21:0	-	-	-	1.33	1.20	0.46	0.41
C22:0	0.92	4.90	0.981	0.12	-	0.33	-
C23:0	0.38	0.36	-	-	-	-	-
C24:0	-	3.69	1.62	-	-	-	-
C16:1n-7	59.20	0.75	4.94	4.52	4.24	1.81	2.08
C17:1n-7	-	-	-	0.68	0.67	0.30	0.37
C18:1n-9	123.00	246.00	145.00	15.77	14.05	18.74	16.95
C20:1n-9	27.90	4.64	-	1.25	1.20	0.32	0.13
C22:1n-9	4.20	-	-	-	-	-	-
C24:1n-9	5.98	-	-	0.45	0.44	-	-
C18:3n-3 (ALA)	8.98	53.70	1.38	5.15	0.89	8.22	0.32
C20:3n-3	1.58	-	-	2.42	2.40	-	-
C20:5n-3 (EPA)	49.90	-	-	9.26	8.62	4.12	3.85
C22:6n-3 (DHA)	141.00	-	-	11.76	10.28	4.37	4.20
C18:2n-6 (LA)	31.50	496.00	591.00	27.73	29.23	50.18	60.84
C18:3n-6	1.04	-	-	-	-	-	-
C20:2n-6	2.35	-	-	-	-	-	-
C20:4n-6 (ARA)	8.88	-	-	0.92	0.77	0.15	0.13
∑SFA	258.67	143.00	209.87	30.04	32.33	24.25	31.41
∑MUFA	220.28	251.39	149.94	22.67	20.60	21.18	19.53
∑n-3PUFA	201.46	53.70	1.38	28.60	22.19	16.71	8.37
∑n-6PUFA	43.77	496.00	591.00	28.65	30.01	50.32	60.97
n-3/n-6PUFA	4.60	0.11	0.00	1.00	0.74	0.33	0.14
PUFA	245.23	549.70	592.38	57.24	52.20	67.03	69.34
EPA+DHA	190.90	-	-	21.03	18.90	8.48	8.06

Note: some fatty acids (including C12:0, C13:0, C22:0, C23:0, C17:1n-7 etc.) content were not detected).

SFA: Saturated FAs; MUFA: Monounsaturated FAs; PUFA: Polyunsaturated FAs.

2.2. Fish rearing conditions

The National Fisheries Technology Extension Center supplied the juvenile fish, which were subsequently transported to the National Aquafeed Safety Assessment Station (Nankou, Beijing), for reaing in a circulating water system. Before the experiment, fish underwent a two-week acclimation period. Following a 24-h fasting period, a total of 480 apparently healthy fish were selected to ensure uniform body weight and randomly assigned to four dietary treatments, each with four replicate tanks (30 fish per tank). The initial body weight was 51.66 ± 0.01 g (mean ± SEM, n = 16 tanks). Fish were reared in 16 cylindrical plastic tanks (0.26 m^3; Figure 3-1) at a stocking density of approximately 5.85 kg/m^3, which falls within the range commonly used in laboratory-scale feeding trials with juvenile fish. The fish were fed experimental diets to satiation twice daily (8:00 am and 4:00 pm). Throughout the experimental period, the water temperature was between 24 and 26 ℃, pH ranged from 7.4 to 7.7, dissolved oxygen was not less than 6.0 mg/L, and ammonia-N was lower than 0.2 mg/L. The fish management adhered to the China National Standard (GB/T35892–2018, 2018).

Figure 3-1. Recirculating aquaculture system in National Aquafeed Safety Assessment Station (Nankou, Beijing).

2.3. Sampling

Before the experiment, 6 fish were anesthetized using MS-222 (60 mg/L), and preserved at -20 °C for initial proximate composition testing. After a 10-week feeding period, the fish in each tank were fasted for 24 h, then individually counted and weighted to determine final body weight (FBW), survival rate (SR), weight gain (WG), specific growth rate (SGR), feeding rate (FR) and feed conversion ratio (FCR). Subsequently, 3 fish from each tank were anesthetized with MS-222 (100 mg/L) and preserved at -20 ℃ for proximate composition evaluation.

Another 3 fish from each tank were sampled and anesthetized for further analysis. Blood was extracted from the tail vein of the fish and then centrifuged (4,000×g, 10 min, 4 ℃) to obtain plasma for further assessments. Body length, body weight, carcass weight (after removing the head, fins, tail, and viscera), liver weight, viscera weight, and visceral adipose tissue weight were recorded to complete the calculation of the condition factor (CF), hepatosomatic index (HSI), viscerosomatic index (VSI), and visceral adipose index (VAI). After the removal of fish scales and skin, the dorsal muscles were collected. Three cubes (3 cm×3 cm×2 cm) were immediately taken for fillet quality analysis, and the remaining portion was preserved at -20 ℃ for fatty acid composition analysis. The sampled liver tissues, underwent histological evaluation on sections near the bile ducts. The remaining tissues were divided into three portions: the first was snap-frozen in liquid nitrogen and stored at -80 ℃ for subsequent gene and protein expression analyses; the second were maintained at -20 ℃ for tissue biochemical analysis; and the third was also stored at -20 ℃ for fatty acid composition analysis.

2.4. Indicator analysis

2.4.1. Growth performance calculations

Survival (SR, %) = final number of fish (per tank)/initial number of fish (per tank) × 100.

Weight gain (WG, %) = (Wf – Wi + Wd)/Wi × 100.

Specific growth rate (SGR, %d^{-1}) = [ln (Wf) – ln (Wi)]/days × 100.

Feeding rate (FR, %) = feed intake/[(Wf + Wi + Wd)/2]/days × 100.

Feed conversion ratio (FCR) = feed intake/(Wf + Wd – Wi).

Fish in: fish out (FIFO)=FCR × (% dietary FO/0.05 + % dietary FM/0.225) (Terpstra, 2015).

Protein efficiency ratio (PER, %) = (Wf – Wi)/protein intake × 100.

Protein productive value (PPV, %) = obtained protein /protein intake × 100.

Lipid productive value (LPV, %) = obtained lipid/lipid intake × 100.

Carcass ratio (CR, %) = carcass weight/fish weight × 100.

Condition factor (CF, g/cm^3) = (fish weight/body length3) × 100.

Viscera somatic index (VSI, %) = viscera weight/fish weight × 100.

Hepatopancreas somatic index (HSI, %) = hepatopancreas weight/fish weight × 100.

Visceral adipose index (VAI, %) = visceral adipose weight/fish weight × 100.

Wi represents the initial weight of all fish (per tank), Wf denotes the final weight of all fish (per tank), and Wd indicates the weight of dead fish (per tank) in cultivation.

2.4.2. Proximate and fatty acid composition

The proximate composition of the experimental diets, whole-body samples, and selected tissues (muscle and liver) was analyzed in accordance with the Official Methods of Analysis (AOAC, 2000). The gross energy of the four experimental diets was measured using an IKA C2000 bomb calorimeter (IKA, Germany), where gross energy was determined by measuring the heat released during the complete combustion of the sample under excess oxygen conditions (Ge et al., 2022). Muscle and liver specimens were freeze-dried under vacuum at low temperature and then ground into powder for the determination of proximate and fatty acid composition.

Fatty acids from oil sources, diets, muscle, and liver were extracted from prepared samples using a hydrolysis procedure in accordance with the China National Standard (GB5009.168-2016, 2016). The procedure was as follows: (1) approximately 1 g sample was weighed and transferred into a 10 mL volumetric flask; (2) 2.5 mL each of petroleum ether and benzene was added, the mixture was shaken thoroughly, and then left to stand for 2 h; (3) 0.5 mL of 2 mol/L potassium hydroxide methanol solution was added and the mixture was allowed to stand for an additional 30 min; (4) saturated sodium chloride solution was added until the level reached 1 cm below the frosted neck of the flask, and the mixture was left to stand until the supernatant became clear; (5) the solution was filtered through a 0.22 µm organic membrane filter; (6) fatty acid composition was analyzed using a gas chromatograph (Agilent GC7890B, Agilent Technologies, USA) method.

2.4.3. Fillet quality

The pH of the muscle sample was obtained using an HM Digital pH-80 device (HM Digital, Korea). The glass electrode was submerged into the fish muscle for a duration of 1 minute at 0 h, 24 h, 48 h, and 72 h.

The experiment evaluated the water holding capacity (WHC) by measuring drip and cooking losses. Drip loss (%) was calculated as the percentage of weight loss during storage. Fillet samples were immediately weighed and placed in aerated plastic bags, hung vertically with iron wires (to prevent contact with bag walls), sealed, and stored at 4 ℃ for 24 h. Subsequently, the samples were removed, gently dried with filter paper, and weighed again. Drip loss was calculated using the formula: Drip loss (%) = [initial sample weight (g) - sample weight after holding (g)]/initial sample weight (g) × 100.

Cooking loss (%) was calculated as the percentage of weight loss after cooking relative to the initial weight. The fresh fillets were first weighed, placed in a sealed plastic bag, and then subjected to heating in a water bath maintained at 70 °C for 10 min. After heating, the samples'surface were wiped dry, and they were reweighed to calculate the moisture loss. Cooking loss was calculated using the formula: Cooking loss (%) = [initial sample weight (g) - sample weight after cooking (g)]/initial sample weight (g) × 100.

A TMS-Pro texture analyzer (FTC, USA) was used to conduct the texture profile analysis (TPA), which included parameters such as hardness, adhesiveness, cohesiveness, springiness, gumminess, and chewiness (Chen et al., 2024b). The fresh and cooked fillet samples (3 cm × 3 cm × 2 cm) were used for the TPA, following methods reported in previous studies (Alak et al., 2024; Yuan et al., 2022a). Both fresh and cooked fillets were subjected to the same TPA procedure; however, only parameters relevant to the structural and mechanical properties of fresh fillets were considered in the subsequent analysis of raw muscle quality.

2.4.4. Biochemical indicators of plasma and liver, and the activity of liver enzymes

The levels of plasma glucose (GLU), triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AKP), total bile acid (TBA) and hepatic total protein (TP), TG, TC, LDL-c were assayed using commercial kits (Nanjing Jiancheng Bioengineering Institute, China).

Additionally, the protein abundances of cyclic adenosine monophosphate (cAMP), AMP-activated protein kinase (AMPK), phosphorylated AMPK (p-AMPK), Δ6 fatty acyl desaturase (Fads), Δ5 Fads, and Δ4 Fads in liver tissue were assessed by ELISA kits following the instruction manual (Jiangsu Meimian Industrial Co., Ltd., China). Similar to Western blot, ELISA kits use specific antibodies to recognize target proteins and, in a sandwich format, capture and detect them with an enzyme-linked secondary antibody that catalyzes a colorimetric reaction, where the color intensity is proportional to protein concentration for quantitative analysis (Nguyen et al., 2025; Zhang et al., 2021).

The liver samples were homogenized with a solution consisting of 9 times the volume of ice-cold phosphate buffer (pH 7.4), then centrifuged at 4, 000 × g for 10 min at 4 ℃. The resulting supernatant was used for the measurement of the above indicators.

2.4.5. Hepatic histopathological examination

Histological analysis was processed according to established protocols (Yu et al., 2019a; Zhang et al., 2019c). Liver specimens were preserved in 4% paraformaldehyde for 24 h, then dehydrated using the Slee MTM I/II tissue processing machine (Slee, Germany) and embedded in paraffin with the Leica EG1150 embedding station (Leica, Germany). Subsequently, tissue sections approximately 5-6 μm thick were prepared using the Leica RM 2255 paraffin slicer (Leica, Germany). After hematoxylin–eosin (H&E) staining, all tissue sections were scanned and analyzed using Tissue FAXS (Tissue Gnostics GmbH, Austria). Histological quantification was conducted using standardized procedures, with three non-overlapping regions of interest (ROIs) randomly selected from each section. Uniform image thresholding parameters were applied across all samples to ensure objective and reproducible measurements. Morphological evaluation included the identification of hepatocellular swelling, nuclear displacement, and severe vacuolization among groups. Scale bars in images represent 50 μm.

2.4.6. Gene expression analysis

The expression of genes in the liver involved in fatty acid synthesis (*acc1*, *fasn*, *pparγ*), triglyceride synthesis (*lpin1*, *dgat1*), triglyceride hydrolysis (*atgl*, *hsl*, *mgl*), triglyceride β-oxidation (*cpt1*, *pparα*), fatty acid desaturation (*fads2*, *delta4 fads*, *fads6*), and fatty acid elongation (*elovl4a*, *elovl5*, *elovl6*, *elovl8a*) were analyzed using the Quantitative Real-Time PCR method with the Bio-Rad CFX96 system (Bio-Rad, USA) (Wei et al., 2019; Xie et al., 2022b). The primer sequences for *fads6* and *elovl5* were designed according to the research on largemouth bass (Liang et al., 2023), and the sequences of other genes were retrieved from the RNA-seq database. RNA quality was verified prior to reverse transcription, with A260/280 ratios between 1.9 and 2.1. Amplification efficiencies ranged from 94% to 105% ($R^2 > 0.99$) based on standard curves. Melt-curve analysis confirmed a single, specific peak for each primer pair, and both no-reverse-transcription (NRT) and no-template (NTC) controls showed no detectable amplification. Table 3-3 presents the primer sequences. The *ef-1α* was identified as the most stable reference gene and was therefore selected for normalizing gene expression data.

Table 3-3. Real-time quantitative PCR primers.

Genes	Primer sequences	Product size (bp)	E-Value (%)	Tm (℃)
ef-1α	F: TGCTGCTGGTGTTGGTGAGTT R: TTCTGGCTGTAAGGGGGCTC	147	102.8	60.4
acc1	F: ATCCCTCTTTGCCACTGTTG R: GAGGTGATGTTGCTCGCATA	121	102.1	57.5
fasn	F: GGGCATCGTTAACCTGGACA R: CATCGTAGTGGCGCTTTTCG	121	99.0	57.5
pparγ	F: CCTGTGAGGGCTGTAAGGGTTT R: TTGTTGCGGGACTTCTTGTGA	103	102.0	59.0
lpin1	F: TCCTACGTTCCCGAGAGAAA R: TACGAGGGAACCACTTCCTG	136	98.8	58.5
dgat1	F: CACGCCTCTTCTTGGAGAAC R: AATGGTACCCACAGCCAGAC	176	105.3	58.5
atgl	F: CCATGATGCTCCCCTACACT R: GGCAGATACACTTCGGGAAA	176	99.1	58.0
hsl	F: ATCAGAGCTGGAGCACCCTA GCAGAGGAGAGCAGAAAGGA	122	99.3	60.0
mgl	F: AAGGTTTTTCTGGCGAAGGT R: CGTGGAAGTTCAGCTCATCA	130	96.8	58.0
cpt1	F: CATGGAAAGCCAGCCTTTAG R: GAGCACCAGACACGCTAACA	128	98.8	60.0

Genes	Primer sequences	Product size (bp)	E-Value (%)	Tm (℃)
pparα	F: CCACCGCAATGGTCGATATG R: TGCTGTTGATGGACTGGGAAA	144	104.3	59.0
fads2	F: ATCATTGCCCTACAATCACCAAC R: AAACCAAGCCAGATCCACCC	133	98.9	62.0
delta4 fads	F: ACTTGGAACCACTCAGCCAG R: ACCCACAAGCGGTGAGAAAT	162	97.0	56.0
fads6	F: TTGGAGAGCCACTGGTTTGT R: TCGTTGAGGAGGGACTGCT	134	91.3	57.5
elovl4a	F: GGCCATTCGTACTTTGGTGC R: CATCGTCTGGGACACGGTTA	165	96.4	57.0
elovl5	F: GGGGACTTCTGCTGCTTGA AGGACAAGAGTGTGACGCCC	159	98.1	59.0
elovl6	F: GTCACGCAAATTCGCCATGT R: CAGCCGAGGCAGTTGATTTG	227	93.6	57.0
elovl8a	F: TGGGGACCAAATCTAGGGGT R: ATCAGGAAAGACTGGCCACC	226	93.6	57.0

Abbservation: *acc1*, acetyl-CoA carboxylase 1; *fasn*, fatty acid synthase; *pparγ*, peroxisomeproliferators-activated receptor gamma; *lpin1*, lipid phosphate phosphohydrolase1; *dgat1*, diacylglycerol acyltransferase 1; *atgl*, adipose triglyceride lipase; *hsl*, hormone-sensitive lipase; *mgl*, monoacylglycerol lipase; *cpt1*, carnitine palmitoyltransferase 1; *pparα*, peroxisomeproliferators-activated receptor alpha; *fads2*, fatty acid desaturase 2; *delta4 fads*, acyl-CoA delta-4 desaturase-like; *fads6*, fatty acid desaturase 6; *elovl4a*, fatty acid elongase 4a; *elovl5*, fatty acid elongase 5; *elovl6*, fatty acid elongase 6; *elovl8a*, fatty acid elongase 8a. F: forward primer; R: reverse primer.

2.4.7. Western blot

Liver samples underwent protein extraction and concentration determination, following a previously reported method (Huang et al., 2019; Yu et al., 2019a). The primary antibody used was Δ5 Fads (FADS1, ab126706, abcam), and secondary antibody was anti-rabbit IgG-HRP (CWBIO, China). Target protein bands were developed using enhanced Clarity™ Western ECL (Bio-Rad, USA). Protein expression was quantified using the total protein normalization method (Hagner-McWhirter et al., 2015; Maloy et al., 2022) and assessed with Image Lab software (Bio-Rad, USA).

2.5. Statistical methods

All findings were reported as mean ± standard error of the mean (mean ± SEM). Statistical analysis and graphical representations were processed using GraphPad Prism 8 and Origin 2021. Differences were considered statistically significant at $P < 0.05$. A two-way ANOVA was conducted to evaluate the effects of oil type (SO and CO), inclusion level (50% and 100%), and their interaction on the measured variables. When no significant interaction was detected, the main effects were reported based on the pooled data, and Tukey's HSD test was applied to significant main effects. When a significant interaction was detected, simple effects were further examined based on tank means. Specifically, comparisons among oil sources within different VO inclusion levels (e.g., SO50, CO50, and SO100, CO100) and VO inclusion level effects within each oil source (SO50 vs. SO100, CO50 vs. CO100). Pairwise comparisons were adjusted using Sidak’s test. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1 Growth and proximate composition

The survival rate for each group exceeded 99%, with no significant differences observed among the treatments (Table 3-4, Table 3-5). Results from the two-way ANOVA indicated that, compared to largemouth bass fed diets with CO, those fed diets with SO exhibited significantly better performance in terms of FBW, WG, SGR, PER, PPV, LPV, CW, VAI and VSI ($P < 0.05$). A 50% inclusion level of VO resulted in an increase in PER, PPV, and LPV, while causing a decrease in HSI and VSI ($P < 0.05$). However, no significant differences were observed in FR, CR, and CF across VO sources and inclusion levels ($P > 0.05$).

There was a significant interaction of oil source and inclusion level on FCR ($P < 0.05$): no significant difference in FCR was observed between SO50 and CO50 groups ($P > 0.05$); however, the CO100 group exhibited significantly higher FCR than both the SO100 group ($P < 0.05$) and the CO50 group ($P < 0.05$). Correspondingly, fish fed SO diets exhibited a significantly lower FIFO ratio than those fed CO diets ($P < 0.05$), while increasing the VO inclusion level from 50% to 100% significantly reduced the FIFO ratio ($P < 0.05$).

Before the experiment commenced, the initial fish contained levels of crude protein, crude lipid, dry matter, and ash at 17.07%, 7.84%, 29.98% and 4.35% (on a wet basis) respectively. At the end of experiment, it was determined that neither the various VO sources and their inclusion levels had a significant effect on the dry matter crude protein, and ash, in fish whole body ($P > 0.05$) (Table 3-6). However, when the VO inclusion level was increased to 100%, there was a significant decrease in the crude lipid content of the whole fish ($P < 0.05$).

Table 3-4. Growth performance of largemouth bass after 10-week culturing with different lipid sources (mean ± SEM, n=4)

Item	SR (%)	FBW (g)	WG (%)	SGR ($\%\cdot d^{-1}$)	FR (%)	FCR	PER (%)	PPV (%)	LPV (%)	FIFO
Individual treatment means										
SO50	100.00±0.00	114.86±5.79	122.32±11.19	1.14±0.07	1.26±0.04	1.18±0.05	1.70±0.07	29.47±1.30	84.59±5.35	1.39±0.06
CO50	99.17±1.67	105.57±6.16	102.57±11.71	1.01±0.08	1.20±0.04	1.26±0.06	1.65±0.07	28.18±1.28	75.29±2.60	1.49±0.07
SO100	100.00±0.00	112.04±5.43	116.92±10.48	1.10±0.07	1.27±0.04	1.22±0.05^{Y}	1.60±0.07	28.30±1.23	76.53±6.57	0.82±0.04
CO100	100.00±0.00	91.42±1.31	76.94±2.57	0.81±0.02	1.19±0.03	1.51±0.02^{X*}	1.32±0.01	22.57±0.48	63.36±2.53	1.01±0.01
Means of main effect										
SO	100.00±0.00	113.45^{a}	119.62^{a}	1.12^{a}	1.26		1.68^{a}	28.89^{a}	80.56^{a}	1.10^{b}
CO	99.58±0.42	98.50^{b}	90.28^{b}	0.92^{b}	1.20		1.46^{b}	25.38^{b}	69.33^{b}	1.25^{a}
Level 50%	99.58±0.42	110.22	112.97	1.08	1.23		1.65^{x}	28.83^{x}	79.94	1.44^{x}
Level 100%	100.00±0.00	101.73	96.93	0.96	1.23		1.49^{y}	25.44^{y}	69.95	0.91^{y}
Two-way ANOVA: *P*-value										
Oil sources	0.337	0.012	0.011	0.010	0.101		0.004	0.009	0.031	0.010
Inclusion level	0.337	0.120	0.126	0.105	0.937		0.022	0.011	0.051	< 0.001
Interaction	0.337	0.286	0.297	0.243	0.848	0.046	0.085	0.073	0.682	0.322

A two-way ANOVA was conducted to evaluate the effects of oil sources (SO and CO) and inclusion levels (50% and 100%) on the measured parameters. Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (SO, CO) and inclusion levels (50%, 100%), respectively ($P < 0.05$). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant inclusion level effect within each oil source.

Note: Fish in:fish out (FIFO) was calculated based on FCR and dietary fish oil and fishmeal levels, assuming yields of 5% for fish oil and 22.5% for fishmeal.

Table 3-5. Morphometric parameters of largemouth bass fed with different lipid sources (mean ± SEM, n = 12).

Item	CW (g)	CR (%)	VAI (%)	HSI (%)	VSI (%)	CF (g/cm^3)
Individual treatment means						
SO50	89.56±5.28	70.73±0.31	3.35±0.18	1.43±0.07	7.61±0.26	1.76±0.10
CO50	75.59±2.15	70.30±0.40	2.65±0.09	1.36±0.06	6.73±0.14	1.62±0.02
SO100	98.92±6.91	71.04±0.48	3.53±0.23	1.61±0.08	7.92±0.28	1.77±0.04
CO100	86.19±5.87	70.30±0.29	3.03±0.19	1.49±0.05	7.55±0.25	1.71±0.03
Means of main effect						
SO	94.24^{a}	70.88	3.44^{a}	1.52	7.76^{a}	1.76
CO	80.89^{b}	70.30	2.84^{b}	1.43	7.14^{b}	1.67
Level 50%	82.57	70.51	3.00	1.40y	7.17^{y}	1.69
Level 100%	92.56	70.67	3.28	1.55x	7.74^{x}	1.74
Two-way ANOVA: *P*-value						
Oil sources	0.017	0.127	0.002	0.183	0.012	0.098
Inclusion level	0.069	0.689	0.132	0.032	0.022	0.098
Interaction	0.908	0.683	0.573	0.714	0.287	0.446

A two-way ANOVA was conducted to evaluate the effects of oil sources (SO and CO) and inclusion levels (50% and 100%) on the measured parameters. Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (SO, CO) and inclusion levels (50%, 100%), respectively ($P <$ 0.05). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant inclusion level effect within each oil source.

Table 3-6. Proximate composition of whole body (%, wet basis) of largemouth bass after 10-week culturing with different lipid sources (mean ± SEM, n = 4)

Item	Dry matter	Crude protein	Crude lipid	Ash
Individual treatment means				
SO50	31.58±0.22	17.22±0.07	9.87±0.24	4.04±0.05
CO50	31.65±0.25	17.36±0.08	9.53±0.08	4.25±0.06
SO100	31.21±0.29	17.09±0.26	9.13±0.39	4.10±0.11
CO100	31.34±0.18	17.06±0.12	9.09±0.17	4.25±0.11
Means of main effect				
SO	31.40	17.15	9.50	4.07
CO	31.49	17.21	9.31	4.25
Level 50%	31.61	17.29	9.70^{x}	4.15
Level 100%	31.27	17.07	9.11^{y}	4.17
Two-way ANOVA: *P*-value				
Oil sources	0.704	0.732	0.455	0.063
Inclusion level	0.182	0.184	0.034	0.768
Interaction	0.907	0.595	0.558	0.768

A two-way ANOVA was conducted to evaluate the effects of oil sources (SO and CO) and inclusion levels (50% and 100%) on the measured parameters. Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (SO, CO) and inclusion levels (50%, 100%), respectively ($P <$ 0.05). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant inclusion level effect within each oil source.

3.2 Fatty acid composition of tissues

The fatty acid profiles of fish muscle are presented in Table 3-7. The levels of C18:1n-9 and C20:1n-9, total MUFA and total n-3 PUFA were notably higher in the SO compared to the CO ($P < 0.05$). When the VO inclusion level reached 100%, the concentrations of EPA, DHA, and total n-3 PUFA dropped significantly, while those of LA, C20:2n-6, and total n-6 PUFA rose notably ($P < 0.05$). Besides, ALA showed significant interactions between oil source and inclusion level ($P < 0.05$). No significant differences in ALA were found between SO50 and CO50 ($P > 0.05$); however, the levels in SO100 were significantly higher than those in CO100 ($P < 0.05$).

The FA profiles of fish liver are also illustrated in Table 3-8. In the CO groups, the levels of C16:0, LA, total saturated FAs (SFA), and total n-6 PUFA were significantly higher ($P < 0.05$). Conversely, total monounsaturated FAs (MUFA), and total n-3 PUFA levels were markedly reduced ($P < 0.05$). Specifically, no considerable difference was observed in the levels of ARA, EPA, DHA, and total n-3 PUFA between the two oil treatments ($P > 0.05$). In the 50% VO groups, the contents of C14:0, C17:0, C16:1n-7, ARA, EPA, DHA, and total n-3 PUFA were considerably increased ($P < 0.05$). In contrast, within the 100% VO groups, the levels of LA, total SFA, total MUFA, total n-6 PUFA and total PUFA exhibited an upward trend ($P < 0.05$). In addition, significant interactions were observed for in the level of C18:0, ALA and C20:2n-6 ($P < 0.05$). Although no differences between SO and CO were found at either the 50% or 100% inclusion level for C18:0 and C20:2n-6 ($P > 0.05$), C18:0 increased with higher SO inclusion, while C20:2n-6 increased with higher CO inclusion ($P < 0.05$). ALA levels was consistently higher in SO than in CO at both inclusion levels, and also increased with SO inclusion ($P < 0.05$).

Table 3-7. Fatty acid compositions (mg/g, wet basis) in the muscle of largemouth bass after 10-week culturing with different lipid sources (mean ± SEM, n=4)

Item	C14:0	C16:0	C17:0	C18:0	C16:1n-7	C18:1n-9	C20:1n-9	C18:2n-6	C20:2n-6	C20:4n-6
Individual treatment means										
SO50	0.30±0.04	2.48±0.19	0.09±0.01	0.82±0.04	0.41±0.06	2.05±0.28	0.13±0.02	3.07±0.28	0.08±0.00	0.16±0.01
CO50	0.28±0.03	2.42±0.17	0.08±0.01	0.85±0.06	0.35±0.03	1.71±0.11	0.12±0.01	3.00±0.28	0.08±0.01	0.16±0.00
SO100	0.23±0.04	2.82±0.35	0.10±0.01	1.03±0.09	0.35±0.08	3.11±0.56	0.11±0.02	5.10±0.52	0.15±0.01	0.12±0.01
CO100	0.15±0.01	2.23±0.07	0.11±0.00	0.81±0.02	0.20±0.01	1.54±0.07	0.07±0.00	4.18±0.17	0.17±0.01	0.11±0.01
Means of main effect										
SO	0.27	2.65	0.10	0.93	0.38	2.58^{a}	0.12^{a}	3.94	0.12	0.14
CO	0.21	2.33	0.10	0.83	0.28	1.63^{b}	0.09^{b}	3.59	0.13	0.14
Level 50%	0.29^{x}	2.45	0.09	0.83	0.38	1.88	0.13^{x}	3.04^{y}	0.08^{y}	0.16^{x}
Level 100%	0.19^{y}	2.53	0.11	0.92	0.28	2.33	0.09^{y}	4.58^{x}	0.16^{x}	0.12^{y}
Two-way ANOVA: *P*-value										
Oil sources	0.133	0.165	0.904	0.122	0.070	0.012	0.040	0.163	0.637	1.000
Inclusion level	0.012	0.747	0.059	0.173	0.070	0.184	0.020	0.001	<0.001	<0.001
Interaction	0.405	0.256	0.904	0.054	0.381	0.079	0.271	0.229	0.280	0.479

Item	C18:3n-3	C20:5n-3	C22:6n-3	C20:5n-3+ C22:6n-3	ΣSFA	ΣMUFA	Σn-6 PUFA	Σn-3 PUFA	n-3/n-6 PUFA	ΣPUFA
Individual treatment means										
SO50	0.36±0.08	0.59±0.03	2.80±0.16	3.39±0.18	3.69±0.26	2.58±0.36	3.32±0.29	3.75±0.25	1.14±0.04	7.07±0.53
CO50	0.21±0.03	0.61±0.03	2.73±0.09	3.34±0.12	3.63±0.24	2.18±0.15	3.25±0.29	3.55±0.10	1.11±0.07	6.79±0.39
SO100	0.64±0.09^{X*}	0.32±0.04	2.23±0.16	2.55±0.19	4.19±0.49	3.57±0.65	5.35±0.53	3.19±0.29	0.56±0.02	8.32±0.74
CO100	0.11±0.01^{Y}	0.29±0.01	1.98±0.07	2.27±0.07	3.30±0.09	1.82±0.08	4.46±0.18	2.38±0.08	0.53±0.00	6.84±0.26
Means of main effect										
SO		0.46	2.51	2.97	3.94	3.08^{a}	4.19	3.47^{a}	0.89	7.60
CO		0.45	2.36	2.80	3.46	1.99^{b}	3.86	2.96^{b}	0.83	6.82
Level 50%		0.60^{x}	2.77^{x}	3.36^{x}	3.66	2.38	3.28^{y}	3.65^{x}	1.13^{x}	6.93
Level 100%		0.31^{y}	2.10^{y}	2.41^{y}	3.74	2.69	4.84^{x}	2.79^{y}	0.54^{y}	7.47
Two-way ANOVA: *P*-value										
Oil sources		0.764	0.226	0.889	0.198	0.015	0.185	0.026	0.607	0.112
Inclusion level		<0.001	<0.001	<0.001	0.791	0.426	0.001	0.001	<0.001	0.227
Interaction	0.011	0.420	0.465	0.380	0.145	0.101	0.248	0.154	0.909	0.259

1. A two-way ANOVA was conducted to evaluate the effects of oil sources (SO and CO) and inclusion levels (50% and 100%) on the measured parameters. Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (SO, CO) and inclusion levels (50%, 100%), respectively ($P < 0.05$). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant inclusion level effect within each oil source.

2. SFA: Saturated FAs; MUFA: Monounsaturated FAs; PUFA: Polyunsaturated FAs.

Table 3-8. Fatty acid compositions (mg/g, wet basis) in the liver of largemouth bass after 10-week culturing with different lipid sources (mean ± SEM, n=4)

Item	C14:0	C16:0	C17:0	C18:0	C16:1n-7	C18:1n-9	C20:1n-9	C18:2n-6	C20:2n-6	C20:4n− 6
Individual treatment means										
SO50	0.72±0.04	5.42±0.22	0.20±0.01	3.01±0.02	0.94±0.06	4.56±0.34	0.33±0.02	6.21±0.34	0.19±0.01	0.70 ± 0.02
CO50	0.76±0.06	6.19±0.62	0.22±0.02	3.55±0.17	0.89±0.09	3.73±0.34	0.28±0.02	7.13±0.61	0.21±0.02	0.66 ± 0.04
SO100	0.47±0.01	5.81±0.12	0.18±0.00	3.75±0.18*	0.66±0.01	6.94±0.46	0.31±0.01	13.14±0.33	0.43±0.01	0.46 ± 0.01
CO100	0.62±0.04	7.72±0.67	0.17±0.01	3.64±0.11	0.77±0.06	4.88±0.35	0.23±0.01	15.90±1.41	0.60±0.04*	0.42 ± 0.02
Means of main effect										
SO	0.60	5.62^{b}	0.19		0.80	5.75^{a}	0.32^{a}	9.68^{b}	0.31	0.58
CO	0.69	6.96^{a}	0.19		0.83	4.30^{b}	0.25^{b}	11.52^{a}	0.40	0.56
Level 50%	0.74^{x}	5.81	0.21^{x}		0.92^{x}	4.14^{y}	0.30	6.67^{y}	0.20	0.68^{x}
Level 100%	0.55^{y}	6.77	0.17^{y}		0.71^{y}	5.91^{x}	0.27	14.52^{x}	0.52	0.44^{y}
Two-way ANOVA: *P*-value										
Oil sources	0.053	0.016	0.762		0.633	0.002	0.003	0.041		0.106
Inclusion level	0.001	0.067	0.008		0.006	0.001	0.098	0.041		<0.001
Interaction	0.280	0.253	0.278	0.031	0.216	0.126	0.369	0.278	0.002	0.924

Item	C18:3n-3	C20:5n-3	C22:6n-3	EPA+DHA	ΣSFA	ΣMUFA	Σn-6PUFA	Σn-3PUFA	n-3/n-6 PUFA	ΣPUFA
Individual treatment means										
SO50	0.78±0.05^{A*}	1.73±0.09	9.23±0.45	10.97±0.53	9.36±0.26	5.82±0.42	7.10±0.35	11.75±0.55	1.66±0.09	18.85±0.77
CO50	0.37±0.05^{B}	1.98±0.12	8.98±0.22	10.96±0.33	10.72±0.71	4.90±0.45	8.00±0.66	11.28±0.35	1.53±0.03	18.64±0.50
SO100	1.30±0.04^{X}	0.95±0.04	7.28±0.13	8.23±0.17	10.21±0.26	7.91±0.48	14.04±0.33	9.53±0.18	0.68±0.01	23.57±0.48
CO100	0.39±0.02^{Y}	1.12±0.12	8.15±0.63	9.27±0.74	12.14±0.80	5.87±0.42	17.71±1.49	9.66±0.75	0.56±0.03	27.56±2.29
Means of main effect										
SO	1.04	1.35	8.26	9.60	9.79^{b}	6.87^{a}	10.57^{b}	10.64	1.17^{a}	21.21
CO	0.38	1.49	8.51	9.99	11.43^{a}	5.39^{b}	12.16^{a}	10.36	1.05^{b}	23.10
Level 50%	0.58	1.84^{x}	9.12^{x}	10.96^{x}	10.04	5.36^{y}	7.55^{y}	11.55^{x}	1.61^{x}	18.76^{y}
Level 100%	0.84	1.04^{y}	7.72^{y}	8.75^{y}	11.18	6.89^{x}	15.61^{x}	9.60^{y}	0.63^{y}	25.28^{x}
Two-way ANOVA: *P*-value										
Oil sources		0.063	0.488	0.349	0.013	0.006	0.015	0.760	0.044	0.164
Inclusion level		<0.001	0.008	0.001	0.067	0.005	<0.001	0.005	<0.001	<0.001
Interaction	<0.001	0.693	0.225	0.343	0.627	0.233	0.109	0.591	0.959	0.125

1. A two-way ANOVA was conducted to evaluate the effects of oil sources (SO and CO) and inclusion levels (50% and 100%) on the measured parameters. Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (SO, CO) and inclusion levels (50%, 100%), respectively ($P < 0.05$). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant inclusion level effect within each oil source.

2. SFA: Saturated FAs; MUFA: Monounsaturated FAs; PUFA: Polyunsaturated FAs.

3.3 Fillet qualities

Table 3-9 reveals that varying oil sources did not significantly influence the drip loss or pH of fish fillet ($P > 0.05$). Compared to the 50% inclusion, 100% inclusion significantly reduced drip loss and increased pH (24h, 48h, 72h) ($P < 0.05$). Moreover, significant interactions between oil source and inclusion level were observed for cooking loss and pH (0h) ($P < 0.05$). No significant differences were found between SO and CO at either the 50% or 100% inclusion level for cooking loss and pH (0h) ($P > 0.05$). However, cooking loss significantly decreased with higher inclusion of CO, and pH (0h) increased at higher inclusion of SO ($P < 0.05$).

However, CO significantly enhanced the cohesiveness of the fresh fillets ($P < 0.05$, Figure 3-2A), whereas inclusion level had no significant effect on fillet texture ($P > 0.05$). Neither lipid sources nor inclusion levels affected the texture of cooked fillets ($P > 0.05$) (Figure 3-2B).

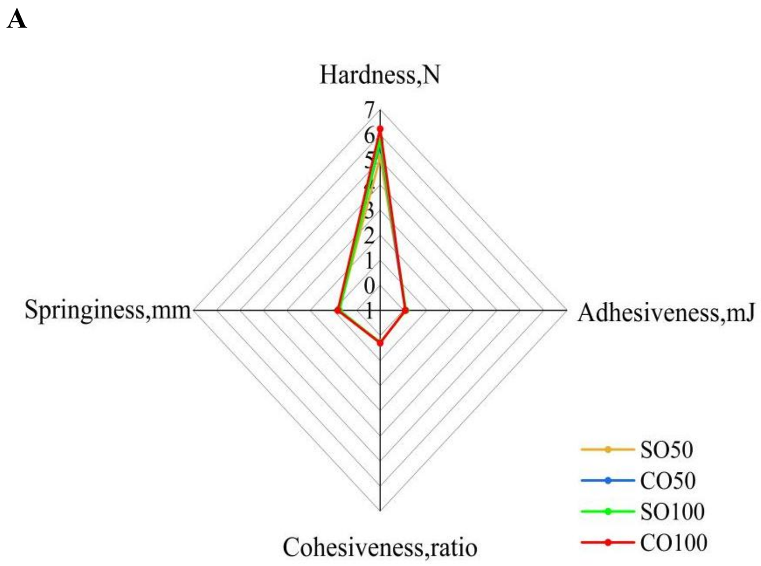
3.4 Biochemical indicators of plasma and liver

CO led to a significant decrease in plasma TC and LDL-c levels ($P < 0.05$) (Table 3-10). Additionally, neither VO source nor inclusion level significantly affected plasma GLU, AST, ALT, or hepatic TP and LDL-c levels ($P > 0.05$). Moreover, significant interaction effects between oil source and inclusion level were observed for plasma TG, HDL-c, HDL-c/LDL-c, AKP, TBA, and hepatic TG ($P < 0.05$). Further analysis showed that the levels of plasma HDL-c did not differ significantly among groups ($P > 0.05$). For plasma TG, HDL-c/LDL-c, AKP and hepatic TG, no significant differences were observed between SO50 and CO50 ($P > 0.05$); however, they were significantly higher in SO100 than in CO100 ($P < 0.05$). TBA levels showed no significant differences between SO and CO at either inclusion level ($P > 0.05$), but increased significantly with higher inclusion of both oils ($P < 0.05$). Preliminary histopathological analysis indicated that there were no significant abnormalities in the hepatic phenotype of largemouth bass (Figure 3-3A).

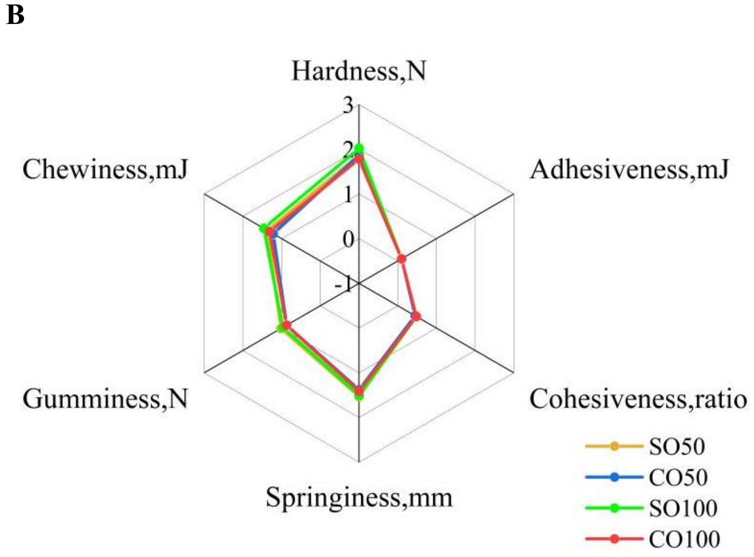
Table 3-9. Fillet quality of largemouth bass after 10-week culturing with different lipid sources (mean ± SEM, n=8)

Item	Drip loss	Cooking loss	pH (0h)	pH (24h)	pH (48h)	pH (72h)
Individual treatment means						
SO50	9.78±0.86	9.83±1.43	6.86±0.07	6.32±0.03	6.39±0.03	6.37±0.03
CO50	11.87±0.81	12.38±0.65*	7.07±0.08	6.40±0.04	6.48±0.03	6.47±0.02
SO100	9.03±0.45	8.46±0.79	7.14±0.06*	6.51±0.04	6.56±0.02	6.54±0.03
CO100	9.26±0.91	6.30±1.18	6.93±0.11	6.51±0.07	6.58±0.06	6.52±0.06
Means of main effect						
SO	9.34			6.43	6.49	6.47
CO	10.66			6.45	6.52	6.50
Level 50%	10.75^{x}			6.36^{y}	6.43^{y}	6.42^{y}
Level 100%	9.11^{y}			6.51^{x}	6.57^{x}	6.53^{x}
Two-way ANOVA: *P*-value						
Oil sources	0.119			0.307	0.091	0.164
Inclusion level	0.028			0.001	<0.001	0.004
Interaction	0.209	0.030	0.009	0.335	0.288	0.110

A two-way ANOVA was conducted to evaluate the effects of oil sources (SO and CO) and inclusion levels (50% and 100%) on the measured parameters. Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (SO, CO) and inclusion levels (50%, 100%), respectively ($P < 0.05$). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant inclusion level effect within each oil source.



Item	*P*-value			Means of main effect			
	Oil sources	Inclusion level	Interaction	SO	CO	Level 50%	Level 100%
Hardness (N)	0.241	0.164	0.877	5.51	5.92	5.40	5.93
Adhesiveness (mJ)	0.846	0.058	0.314	0.10	0.07	0.08	0.09
Cohesiveness (Ratio)	0.001	0.940	0.413	0.27^{b}	0.30^{a}	0.29	0.28
Springiness (mm)	0.224	0.958	0.636	0.75	0.79	0.77	0.76



Item	*P*-value			Means of main effect			
	Oil sources	Inclusion level	Interaction	SO	CO	Level 50%	Level 100%
Hardness (N)	0.360	0.793	0.395	2.05	1.83	1.98	1.94
Adhesiveness (mJ)	0.602	0.533	0.800	0.11	0.11	0.10	0.11
Cohesiveness (Ratio)	0.665	0.443	0.497	0.48	0.46	0.46	0.48
Springiness (mm)	0.125	0.989	0.671	1.51	1.39	1.46	1.47
Gumminess (N)	0.254	0.853	0.742	0.99	0.88	0.94	0.94
Chewiness (mJ)	0.345	0.579	0.979	1.54	1.25	1.44	1.41

Figure 3-2. Impact of different lipid sources on texture of fresh and cooked fillets of largemouth bass (n=8). (A) Hardness, adhesiveness, cohesiveness, and springiness of fresh fillets; (B) hardness, adhesiveness, cohesiveness, springiness, gumminess, and chewiness of cooked fillets.

Table 3-10. Biochemical indicators of plasma and liver of largemouth bass after 10-week culturing with different lipid sources (mean ± SEM, n=12)

Item	Plasma						Liver
	TG (mmol/L)	TC (mmol/L)	HDL-c (mmol/L)	LDL-c (mmol/L)	HDL-c/LDL-c	TBA (μmol/L)	TG (mmol/g prot)
Individual treatment means							
SO50	6.60±0.72	4.33±0.26	3.43±0.30	1.18±0.13	3.46±0.57	11.39±0.58	0.17±0.02
CO50	5.15±0.58	3.67±0.27	2.70±0.26	1.05±0.06	2.70±0.33	19.82±1.36	0.13±0.01
SO100	9.12±0.78^{X}	4.30±0.26	2.69±0.21	1.38±0.13	2.22±0.30^{Y}	30.52±0.23*	0.28±0.02^{X*}
CO100	4.48±0.76^{Y}	3.80±0.26	3.28±0.25	0.91±0.07	3.77±0.39^{X}	31.62±0.56*	0.15±0.02^{Y}
Means of main effect							
SO		4.32±0.18^{a}		1.28±0.09^{a}			
CO		3.73±0.19^{b}		0.99±0.05^{b}			
Level 50%		3.99±0.20		1.11±0.07			
Level 100%		4.09±0.19		1.17±0.09			
Two-way ANOVA: *P*-value							
Oil sources		0.034		0.008			
Inclusion level		0.857		0.798			
Interaction	<0.001	0.782	0.014	0.119	0.007	<0.001	0.014

A two-way ANOVA was conducted to evaluate the effects of oil sources (SO and CO) and inclusion levels (50% and 100%) on the measured parameters. Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (SO, CO) and inclusion levels (50%, 100%), respectively ($P < 0.05$). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant inclusion level effect within each oil source.

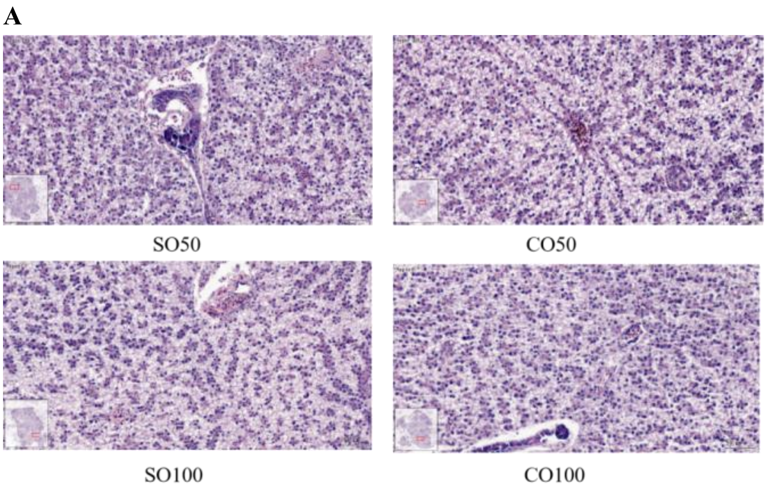
3.5 Expression of genes and protein related to lipid metabolism and energy metabolism in liver

The VO sources as well as VO inclusion levels did not significantly affect the expression of genes related to fatty acid synthesis, β-oxidation, and triglyceride synthesis related genes ($P > 0.05$) (Figure 3-3B). Fish in the SO groups presented a decreased expression level of *atgl* compared to fish in CO groups ($P < 0.05$). Furthermore, a higher VO inclusion level significantly reduced the expression level of *atgl* ($P < 0.05$), which is associated with triglyceride hydrolysis. Liver p-AMPK protein abundance in the CO groups was significantly higher than that in the SO groups ($P < 0.05$) (Figure 3-3C). At the 100% VO inclusion level, the protein abundances of cAMP, and p-AMPK, and the p-AMPK/AMPK ratio were significantly increased ($P < 0.05$).

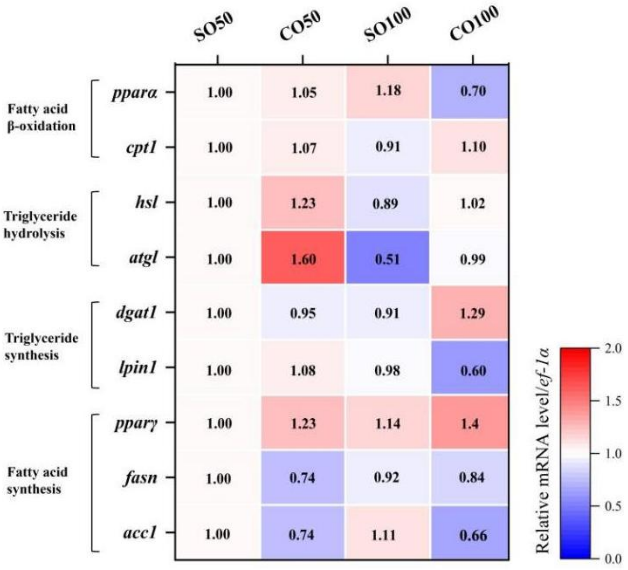
3.6 Expression of genes and protein related to LC-PUFA biosynthesis in liver.

The SO and a 100% VO inclusion level markedly enhanced the expression levels of *fads2*, *delta4 fads*, which are related to fatty acid desaturation ($P < 0.05$) (Figure 3-4A). Moreover, a significant interaction effect between oil source and inclusion level was observed for *fads6* ($P < 0.05$). No significant difference was found between SO50 and CO50 ($P > 0.05$); however, *fads6* expression was significantly higher in SO100 compared to CO100 ($P < 0.05$). The expression levels of elongase genes (*elovl5* and *elovl8a*) remained unchanged irrespective of the VO sources or their inclusion levels ($P > 0.05$).

Besides, ELISA analysis showed that hepatic Δ6 Fads and Δ5 Fads protein abundances did not differ significantly between SO and CO at either inclusion level ($P > 0.05$; Figure 3-4B). In contrast, Δ4 Fads protein abundance was significantly elevated in response to CO and to 100% VO inclusion ($P < 0.05$). Given the pivotal role of Δ5 Fads in downstream LC-PUFA biosynthesis, Western blot was further conducted to validate its protein expression pattern (Figure 3-4C). Consistent with the ELISA results, WB revealed that Δ5 Fads expression was not affected by oil source ($P > 0.05$). However, a significant increase was observed at the higher VO inclusion level ($P < 0.05$), suggesting that dietary VO inclusion level, rather than oil soures, influenced Δ5 Fads protein expression.

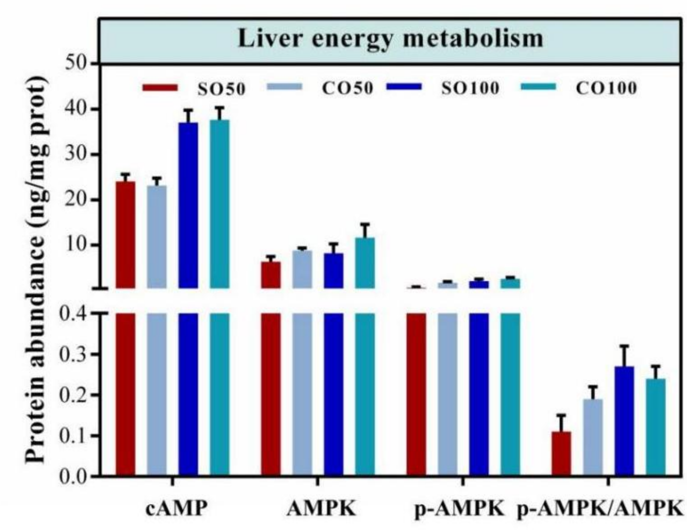


B



Item	*P*-value			Means of main effect			
	Oil sources	Inclusion level	Interaction	SO	CO	Level 50%	Level 100%
acc1	0.063	0.933	0.605	1.06	0.70	0.86	0.89
fasn	0.278	0.957	0.587	0.96	0.79	0.87	0.88
pparγ	0.027	0.146	0.904	1.07^b	1.32^a	1.12	1.27
lpin1	0.581	0.364	0.407	0.99	0.82	1.04	0.79
dgat1	0.184	0.341	0.093	0.95	1.12	0.98	1.10
atgl	0.001	0.001	0.675	0.74^b	1.29^a	1.32^x	0.75^y
hsl	0.120	0.165	0.663	0.94	1.13	1.12	0.95
cpt1	0.640	0.704	0.926	0.95	1.08	1.04	1.00
pparα	0.114	0.523	0.054	1.09	0.88	1.02	0.94

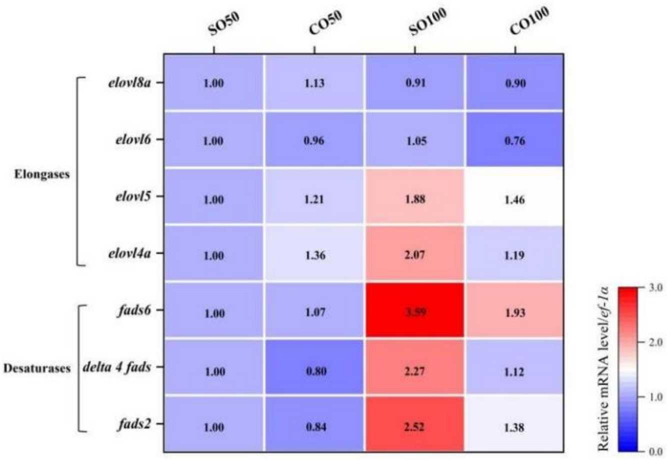
C



Item	*P*-value			Means of main effect			
	Oil sources	Inclusion level	Interaction	SO	CO	Level 50%	Level 100%
cAMP	0.943	<0.001	0.728	29.6	29.72	23.57^y	37.35^x
AMPK	0.103	0.185	0.776	7.15	10.05	7.59	9.95
p-AMPK	0.023	0.002	0.331	1.24^b	2.06^a	1.14^y	2.33^x
p-AMPK/AMPK	0.650	0.030	0.174	0.18	0.21	0.15^y	0.25^x

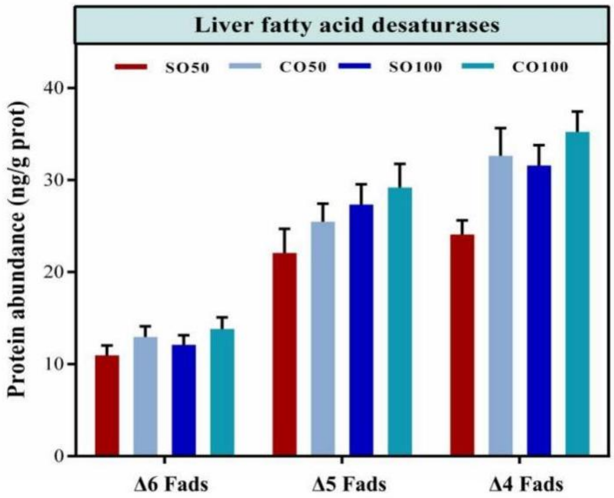
Figure 3-3. Impact of different lipid sources on lipid metabolism in the liver of largemouth bass. (A) hepatic histopathological examination of largemouth bass. H&E staining for histology examination, Statistical results of liver phenotypes (n = 12); (B) mRNA levels of hepatic fatty acid synthesis (*acc1* and *fasn*), TG hydrolysis (*atgl* and *hsl*), β-oxidation (*cpt1* and *pparα*) and triglyceride synthesis (*dgat1* and *lpin1*) related genes (n = 8); (C) Protein abundance of liver cAMP, AMPK, p-AMPK, p-AMPK/AMPK (mean ± SEM, n = 4).

A



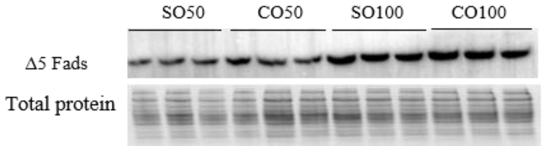
Item	*P*-value			Means of main effect			
	Oil sources	Inclusion level	Interaction	SO	CO	Level 50%	Level 100%
fads2	0.025	0.001	0.083	1.76^{a}	1.11^{b}	0.92^{y}	1.95^{x}
delta4 fads	0.007	0.002	0.051	1.64^{a}	0.96^{b}	0.90^{y}	1.70^{x}
fads6			0.023				
eovl4a	0.478	0.221	0.100	1.54	1.27	1.18	1.63
elovl5	0.761	0.120	0.375	1.44	1.33	1.1	1.67
elovl6	0.483	0.766	0.601	1.03	0.86	0.98	0.91
elovl8a	0.498	0.094	0.439	0.96	1.02	1.07	0.91

B



Item	*P*-value			Means of main effect			
	Oil sources	Inclusion level	Interaction	SO	CO	Level 50%	Level 100%
Δ6 Fads	0.118	0.386	0.901	11.57	13.38	12.02	12.96
Δ5 Fads	0.274	0.066	0.744	24.89	27.33	23.89	28.27
Δ4 Fads	0.041	0.016	0.305	28.65^{b}	33.41^{a}	28.10^{y}	33.93^{x}

C



P-Values	Δ5 Fads
Oil source	0.986
Inclusion level	0.014
Interaction	0.797

Figure 3-4. Impact of different lipid sources on LC-PUFA biosynthesis in the liver of largemouth bass. (A) Relative mRNA expression of fatty acyl desaturase *(fads*) and elongase (*elovl*) genes (n = 8); (B) Protein abundances of liver Fads (mean ± SEM, n = 12); (C) Western blot of Δ5 Fads (convert C20:4n-3 to C20:5n-3) (n=3).

4. Discussion

4.1 Impact of different lipid sources on LC-PUFA biosynthesis

Moreover, ALA and LA are crucial FA for freshwater fish species and serve as substrates for LC-PUFA biosynthesis (Tocher, 2010; Xie et al., 2021). Desaturase and elongase are two important enzymes in the biosynthesis of LC-PUFA. As Δ6 and Δ5 desaturases are essential for the bioconversion of C18 PUFAs to LC-PUFAs (Xie et al., 2021). In largemouth bass, the biosynthesis of n-3 LC-PUFAs from ALA involves key desaturase and elongase genes, including *fads2*, *fads6*, and *delta 4 fads*, as well as elongases such as *elovl4a*, *elovl5*, *elovl8a*, and *elovl6*. Among them, *fads2* and *delta4 fads* play central roles in desaturation steps, while *elovl5* and *elovl8a* are mainly involved in elongating intermediate fatty acids (Li et al., 2025; Liang et al., 2023; Zhang et al., 2023b). Although there is limited research on *fads6*, and its function in largemouth bass remains unknown (Zhang et al., 2023b), it has been identified to possess Δ4, Δ5, and Δ8 desaturase activities toward LC-PUFAs in golden pompano *Trachinotus ovatus* (Zhu et al., 2018). Moreover, *fads6* has also been investigated in relation to LC-PUFA biosynthesis in several other fish species (Wang et al., 2025; Xing et al., 2023; Zhu et al., 2021).

Studies have shown that a reduction of n-3 LC-PUFA or an elevation of the ALA/LA ratio in the fish diet regulates desaturase-related genes and enzyme activities thereby promoting the n-3 LC-PUFA biosynthetic capacity (Jin et al., 2017b; Mohammad Ali Jalali et al., 2021; Nasopoulou & Zabetakis, 2012; Qin et al., 2013; Tocher et al., 2002a; Xie et al., 2021; Xie et al., 2022a; Xie et al., 2013). However, in this study, largemouth bass showed no significant differences in n-3 LC-PUFA content in the muscle and liver among diets containing SO and CO with varying levels of ALA, which is consistent with previous reports (Yadav, 2020; Zhang et al., 2019a). Expression of *fads2 and delta4 fads* in liver were upregulated in SO groups, while *elovl5*, *elovl8a* and Δ5 Fads protein levels showed no significant differences between SO and CO. The increase in *fads6* expression at SO100 suggests its regulation may depend on the level of ALA, highlighting substrate-specific responses in LC-PUFA biosynthesis. However, unchanged Δ6 Fads and Δ5 Fads protein abundance as well as Δ5 Fads protein expression in the liver of SO groups and CO groups. Although the 100% VO inclusion level significantly upregulated the desaturase gene expression and Δ5Δ4 Fads protein abundance, tissue EPA and DHA levels declined at complete VO replacement. Taken together, these resluts suggest that the upregulation of desaturase-related gene expression does not necessarily lead to substantial changes in enzyme activity during the conversion process (Cerone & Smith, 2022). On the other hand, the similarly high LA levels in both oils compete with ALA for the same rate-limiting enzymes, thereby constraining the conversion efficiency to EPA and DHA (Vagner et al., 2009). Overall, these results suggested that largemouth bass have limited capacity for LC-PUFA biosynthesis from dietary ALA (Ruyter et al., 2000; Yadav, 2020; Zhang et al., 2019a).

4.2 Impact of different lipid sources on tissue fatty acid composition

Additionally, VO source and inclusion level have been reported to significantly influence fatty acid composition in fish muscle and liver tissues (Qin et al., 2013; Turchini et al., 2010; Yadav, 2020), which was also observed in the present study. Despite the expression of desaturase-related genes and Δ5 Fads protein being up-regulated in 100% VO groups, the accumulation of n-3 LC-PUFA content decreased in the tissues. This suggests that the dietary availability of these fatty acids plays a more decisive role than endogenous biosynthesis (Tocher, 2003; Turchini et al., 2009; Yadav, 2020). Previous study has shown that freshwater fish fed FO-based diets preferentially accumulate n-3 LC-PUFA in their tissues, which is consistent with the higher whole-body crude lipid content observed in the 50% VO groups (Ji & Tian, 2014). In addition to the reduction in n-3 LC-PUFA, a 100% VO inclusion led to increased n-6 PUFA levels in both muscle and liver tissues. Compared with CO, SO resulted in higher levels of beneficial fatty acids, including ALA and MUFA in muscle, and significantly increased hepatic ALA levels. In contrast, CO was associated with elevated levels of LA, SFA, and n-6 PUFAs in the liver. These findings underscore the importance of carefully selecting and regulating the VO source and inclusion level to maintain optimal tissue fatty acid profiles, preventing n-3 LC-PUFA deficiency and excessive accumulation of n-6 PUFAs in farmed fish (Emre et al., 2016; Güler & Yildiz, 2011; Turchini et al., 2009).

Findings have indicated that certain fish species are unable to biosynthesize sufficient amount of EPA and DHA from dietary ALA, necessitating the dietary supplementation of EPA and DHA in fish diet to support rapid growth and physiological functions. Cold-water carnivorous fish like rainbow trout require 0.7%-1.0% ALA and 0.4%-0.5% n-3 LC-PUFA, while channel catfish *Ictalurus punctatus* need 1.0%-2.0% ALA and 0.5%-0.75% n-3 LC-PUFA to support optimal growth (Ji & Tian, 2014). Similarly, although grass carp can biosynthesize EPA and DHA, a diet containing 0.52% n-3 LC-PUFA can support better growth and lipid metabolism (Ji et al., 2011). Largemouth bass show optimal growth at 0.76% n-3 LC-PUFA with 0.15% ALA (An et al., 2023), or at 1% n-3 LC-PUFA with 3.2% ALA (Yadav, 2020). In this study, 30.2% FM likely provided sufficient n-3 LC-PUFA to meet the needs of largemouth bass, which may explain the lack of growth differences between the 50% VO (2.0% n-3 LC-PUFA) and 100% VO (0.8% n-3 LC-PUFA) groups. Nonetheless, further research is needed to determine the optimal combination levels of ALA and n-3 LC-PUFAs in the diet of this species.

The adverse physiological effects of VO-based diets in fish are largely attributed to an imbalanced n-3 to n-6 PUFA ratio (Hamad et al., 2024). This imbalance can potentially lead to lipid deposition and disrupt liver lipid metabolism (Bandarra et al., 2011; Li et al., 2015; Liu et al., 2022c; Menoyo et al., 2004). The VAI and VSI served as indicators to evaluate the degree of lipid accumulation in fish. In this study, changes in the VAI and VSI generally aligned with growth performance trends, suggesting that these variations were primarily growth-related. The HSI is a key metric for assessing the impact of diet on liver health. An elevated HSI (normally 1% to 2% for osteichthyes), may signal metabolic imbalances (Dernekbaşı, 2012) and a risk of fatty liver. In this study, the HSI values varied between 1.36% and 1.61%, which is within the normal range, indicating no significant liver abnormalities.

4.3 Impact of different lipid sources on lipid and energy metabolism

Additionally, the impact of VO on lipid deposition in largemouth bass is also reflected in hematological indices, including TG, TC, LDL-c etc. (Guo et al., 2019; Li et al., 2014; Ye et al., 2011), which serve as indicators of lipid levels in metabolic circulation (Gong et al., 2024; Yun et al., 2012; Zhang et al., 2022). In the present study, fish fed SO exhibited elevated plasma TC and LDL-c levels. Besides, a significant interaction effect was observed for plasma TG, HDL-c/LDL-c, AKP, and hepatic TG, suggesting that the effects of SO become pronounced only at higher inclusion levels. When considered alongside the HSI and VSI data from the SO100 group, the findings indicate that excessive SO intake may contribute to visceral lipid accumulation and disrupt the balance of lipid metabolism (Güler & Yildiz, 2011; Piedecausa et al., 2007). However, the hepatic histopathological examination revealed no significant effects in fish fed various experimental diets, suggesting that the increased liver lipid accumulation did not lead to hepatocyte vacuolization.

Several published studies have demonstrated that gene expression data can effectively reflect changes in lipid metabolism in fish (Gong et al., 2024; Tang et al., 2025; Wang et al., 2022a). We further investigated parameters of hepatic lipid metabolism and gene expression associated with several pathways, including the absorption of FA into tissues, lipid synthesis, and their subsequent breakdown (Ipsen et al., 2018; Musso et al., 2009; Saponaro et al., 2015). ATGL, primarily expressed in the liver of fish, is crucial for TG hydrolysis, and its inhibition is linked to excessive lipid accumulation (Han et al., 2021). The upregulated expression of *atgl* in the liver of fish fed CO diets or 100% VO diets suggests a regulatory response to enhance the hydrolysis of TG, thereby influencing the cellular oxidative metabolism and energy storage (Roque-Jiménez et al., 2021). As ATGL is functionally linked to lipid mobilization and energy balance, its activation may interact with key signaling pathways involved in energy sensing (Shi et al., 2018c).

AMPK is an essential protein kinase that regulates cellular energy metabolism. When an organism's energy level are insufficient, it is activated via phosphorylation (p-AMPK), which promotes lipid degradation in fish to supply energy (Nie et al., 2020; Ran et al., 2021). Furthermore, cAMP, a second messenger, regulates mitochondrial biogenesis and participates in energy metabolism (Cheng, 2012). High-VO diets, rich in PUFA, have been associated with increased energy metabolism (Glencross et al., 2016; Henderson, 1996; Jiang et al., 2013). In this study, the 100% VO groups exhibited a high basal metabolic rate, characterized by upregulated cAMP and p-AMPK levels, especially with significantly higher p-AMPK observed in the CO100 group. Our findings suggest that largemouth bass utilize lipid catabolism as a source of energy for growth; however, excessive lipid catabolism, as noted in this study, can resulted in emaciation, which may contribute to the impaired growth observed in the fish of CO100 group (Tocher, 2003).

4.4 Impact of different lipid sources on fillet quality

Fish fillet quality serves as a critical indicator when assessing dietary oil sources, given that consumers favor flesh with desirable textural attributes such as firmness, springiness, and juiciness (Yuan et al., 2022b). A gradual decline in pH is generally associated with fillet quality deterioration (Liu et al., 2018). As time passed, the pH value of the fillets gradually decreased and showed a gentle change at 72 h post-sampling. Previous studies have reported that neither the type of VO source nor the content of VO significantly affects fillet pH values (Álvarez et al., 2020; Duan et al., 2014; Izquierdo et al., 2005). However, in this study, higher dietary VO inclusion was associated with increased muscle pH at 24h, 48h, and 72h, and pH at 0h was also elevated with higher SO inclusion. These findings align with observations in Atlantic salmon, where high VO inclusion in the diet has been shown to reduce lipid oxidation (Tocher et al., 2003), potentially lowering the production of acidic metabolites such as lactic acid and contributing to a higher fillet pH.

WHC reflects the ability of muscle to retain water under external forces and is largely influenced by myofibrillar protein integrity and muscle structure. In this study, drip loss was higher in the 100% VO groups, indicating reduced WHC during storage, whereas cooking loss decreased with increasing dietary CO inclusion, suggesting improved water retention during heating. This difference likely reflects the distinct mechanisms of water loss in fresh and cooked muscle. In addition, reduced lipid deposition in the 100% VO groups may have altered muscle structure and water distribution, thereby affecting WHC (Hocquette et al., 2010; Roth et al., 2021). Moreover, higher pH may enhance the WHC of muscle, thereby reducing water loss during processing and preservation (Andrés-Bello et al., 2013), which may also contribute to the higher WHC observed in the 100% VO group.

Texture is an important indicator of fillet quality because it reflects the structural integrity and physicochemical characteristics of muscle tissue, which can influence processing performance, storage stability, and consumer perception of fish products. (Szczesniak, 2002; Yang et al., 2020). In the present study, dietary oil sources and inclusion levels affected the texture characteristics of fresh fillets, whereas no significant differences were observed in cooked fillets. These results suggest that fresh fillet texture is more sensitive to variations in dietary lipid composition, while heat-induced protein denaturation and muscle structural rearrangement during cooking may reduce the differences originally present in raw muscle (Dhanapal et al., 2012). Therefore, although the dietary treatments influenced the physical properties of fresh fillets, their effects on the texture of cooked products appeared limited.

Fresh fillets from the CO groups exhibited higher cohesiveness than those from the other treatments, which primarily reflect the integrity of the muscle matrix and the resistance of the tissue structure to deformation. The improved structural properties observed in the CO groups may be associated with the presence of natural antioxidants, such as vitamin E, which can alleviate lipid oxidation and help preserve muscle protein integrity and tissue organization (Riaz et al., 2023; Zia et al., 2021). Similar improvements in the mechanical properties of fresh fillets have been reported in grass carp and triploid rainbow trout fed vegetable oil-based diets (Song et al., 2024b; Wang et al., 2022b). These findings indicate that dietary lipid sources can influence the structural characteristics of fresh fillets, which may be relevant for fresh product quality evaluation and processing applications.

4.5 Impact of different lipid sources on growth and proximate composition

In this study, since all treatments were conducted under identical stocking densities, the observed differences are attributed to dietary factors. Growth performance of largemouth bass was significantly affected by the oil sources (SO and CO). A meta-analysis revealed that SO, rapeseed oil, palm oil, and flaxseed oil noticeably increased the FCR, whereas CO, sunflower seed oil, corn oil, and rapeseed oil exhibited a milder effect on the FCR of major cultured fish species (Qian et al., 2024). The findings of this study were consistent with previous research, indicating that oil sources significantly affected growth of largemouth bass (Gong et al., 2024; Liang et al., 2022; Zhang et al., 2019a). In this study, the effect of CO on the FCR of largemouth bass was dependent on its inclusion level in the diet. The highest FCR was observed in the 100% CO group, which, in combination with the lowest FBW, WG, and SGR, indicated the poorest growth performance. A study on channel catfish suggested that CO is unsuitable for aquafeed applications because of the presence of anti-nutritional factors and potentially harmful compounds, including free gossypol (Yin et al., 2013). However, the content of gossypol in the feed ranged from 98.00 to 99.22 mg/kg, which is below the limit of the China National Standard (≤150mg/kg) (GB13078-2017). Therefore, this was not recognized as the main factor accounting for the growth differences in largemouth bass.

ALA is renowned for its role in maintaining the body's homeostasis (Yu et al., 2023). Studies have shown that appropriate dietary supplementation of ALA can improve the lipid metabolic function of fish, enhance the ability of digestion and absorption and therefore contribute to promoting growth performance and overall health (Castell et al., 1972; Mourente et al., 2005; Xu et al., 2015; Yu et al., 2023). From this research, SO primarily contained LA (496.00 mg/g) and ALA (53.70 mg/g), whereas CO had a higher content of LA (591.00 mg/g) but significantly less ALA (1.38 mg/g). In these two oils, LA constituted over 90% of the C18 PUFA. Compared with the other diets, the CO100 diet contained lower levels of ALA, which may be one of the reasons for the poorest growth observed among all experimental groups.

Consistent with growth performance and FCR, fish fed SO diets exhibited comparable growth efficiency to those fed CO diets, while showing a lower FIFO ratio. This indicates that comparable growth performance across treatments establishes a fundamental prerequisite for sustainability evaluation, as reductions in the FIFO ratio are meaningful only when production output is maintained (Tacon & Metian, 2008). In the present study, the lower FIFO observed in the SO group can be attributed to the combined effects of improved FCR and reduced reliance on marine-derived ingredients. This integrated effect indicates that VO substitution enhances aquafeed sustainability not only by reducing the use of finite marine resources at the formulation level, but also by optimizing the efficiency of converting wild fish inputs into farmed fish production (Gu et al., 2025; Piedecausa et al., 2007; Teoh & Ng, 2016).

5. Conclusion

Despite higher tissue n-3 LC-PUFA levels in fish fed 50% VO diets, the VO replacement level (50% vs. 100%) was not the primary determinant of growth performance, likely because

~0.8% dietary n-3 LC-PUFA supplied by FM met the requirement of largemouth bass. Instead, growth differences were associated with ALA availability: SO supported superior performance, whereas ALA-deficient CO, particularly at full replacement, impaired growth. These findings indicate that limited biosynthetic capacity and ALA deficiency, rather than total n-3 LC-PUFA levels alone, constrain growth, with 50% CO representing a practical compromise. Importantly, increased expression of desaturase-related genes did not result in enhanced n-3 LC-PUFA accumulation, suggesting downstream metabolic constraints in the biosynthetic pathway. As background n-3 LC-PUFA derived from FM may obscure endogenous capacity, further studies using FM- and FO- free diets are warranted.

4

Chapter IV LC-PUFA biosynthetic capacity and nutritional regulation in largemouth bass fed fishmeal- and fish oil-free diets

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Abstract

A feeding trial with diets free of fishmeal (FM) and fish oil (FO) was conducted in largemouth bass *Micropterus salmoides* to assess long-chain n-3 polyunsaturated fatty acid (n-3 LC-PUFA) biosynthetic capacity and the roles of dietary α-linolenic acid (ALA, C18:3n-3) and docosahexaenoic acid (DHA, C22:6 n-3) in growth and tissue deposition. In total, 300 juvenile fish (17.99 ± 0.02 g, mean ± SEM) were randomly assigned to four dietary treatments in a 2 × 2 factorial design, with three replicate tanks per treatment. The two factors were oil source (linseed oil, LO; 54.24% ALA and 16.95% LA, and cottonseed oil, CO; 0.14% ALA and 63.61% LA) and dietary DHA supplementation (with or without 3% *Schizochytrium sp.* meal, SCM; 43.27% DHA). Two-way ANOVA revealed that LO-fed fish exhibited significantly higher growth indicators and greater tissue n-3 PUFA content than CO-fed fish ($P < 0.05$). However, *delta4 fads* and *elovl5* expression was lower in the LO than CO ($P < 0.05$). By comparison, CO-fed fish showed the lowest liver triglycerides (TG) and total cholesterol (TC) content, as well as the ratio of High-density lipoprotein cholesterol and Low-density lipoprotein cholesterol (HDL-c/LDL-c) in plasma under DHA inclusion compared with LO, along with increased monoacylglycerol lipase (*mgl*) expression ($P < 0.05$) in liver. Dietary DHA significantly improved the growth performance and tissue DHA levels in CO-fed fish while reducing liver crude lipid and TG. DHA also lowered Δ5 and Δ4 Fads contents in the liver ($P < 0.05$). Overall, these findings indicate that ALA, as a key essential fatty acid for this species, sufficient dietary ALA can promote the endogenous biosynthesis of EPA and DHA, whereas dietary DHA supplementation alleviated growth depression under ALA-deficient conditions.

Keywords: largemouth bass, n-3 LC-PUFA biosynthesis, oil sources, DHA inclusion

1. Introduction

The endogenous conversion of docosahexaenoic acid (DHA, C22:6n-3) from precursors in largemouth bass appears limited by downstream metabolic constraints, as increased desaturase expression does not translate into higher tissue n-3 long-chain polyunsaturated fatty acid (n-3 LC-PUFA) levels. However, the use of fishmeal (FM)-containing diets in previous studies may have masked this intrinsic biosynthetic capacity, leaving the specific roles of dietary α-linolenic acid (ALA, C18:3n-3) and DHA unresolved.

To address this limitation, diets devoid of marine n-3 LC-PUFAs are required, in which vegetable oils (VO) provide defined and contrasting fatty acid substrates. Moreover, the fatty acid composition of VO directly influence growth performance, tissue lipid composition, and the regulation of n-3 LC-PUFA biosynthesis in fish (Gu et al., 2025; Regost et al., 2003). Furthermore, the fatty acid composition of these oils sources directly influences the fish growth performance, tissue fatty acid composition, and the level or capacity of n-3 LC-PUFA biosynthesis (Liang et al., 2022; Liu et al., 2018). Therefore, to provide insight into the endogenous LC-PUFA biosynthetic pathway of largemouth bass in a context free from dietary preformed EPA and DHA, it is necessary to select oil sources with distinctly different fatty acid compositions.

Linseed oil (LO), rich in ALA, the biosynthesis precursor of n-3 LC-PUFA, has been evaluated as a partial replacement for FO in aquafeeds across multiple farmed fish species (Asghar et al., 2025; Li et al., 2016; Turchini et al., 2009). Dietary LO has been shown to alter tissue fatty acid composition and modulate the expression of desaturase and elongase genes involved in LC-PUFA biosynthesis in species such as silver barb and rainbow trout (Nayak et al., 2020; Turchini et al., 2018). In largemouth bass, LO significantly affected growth and whole-body fatty acid composition, with dietary ALA influencing tissue EPA and DHA levels (Liang et al., 2022; Shi et al., 2019; Tidwell et al., 2007). Overall, LO represents an appropriate dietary oil source for studying LC-PUFA metabolism.

Cottonseed oil (CO), characterized by high LA content, and relatively low in n-3 PUFA, was therefore selected as an n-6-dominant dietary lipid source to contrast with n-3 precursor-rich oils. Although CO is nutritionally and compositionally comparable to widely used vegetable oils such as soybean and corn oils, it has been far less extensively evaluated in fish nutrition studies (Eroldoğan et al., 2012). Previous studies have reported variable effects on growth performance, tissue fatty acid composition, and lipid metabolism in largemouth bass (Liu et al., 2025) and other species (Hou et al., 2024; Wassef et al., 2015). Accordingly, using CO as a high-LA background diet, in contrast to a high-ALA diet, offers a model for elucidating n-3 PUFA–dependent metabolic responses in fish.

Moreover, previous studies have demonstrated that dietary inclusion of approximately 1% EPA + DHA (of total fatty acids) can enhance the growth performance and n-3 LC-PUFA deposition of largemouth bass (An et al., 2023; Yadav, 2020). In this study, in order to further investigate the interactive effects of dietary precursor supply and end-product feedback on LC-PUFA biosynthesis, we therefore included *Schizochytrium sp.* meal (SCM) in selected diets. The inclusion level was calculated to achieve approximately 1% dietary EPA + DHA, based on the established requirement for this species and the specific fatty acid profile of the SCM used. In addition, studies in fish species, such as rainbow trout (Bélanger et al., 2021; Santigosa et al., 2020) and Atlantic salmon (Zatti et al., 2023), have demonstrated that

DHA-rich microbial sources, including SCM, when used at practical inclusion level, can improve both nutritional quality and sensory attributes.

To sum up, this chapter aimed to evaluate, under FM- and FO -free dietary conditions, the combined effects of contrasting oil sources (LO vs. CO) with or without DHA inclusion on growth performance, lipid metabolism, and LC-PUFA biosynthesis in largemouth bass, thereby providing a clearer assessment of its endogenous conversion capacity and regulatory mechanisms.

2.Materials and methods

2.1 Diet preparation

A 2 × 2 factorial design was employed to evaluate the effects of different dietary lipid sources and the inclusion of DHA (from SCM) on fish growth performance and fatty acid biosynthesis. Two different oil sources were used as the primary oil sources: LO (Xilin Gol League Hongjingyuan Oil Co., Ltd., China), CO (Xinjiang Jinlan Plant Protein Co., Ltd., China). For each oil source, two levels of DHA inclusion were applied: no SCM (D-) or 3% SCM (D+), resulting in four isonitrogenous and isoenergetic experimental diets, designated LO, LOD, CO, COD. The primary protein sources consisted of 35% cottonseed protein concentrate and 5.5% yeast extract (FA28, Guangxi, China). The four experimental diets corresponding to the four treatments were formulated according to Table 4-1 and prepared according to the procedures described in earlier studies (Liu et al., 2022a; Xie et al., 2022b). Detailed fatty acid composition of the oil sources and diets are presented in Table 4-2.

Table 4-1. Formulation and proximate composition of experimental diets.

Ingredients (%, as-is basis)	LO	CO	LOD	COD
Yeast extract[1]	5.50	5.50	5.50	5.50
Cottonseed protein concentrate	35.00	35.00	35.00	35.00
Defatted superworm meal	5.00	5.00	5.00	5.00
Wheat gluten	4.00	4.00	3.50	3.50
Clostridium autoethanogenum protein	15.00	15.00	15.00	15.00
Wheat flour	8.00	8.00	8.00	8.00
Cassava tuber meal	6.00	6.00	6.00	6.00
Linseed oil	11.60	-	10.00	-
Cottonseed oil	-	11.60	-	10.00
Schizochytrium sp. meal, 43.27% DHA[2]	-	-	3.00	3.00
Permix[3]	1.10	1.10	1.10	1.10
DL-Met	0.50	0.50	0.50	0.50
L-Lys HCl	1.00	1.00	1.00	1.00
$Ca(H_2PO_4)_2H_2O$	2.45	2.45	2.45	2.45
Taurine	0.50	0.50	0.50	0.50
Choline chloride (60% choline)	0.40	0.40	0.40	0.40
Microcrystalline cellulose	3.95	3.95	3.05	3.05
Total	100	100	100	100
Proximate composition (dry matter basis)				
Dry matter (%)	94.26	94.25	94.35	94.24
Crude protein (%)	52.42	51.99	51.85	51.71
Crude lipid (%)	11.91	12.03	12.08	11.88
Ash (%)	7.71	7.55	7.51	7.41
Gross energy (MJ/Kg)	21.93	21.98	21.92	22.09
E/P (kJ/g protein)[4]	41.83	42.29	42.27	42.72

1. Yeast extract (FA28, protein content > 68.7%), was obtained from Angel Yeast CO., Ltd.

2. *Schizochytrium sp.* meal (SCM, ALGAMAC-3050), protein content >17.6%, Oil content >56.2%,, was obtained from Aquafauna Bio-Marine, Inc.

3. The premix provides the following per kg of diets: vitamin premix: VA 20 mg, VD3 10 mg, VE 400 mg, VB1 10 mg, VB2 15 mg, VB6 15 mg, niacin 100 mg, VC ester 1000 mg, calcium pantothenate 40 mg, VK3 20 mg, biotin 2 mg, VB12 8 mg, folic acid 10 mg, corn gluten powder 150 mg; inositol 200 mg; mineral premix: $MgSO_4$ 2040 mg, $FeSO_4·H_2O$ 300 mg, $ZnSO_4·H_2O$ 200 mg, $MnSO_4·H_2O$ 100 mg, Na_2SeO_3 10 mg, $CoCl_2·6H_2O$ 5 mg, KI 80 mg, zeolite powder 705 mg; mould inhibitor 3400 mg; tert-butylhydroquinone (TBHQ) 200 mg.

4. E/P(kJ/g protein), the ratio of diet gross energy(MJ/Kg) / diet crude protein (%)×100.

Table 4-2. Fatty acid composition of linseed oil, cottonseed oil, *Schizochytrium sp*. meal, and experimental diets.

Items	Ingredients (%TFA)			Diets (mg/g, dry matter basis)			
	SCM	LO	CO	LO	CO	LOD	COD
C14:0	8.85	ND	0.51	0.35	1.24	1.70	2.24
C16:0	26.6	5.41	19.84	13.23	33.22	15.98	29.81
C17:0	ND	0.05	0.05	0.28	0.33	0.26	0.28
C18:0	0.64	4.12	1.52	6.20	3.68	5.16	2.77
C20:0	ND	0.14	0.16	0.25	0.35	0.24	0.27
C16:1n-7	0.42	0.05	0.36	0.63	1.10	0.60	1.00
C18:1n-9	0.11	17.93	13.54	23.17	21.05	20.13	16.60
C18:2n-6 (LA)	ND	16.95	63.61	24.99	80.44	22.09	61.18
C18:3n-6	ND	0.73	ND	0.96	ND	0.75	ND
C18:3n-3 (ALA)	ND	54.24	0.14	59.66	0.59	48.86	0.47
C20:5n-3 (EPA)	2.88	ND	ND	ND	ND	0.36	0.33
C22:6n-3 (DHA)	43.27	ND	ND	ND	ND	6.97	6.81
∑SFA	36.09	9.98	22.30	27.72	46.74	32.31	43.43
∑MUFA	0.53	18.10	13.96	24.06	22.45	20.98	17.88
SFA/MUFA	68.09	0.55	1.60	1.15	2.08	1.54	2.43
∑n-3PUFA	46.15	54.24	0.14	59.66	0.59	56.44	7.86
∑n-6PUFA	17.26	17.68	63.61	25.95	80.44	22.85	61.18
n-3/n-6PUFA	2.67	3.07	0.00	2.30	0.01	2.47	0.13
∑PUFA	63.41	71.93	63.75	85.61	81.03	79.29	69.04

Note: ND means the fatty acid were not detected.

Abbservation: SCM, *Schizochytrium sp.* meal; LO, linseed oil; CO, cottonseed oil; SFA, Saturated FAs; MUFA, Monounsaturated FAs; PUFA, Polyunsaturated FAs

2.2 Rearing conditions

The experimental fish sourced from the National Fisheries Technology Extension Center were adapted in a recirculating aquaculture system housed at the National Aquatic Feed Safety Evaluation Base (Nankou, Beijing) and acclimatized for 2 weeks using a commercial diet before the feeding trial. After 24 h of starvation, 300 healthy fish were randomly selected and assigned to 12 tanks (0.26 m^3 each) at a density of 25 fish per tank, with three replicate tanks per treatment. The initial body weight was 17.99 ± 0.02g (mean ± SEM, n=12). During the 8-week trial, fish were fed to apparent satiation status twice daily, at 08:00 and 16:00. Daily feed intake was recorded for each tank throughout the feeding trial. The recirculating aquaculture system was kept at a stable temperature of 24.3± 0.3 °C, pH 7.2-7.8, dissolved oxygen ≥ 7mg/L, and ammonia nitrogen < 0.2 mg/L, with water quality parameters regularly monitored to ensure optimal fish health. The feeding trial complied with the guidelines for animal welfare and was approved by the Animal Ethics Committee of Chinese Academy of Agricultural Sciences (Ethics Approval ID: IFR-CAAS20240611).

2.3 Sampling

Samples were collected according to the established protocol (Liu et al., 2025). Before the trial, six fish were sedated with 60 mg/L MS-222 and stored at -20 °C for baseline analysis of proximate composition. Following the 8-week feeding trial, fish in each tank underwent a 24-hour fasting period before being individually enumerated and weighed to assess the growth performance (n = 3 per treatment). After these measurements, three fish from each tank were anesthetized using 100 mg/L MS-222 and subsequently frozen at -20 °C, and analyzed as one pooled sample (n = 3 per treatment) for proximate composition analysis.

Four fish were collected from each tank, anesthetized and individually sampled (n = 12 per treatment). Measurements including body length, body weight, carcass weight (CW, after removal of the head, fins, tail, and internal organs), liver weight, total viscera weight, and visceral adipose weight were recorded. These data were used to calculate the morphometric parameters. Blood samples were drawn from the caudal vein and spun (4,000 × g, 10 minutes, 4 °C) to isolate plasma for biochemical determinations. Liver tissues were sampled and histologically evaluated on sections adjacent to the bile ducts. The residual liver tissue was divided into two distinct portions: one was rapidly frozen in liquid nitrogen and preserved at -80 °C for gene expression assays; a second was stored at -20 °C for biochemical determinations. An additional four fish were collected from each tank, and their muscle and liver tissues were pooled within each tank and stored at -20 °C for lipid content and fatty acid composition (n = 3 per treatment).

2.4 Analytical procedures

Growth performance. Parameters such as survival rate (SR), initial and final body weight (FBW), weight gain (WG), specific growth rate (SGR), feeding rate (FR), feed conversion ratio (FCR), and morphometric parameters including ,carcass weight (CW), carcass ratio (CR), condition factor (CF), visceral adipose index (VAI), hepatopancreas somatic index (HSI), and viscerosomatic index (VSI) were calculated as described in Section 2.4.1 of Chapter III.

Proximate and fatty acid composition. The proximate composition of experimental diets and biological samples was analyzed according to AOAC (2000). Fatty acid composition was determined following the Chinese National Standard method (GB5009.168-2016, 2016), as described in Section 2.4.2 of Chapter III.

Biochemical indicators. Plasma biochemical parameters, including triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), and high-density lipoprotein cholesterol (HDL-c), as well as liver total protein (TP), TG, TC, and hepatic Δ6, Δ5, and Δ4 Fads contents, were determined using commercial assay kits following the procedures described in Section 2.4.4 of Chapter III.

Hepatic histology examination. Liver histology, including fixation, paraffin embedding, hematoxylin–eosin (H&E) staining, imaging, and quantitative analysis, was performed according to the procedures described in Section 2.4.5 of Chapter III.

Quantitative real-time PCR. The expression of genes related to lipid metabolism and fatty acid biosynthesis was quantified by qRT-PCR according to the procedures described in

Section 2.4.6 of Chapter III. Primer information is provided in Table 3-3, and ef-1α was used as the reference gene for normalization.

2.5 Statistical analyses

All data are presented as means ± standard error of the mean (SEM). Statistical analyses and figure generation were performed using GraphPad Prism 8 and Origin 2021. The effects of dietary oil sources and DHA inclusion, as well as their interaction, were analyzed using a two-way ANOVA. When no significant interaction was detected, the main effects were reported based on the pooled data, and Tukey's HSD test was applied to significant main effects. When a significant interaction was observed, simple effects were examined based on tank means. Specifically, comparisons among oil sources within different DHA levels (e.g., LO, CO, and LOD, COD) and DHA inclusion effects within each oil source (LO vs. LOD, CO vs. COD). Pairwise comparisons were adjusted using Sidak's test. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1 Growth performance and proximate composition

No significant effects of either dietary oil source or DHA inclusion were observed on the SR of largemouth bass ($P > 0.05$, Table 4-3). Feeding diets based on CO significantly decreased FR ($P < 0.05$), while DHA inclusion markedly enhanced FR ($P < 0.05$). The Oil sources × DHA inclusion interaction effect was significant for FBW, WG, and SGR ($P < 0.05$). Particularly, FBW, WG, and SGR were notably higher in LO-fed fish than in CO-fed fish ($P < 0.05$), regardless of DHA inclusion. Moreover, DHA inclusion further enhanced growth in the COD group compared with its respective basal diet ($P < 0.05$). The interaction effect was also significant for FCR ($P < 0.05$). Specifically, in the absence of DHA, FCR was significant higher in the CO group than in the LO group ($P < 0.05$), and was further elevated compared with the COD group ($P < 0.05$).

Oil source and DHA inclusion had no significant effects on CR, CF, VAI, or VSI and HSI ($P > 0.05$, Table 4-4). A significant interaction effect was observed for CW ($P < 0.05$). Without DHA, the CO group exhibited lower CW than the LO group ($P < 0.05$) and was further reduced compared with the COD group ($P < 0.05$). In contrast, CW did not differ among treatments under DHA-inclusion conditions ($P > 0.05$).

Dietary oil source had no significant effect on the content of whole-body dry matter, crude protein, crude lipid, or ash contents, nor on lipid content in muscle or liver tissues ($P > 0.05$, Table 4-5). Dietary DHA inclusion did not affect the content of whole-body crude protein or ash, or muscle lipid ($P > 0.05$), but significantly increased the whole-body lipid content, and reduced liver lipid content ($P < 0.05$).

Table 4-3. Growth performance of largemouth bass fed with different lipid sources (mean ± SEM, n=3).

Item	SR (%)	FBW (g)	WG (%)	SGR (%·d^{-1})	FR (%)	FCR
Individual treatment means						
LO	97.33±2.67	54.30±0.88^{A}	198.20±4.23^{A}	2.01±0.03^{A}	2.14±0.01	1.18±0.01^{B}
CO	100.00±0.00	38.99±1.90^{B}	116.24±9.12^{B}	1.34±0.07^{B}	2.00±0.02	1.71±0.06^{A*}

LOD	98.67±1.33	55.26±1.11^{X}	205.76±5.35^{X}	2.04±0.04^{X}	2.21±0.03	1.17±0.00
COD	98.67±1.33	50.69±0.93^{Y*}	179.27±4.47^{Y*}	1.87±0.04^{Y*}	2.12±0.03	1.25±0.02
Means of main effect						
LO	98.00				2.05^{a}	
CO	99.33				1.53^{b}	
D–	98.67				1.61^{x}	
D+	98.67				1.97^{y}	
Two-way ANOVA: *P*-value						
Oil sources	0.438				0.001	
DHA inclusion	1				0.006	
Interaction	0.438	0.001	< 0.001	< 0.001	0.233	< 0.001

1. FBW, WG and SGR are FR-adjusted marginal means (ANCOVA); all other variables are raw means (ANOVA).

2. Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (LO, CO) and DHA inclusion (D−, D+), respectively ($P < 0.05$). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant DHA inclusion effect within each oil source.

Table 4-4. Morphometric parameters of largemouth bass fed with different lipid sources (mean ± SEM, n=12).

Item	CW (g)	CR (%)	CF (g/cm^{3})	VAI (%)	HSI (%)	VSI (%)
Individual treatment means						
LO	43.31±3.06^{A}	62.64±0.64	1.75±0.02	3.82±0.16	2.08±0.11	9.78±0.23
CO	29.17±0.71^{B}	62.63±0.68	1.75±0.02	3.48±0.14	1.77±0.10	9.95±0.06
LOD	42.39±1.58	63.33±0.25	1.71±0.01	3.63±0.23	2.03±0.05	9.50±0.24
COD	39.81±2.26*	63.33±0.39	1.74±0.05	3.21±0.13	1.92±0.20	9.61±0.37
Means of main effect						
LO		62.99	1.73	3.73	2.06	9.64
CO		62.98	1.74	3.34	1.84	9.78
D–		62.64	1.75	3.65	1.92	9.86
D+		63.33	1.72	3.42	1.98	9.55
Two-way ANOVA: *P*-value						
Oil sources		0.998	0.592	0.070	0.087	0.598
DHA inclusion		0.339	0.333	0.269	0.677	0.252
Interaction	0.002	0.995	0.637	0.839	0.392	0.901

Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (LO, CO) and DHA inclusion (D−, D+), respectively ($P < 0.05$). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant DHA inclusion effect within each oil source.

Table 4-5. Proximate composition of largemouth bass fed with different lipid sources (%, wet basis) (mean ± SEM, n=3).

Item	Whole-body				Muscle	Liver
	Dry matter	Crude protein	Crude lipid	Ash	Crude lipid	Crude lipid
Individual treatment means						
LO	29.87±0.35	17.40±0.05	7.59±0.39	4.81±0.14	1.09±0.15	4.02±0.70
CO	28.17±0.17	17.11±0.25	8.56±0.81	4.83±0.22	0.90±0.12	4.56±0.36
LOD	27.71±0.85	17.32±0.22	12.36±0.69	5.24±0.11	0.85±0.08	2.03±0.39
COD	28.14±0.61	17.61±0.25	11.18±0.94	4.83±0.14	0.70±0.14	1.95±0.37
Means of main effect						
LO	28.79	17.36	9.98	5.02	0.97	3.02
CO	28.16	17.36	9.87	4.83	0.80	3.25
D–	29.02	17.26	8.08^{y}	4.82	1.00	4.29^{x}
D+	27.93	17.47	11.77^{x}	5.03	0.78	1.99^{y}
Two-way ANOVA: *P*-value						
Oil sources	0.293	0.983	0.887	0.262	0.230	0.646
DHA inclusion	0.088	0.343	0.001	0.216	0.122	0.001
Interaction	0.095	0.204	0.182	0.217	0.865	0.537

Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (LO, CO) and DHA inclusion (D−, D+), respectively ($P < 0.05$). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant DHA inclusion effect within each oil source.

3.2 Fatty acid composition of muscle and liver tissue

3.2.1 Muscle fatty acid composition

Oil sources significantly affect the contents of C16:0, C17:1n-9, C18:1n-9, and ΣMUFA in muscle (P < 0.05, Table 4-6). The contents of C16:1n-7, C18:1n-9, C20:1n-9, and ΣMUFA were significantly affected by DHA inclusion (P < 0.05).

For n-6 PUFAs, oil sources significantly influenced the contents of LA, C20:4n-6, and Σn-6 PUFA, with CO-based diets resulting in markedly higher contents of LA and Σn-6 PUFA than the other oil sources (P < 0.05). Moreover, DHA inclusion significantly reduced the contents of LA and Σn-6 PUFA in muscle tissue (P < 0.05). Specifically, a significant interaction was observed for C20:2n-6: its content was higher in CO-fed fish than in those fed other oil sources under both non-DHA and DHA-inclusion conditions, and was also higher in the CO group than in the COD group (P < 0.05).

For n-3 PUFAs, oil sources significantly influenced ALA content, Σn-3PUFA content and n-3/n-6 ratio, with LO-fed fish showing the highest values compared with CO-fed fish (P < 0.05). DHA inclusion had no effect on the ALA content (P > 0.05), but influenced the n-3/n-6 ratio. Moreover, the interaction for the contents of EPA, DHA, DHA + EPA was significant (P < 0.05). Based on simple effect analyses, under non-DHA condition, LO-fed fish showed the highest contents of EPA compared with CO-fed fish (P < 0.05). Notably, the contents of DHA and DHA + EPA were higher only in the LO group compared with the CO group (P < 0.05). The effects of DHA inclusion within each oil sources were variable across individual n-3PUFAs. Across all oil sources, DHA inclusion consistently reduced EPA but concurrently elevated the contents of DHA and DHA + EPA (P < 0.05).

3.2.2 Liver fatty acid composition

Oil source significantly influenced the contents of liver SFAs (P > 0.05, Table 4-7). CO inclusion significantly decreased the contents of C18:1n-9 compared to LO inclusion (P < 0.05). However, noticeable declines in the contents of C14:0, C17:0, C18:0, C16:1n-7, C17:1n-9, C18:1n-9, and ΣMUFA were observed when DHA was included in the diets (P < 0.05). The interaction effects were significant for the contents of C16:0, C20:0, C20:1n-9 and ΣSFA (P < 0.05). Under non-DHA condition, CO-fed fish showed higher contents of C16:0, C20:0, and ΣSFA than LO-fed fish (P < 0.05). The contents of C16:0, C20:0, C20:1n-9 and ΣSFA significantly declined with DHA inclusion (P < 0.05).

Fish fed LO diets showed significantly lower contents of LA, C20:2n-6, C20:3n-6, and Σn-6 PUFA than those fed the CO diets (P < 0.05). DHA inclusion markedly reduced the contents of LA, C20:2n-6, and Σn-6 PUFA (P < 0.05). Fish fed LO diets exhibited a significantly higher ALA and DHA content, and the n-3/n-6 PUFA ratio than those fed the CO diets (P < 0.05). In addition, DHA inclusion significantly increased the contents of DHA as well as the n-3/n-6 PUFA ratio, whereas ΣPUFA content decreased under DHA inclusion (P < 0.05). The interaction effects for the contents of EPA, DHA+EPA, ∑n-3PUFA were significant (P < 0.05). LO-fed groups exhibited significantly higher contents of DHA+EPA and ∑n-3PUFA compared with the CO-fed group under non-DHA inclusion, and higher EPA content regardless of DHA inclusion (P < 0.05). Notably, EPA declined in the LOD group compared with the LO group following DHA inclusion (P < 0.05).

Table 4-6. Muscle fatty acid composition of largemouth bass fed different lipid sources (mg/g, wet basis) (mean ± SEM, n=3).

Item	C11:0	C14:0	C16:0	C17:0	C18:0	C16:1n-7	C17:1n-9	C18:1n-9	C20:1n-9	C18:2n-6	C20:2n-6
Individual treatment means											
LO	2.02±0.06	0.09±0.01	2.22±0.19	0.05±0.00	1.07±0.06	0.13±0.02	0.06±0.00	2.85±0.36	0.09±0.01	2.61±0.36	0.08 ± 0.01^{B}
CO	1.98±0.05	0.15±0.01	2.79±0.19	0.05±0.00	1.06±0.04	0.09±0.01	0.05±0.01	1.68±0.17	0.07±0.01	4.92±0.44	$0.36\pm0.02^{A*}$
LOD	1.88±0.05	0.14±0.00	2.17±0.05	0.05±0.00	0.98±0.03	0.07±0.00	0.06±0.00	1.85±0.11	0.06±0.00	1.74±0.14	0.07 ± 0.00^{Y}
COD	1.98±0.08	0.15±0.03	2.64±0.23	0.05±0.00	1.09±0.05	0.06±0.01	0.05±0.01	1.25±0.19	0.05±0.01	3.71±0.59	0.26 ± 0.03^{X}
Means of main effect											
LO	1.95	0.12	2.19^{b}	0.05	1.03	0.10	0.06^{a}	2.35^{a}	0.07	2.17^{b}	
CO	1.98	0.15	2.71^{a}	0.05	1.08	0.07	0.04^{b}	1.46^{b}	0.06	4.32^{a}	
D–	2.00	0.12	2.50	0.05	1.06	0.11^{x}	0.05	2.26^{x}	0.08^{x}	3.76^{x}	
D+	1.93	0.15	2.40	0.05	1.04	0.06^{y}	0.04	1.55^{y}	0.06^{y}	2.73^{y}	
Two-way ANOVA: *P*-value											
Oil sources	0.640	0.092	0.021	0.622	0.313	0.135	0.003	0.005	0.310	0.001	
DHA inclusion	0.272	0.117	0.589	0.083	0.634	0.011	0.050	0.014	0.027	0.038	
Interaction	0.261	0.098	0.788	0.749	0.231	0.482	0.637	0.240	0.791	0.694	0.031

Item	C20:4n-6	C18:3n-3	C20:5n-3	C22:6n-3	C20:5n-3 + C22:6n-3	∑SFA	∑MUFA	∑n-3PUFA	∑n-6PUFA	n-3/n-6PUFA	∑PUFA
Individual treatment means											
LO	0.10±0.01	3.48±0.56	0.20±0.01^{A*}	0.88±0.05^{A}	1.09±0.06^{A}	5.62±0.30	3.29±0.41	4.78±0.65	2.78±0.38	1.72±0.03	7.56±1.03
CO	0.15±0.04	0.14±0.02	0.09±0.00^{B*}	0.65±0.02^{B}	0.74±0.03^{B}	6.03±0.27	1.89±0.20	0.87±0.04	5.56±0.51	0.16±0.01	6.43±0.55
LOD	0.09±0.00	2.21±0.20	0.11±0.00^{X}	1.37±0.03*	1.47±0.03*	5.27±0.12	2.08±0.13	3.88±0.23	1.90±0.15	2.05±0.04	5.78±0.38
COD	0.11±0.01	0.06±0.01	0.06±0.00^{Y}	1.45±0.07*	1.51±0.07*	5.91±0.34	1.40±0.21	1.57±0.07	4.16±0.62	0.39±0.04	5.73±0.69
Means of main effect											
LO	0.10	2.85^{a}				5.45	2.68^{a}	4.33^{a}	2.34^{b}	1.88^{a}	6.67
CO	0.13	0.10^{b}				5.97	1.64^{b}	1.22^{b}	4.86^{a}	0.27^{b}	6.08
D–	0.12	1.81				5.82	2.59^{x}	2.83	4.17^{x}	0.94^{y}	7.00
D+	0.10	1.13				5.59	1.74^{y}	2.73	3.03^{y}	1.22^{x}	5.75
Two-way ANOVA: *P*-value											
Oil sources	0.214	< 0.001				0.092	0.004	< 0.001	< 0.001	< 0.001	0.425
DHA inclusion	0.322	0.052				0.416	0.011	0.779	0.036	< 0.001	0.116
Interaction	0.430	0.078	< 0.001	0.009	0.005	0.688	0.205	0.050	0.582	0.170	0.464

1. Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (LO, CO) and DHA inclusion (D–, D+), respectively ($P < 0.05$). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant DHA inclusion effect within each oil source.

2. SFA: Saturated FAs; MUFA: Monounsaturated FAs; PUFA: Polyunsaturated FAs.

Table 4-7. Liver fatty acid composition of largemouth bass fed different lipid sources (mg/g, wet basis) (mean ± SEM, n=3).

Item	C11:0	C14:0	C16:0	C17:0	C18:0	C20:0	C16:1n-7	C17:1n-9	C18:1n-9	C20:1n-9	C18:2n-6	C20:2n-6
Individual treatment means												
LO	2.34±0.12	0.33±0.05	5.76±0.87^{B}	0.16±0.03	3.64±0.35	0.16±0.01^{B*}	0.38±0.05	0.12±0.05	9.75±1.54	0.56±0.10*	6.68±1.45	0.34±0.06
CO	2.28±0.08	0.50±0.01	11.40±0.34^{A*}	0.23±0.01	4.80±0.20	0.19±0.00^{A*}	0.33±0.02	0.22±0.00	7.09±0.29	0.44±0.02*	20.44±5.32	1.58±0.37
LOD	2.39±0.09	0.23±0.03	3.40±0.61	0.10±0.02	2.36±0.41	0.10±0.02	0.14±0.01	0.06±0.01	3.52±0.73	0.20±0.03	2.97±0.68	0.18±0.03
COD	2.42±0.21	0.24±0.05	4.03±0.80	0.10±0.01	2.39±0.13	0.09±0.00	0.09±0.02	0.06±0.01	1.97±0.47	0.11±0.01	6.32±1.68	0.61±0.05
Means of main effect												
LO	2.37	0.28		0.13	3.00		0.26	0.09	6.63^{a}		4.82^{b}	0.26^{b}
CO	2.35	0.37		0.16	3.59		0.21	0.14	4.53^{b}		13.38^{a}	1.09^{a}
D–	2.31	0.41^{x}		0.19^{x}	4.22^{x}		0.35^{x}	0.17^{x}	8.42^{x}		13.56^{x}	0.96^{x}
D+	2.40	0.24^{y}		0.10^{y}	2.38^{y}		0.12^{y}	0.06^{y}	2.74^{y}		4.64^{y}	0.40^{y}
Two-way ANOVA: *P*-value												
Oil sources	0.891	0.051		0.088	0.082		0.141	0.058	0.047		0.019	0.002
DHA inclusion	0.495	0.001		0.001	< 0.001		< 0.001	0.003	< 0.001		0.015	0.018
Interaction	0.770	0.070	0.007	0.070	0.091	0.046	0.921	0.085	0.554	< 0.001	0.110	0.064

Item	C20:3n-6	C20:4n-6	C18:3n-3	C20:5n-3	C22:6n-3	C20:5n-3+ C22:6n-3	∑SFA	∑MUFA	∑n-3PUFA	∑n-6PUFA	n-3/n-6PUFA	∑PUFA
Individual treatment means												
LO	0.14±0.01	0.09±0.01	7.77±1.88	0.78±0.06^{A*}	2.46±0.17	3.24±0.22^{A}	12.88±1.51^{B*}	10.81±1.72	12.00±2.15^{A}	7.25±1.51	1.68±0.07	19.24±3.66
CO	0.57±0.22	0.17±0.09	0.16±0.08	0.13±0.02^{B}	0.81±0.31	0.94±0.33^{B}	19.40±0.18^{A*}	8.08±0.32	1.10±0.41^{B}	22.76±5.99	0.05±0.01	23.87±6.36
LOD	0.08±0.01	0.25±0.03	3.24±0.72	0.36±0.05^{X}	3.31±0.36	3.67±0.40	8.69±1.06	3.92±0.77	7.46±1.21	3.49±0.75	2.19±0.12	10.95±1.95
COD	0.19±0.01	0.24±0.05	0.07±0.02	0.04±0.01^{Y}	2.80±0.22	2.84±0.23*	9.25±1.10	2.24±0.51	2.91±0.25	7.36±1.76	0.42±0.06	10.27±2.00
Means of main effect												
LO	0.11^{b}	0.17	5.51^{a}		2.89^{a}			7.36		5.37^{b}	1.94^{a}	15.10
CO	0.38^{a}	0.21	0.12^{b}		1.81^{b}			5.16		15.06^{a}	0.23^{b}	17.07
D–	0.36	0.13	3.97		1.64^{y}			9.44^{x}		15.01^{x}	0.87^{y}	21.56^{x}
D+	0.14	0.25	1.66		3.06^{x}			3.08^{y}		5.42^{y}	1.31^{x}	10.61^{y}
Two-way ANOVA: *P*-value												
Oil sources	0.037	0.513	< 0.001		0.004			0.057		0.017	< 0.001	0.629
DHA inclusion	0.075	0.071	0.051		< 0.001			< 0.001		0.018	< 0.001	0.024
Interaction	0.176	0.444	0.058	0.003	0.073	0.044	0.024	0.609	0.036	0.110	0.415	0.519

1. Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (LO, CO) and DHA inclusion (D–, D+), respectively ($P < 0.05$). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant DHA inclusion effect within each oil source.

2. SFA: Saturated FAs; MUFA: Monounsaturated FAs; PUFA: Polyunsaturated FAs.

3.3 Plasma and liver lipid metabolism

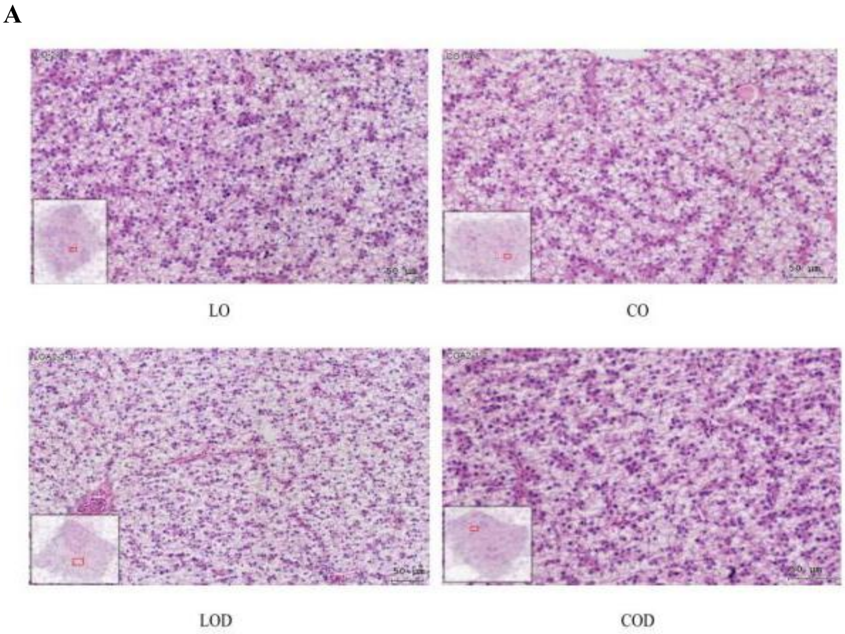
Oil sources did not influence the plasma TG, TC, HDL-c; in contrast, LO inclusion led to higher liver TG and TC levels than CO ($P < 0.05$, Table 4-8). DHA inclusion significantly increased plasma HDL-c and reduced liver TG levels ($P < 0.05$). A significant interaction between dietary oil source and DHA inclusion was observed for plasma LDL-c levels and the HDL-c/LDL-c ratio ($P < 0.05$). Without DHA, the CO group exhibited the lowest LDL-c levels compared with LO. Under DHA-inclusion conditions, LOD group showed the highest HDL-c/LDL-c ratio compared with COD ($P < 0.05$). Comparing diets with and without DHA within the same oil source, DHA inclusion reduced the LDL-c levels in the LOD group and increased the HDL-c/LDL-c ratio in LOD group ($P < 0.05$). Histopathological examination of the liver revealed no obvious hepatocellular swelling, nuclear displacement, or severe vacuolization among different groups (Figure 4-1A).

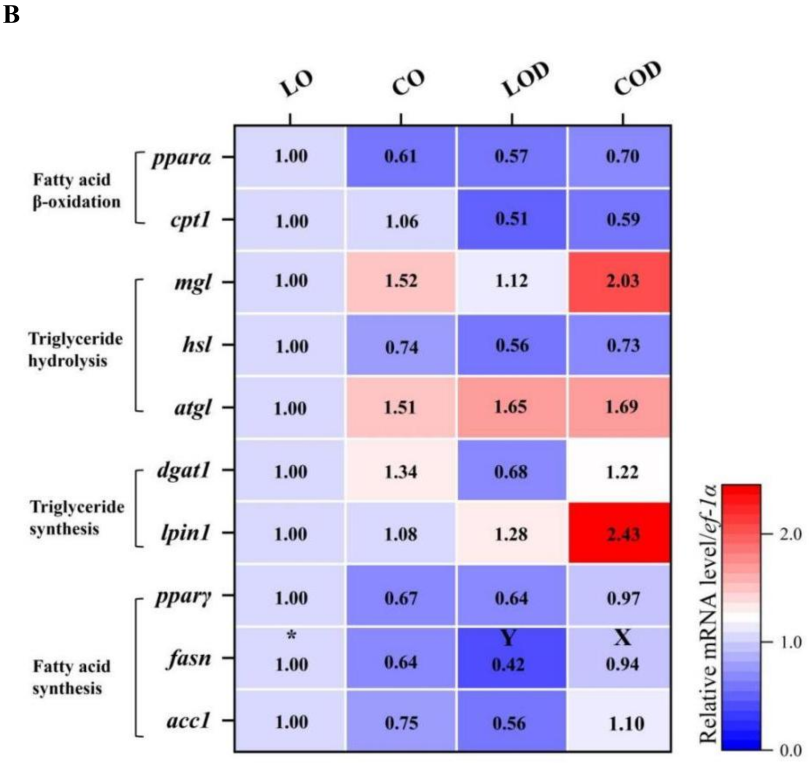
Fish fed CO diets exhibited significantly higher *mgl* expression than LO ($P < 0.05$; Figure 4-1B). DHA inclusion significant upregulated the expression of lipid *lipin1* ($P > 0.05$). However, significant interaction effects were observed for *acc1*, *fasn*, and *pparγ* ($P < 0.05$). Without DHA, the expression of *acc1* and *fasn* did not differ significantly among the LO and CO groups ($P > 0.05$). Under DHA-inclusion conditions, while *fasn* expression was elevated in COD relative to LOD ($P < 0.05$). DHA inclusion reduced the *fasn* expression in LOD group compared with LO group ($P < 0.05$).

Table 4-8. Biochemical indicators in the plasma and liver of largemouth bass fed with different lipid sources (mean ± SEM, n=3).

Item	Plasma					Liver	
	TG (mmol/L)	TC (mmol/L)	HDL-c (mmol/L)	LDL-c (mmol/L)	HDL-c/LDL-c	TG (mmol/g prot)	TC (mmol/g prot)
Individual treatment means							
LO	4.67±0.93	5.16±0.63	8.14±0.65	0.74±0.08^{A*}	11.89±1.34	0.23±0.03	0.08±0.02
CO	3.32±0.47	4.74±0.39	8.29±1.77	0.59±0.04^{B}	15.18±1.56	0.18±0.01	0.03±0.01
LOD	3.62±0.16	5.33±0.12	11.37±0.39	0.54±0.04^{Y}	21.65±0.88^{X*}	0.17±0.02	0.05±0.01
COD	3.87±0.27	5.43±0.18	11.87±0.32	0.73±0.04^{X}	16.66±0.82^{Y}	0.13±0.02	0.04±0.01
Means of main effect							
LO	4.15	5.25	9.75			0.20^{a}	0.07^{a}
CO	3.60	5.08	10.08			0.15^{b}	0.04^{b}
D–	4.00	4.95	8.22^{y}			0.21^{x}	0.06
D+	3.75	5.38	11.62^{x}			0.15^{y}	0.05
Two-way ANOVA: *P*-value							
Oil sources	0.352	0.586	0.637			0.027	0.008
DHA inclusion	0.672	0.152	< 0.001			0.009	0.484
Interaction	0.176	0.385	0.800	< 0.001	0.007	0.883	0.081

1. Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (LO, CO) and DHA inclusion (D−, D+), respectively ($P < 0.05$). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant DHA inclusion effect within each oil source.
2. The unit (mmol/g prot) reflects the lipid content per unit of protein rather than per tissue weight, allowing for normalization across samples with variable liver sizes.





		LO	CO	LOD	COD
Fatty acid β-oxidation	*pparα*	1.00	0.61	0.57	0.70
	cpt1	1.00	1.06	0.51	0.59
Triglyceride hydrolysis	*mgl*	1.00	1.52	1.12	2.03
	hsl	1.00	0.74	0.56	0.73
	atgl	1.00	1.51	1.65	1.69
Triglyceride synthesis	*dgat1*	1.00	1.34	0.68	1.22
	lpin1	1.00	1.08	1.28	2.43
Fatty acid synthesis	*pparγ*	1.00	0.67	0.64	0.97
	fasn	* 1.00	0.64	Y 0.42	X 0.94
	acc1	1.00	0.75	0.56	1.10

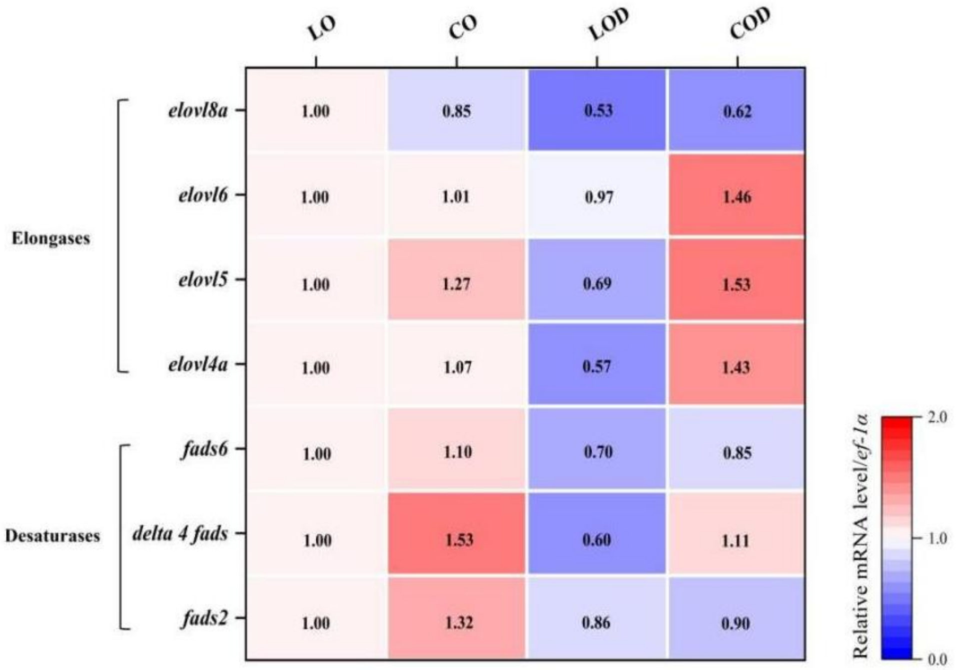
Item	*P*-value			Means of main effect			
	Oil sources	DHA inclusion	Interaction	LO	CO	D–	D+
acc1			0.027				
fasn			0.003				
pparγ			0.023				
lpin1	0.124	0.046	0.181	1.14	1.76	1.04^{y}	1.86^{x}
dgat1	0.113	0.417	0.709	0.84	1.28	1.16	0.95
atgl	0.579	0.395	0.632	1.33	1.60	1.25	1.67
hsl	0.663	0.039	0.058	0.77	0.74	0.86^{x}	0.64^{y}
mgl	0.015	0.265	0.486	1.06^{b}	1.77^{a}	1.26	1.57
cpt1	0.824	0.135	0.984	0.74	0.81	1.03	0.55
pparα	0.335	0.207	0.055	0.79	0.66	0.81	0.64

Figure 4-1. Effect of different lipid sources on liver lipid metabolism of largemouth bass. (A) Representative H&E-stained liver sections (n = 12), no marked hepatocellular swelling, nuclear displacement, or severe vacuolization observed; (B) Relative mRNA levels of liver lipogenesis/proliferation (*acc1, fasn, pparγ*, *lpin1, dgat1*) and lipolysis/β-oxidation (*atgl, hsl, mgl, cpt1, pparα*) related genes (mean ± SEM, n = 8). Lowercase letters (a, b; x, y) denote significant main effects of oil source and DHA inclusion, respectively; uppercase letters (A, B; X, Y) denote differences under significant interaction, and * indicates DHA effect within each oil source ($P < 0.05$).

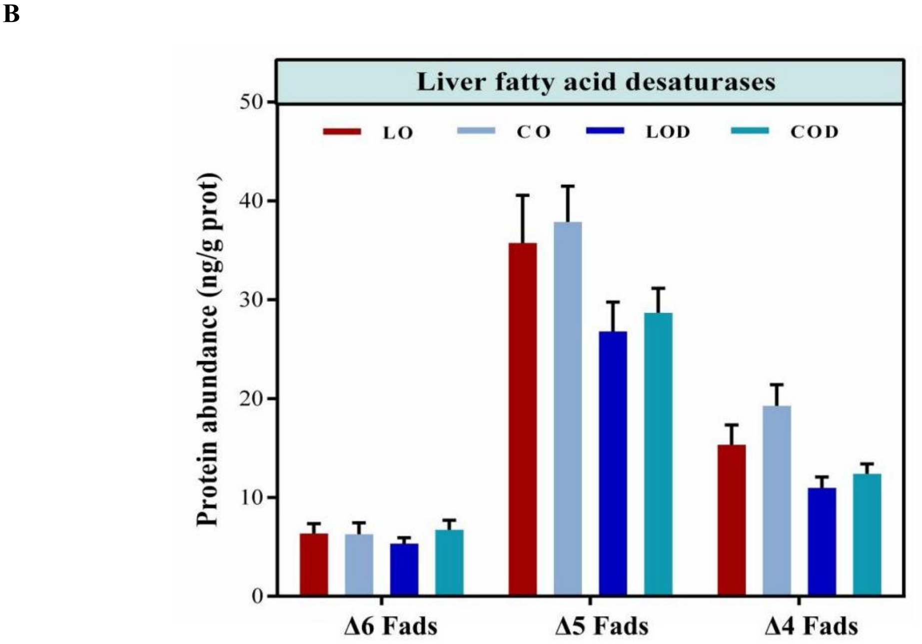
3.4 Liver LC-PUFA biosynthesis

Relative to CO, LO markedly suppressed liver *delta4 fads* and *elovl5* expression ($P < 0.05$, Figure 4-2A). DHA inclusion further downregulated the expression of *elovl8a* ($P < 0.05$). The liver protein abundance of Δ6, Δ5 and Δ4 Fads were not affected by dietary oil sources ($P > 0.05$, Figure 4-2B). DHA inclusion did not significantly influence protein abundance of Δ6 Fads ($P > 0.05$), but markedly reduced that of Δ5 and Δ4 Fads ($P < 0.05$).

A



Item	*P*-value			Means of main effect			
	Oil sources	DHA inclusion	Interaction	LO	CO	D–	D+
fads2	0.550	0.128	0.366	0.93	1.08	1.16	0.83
delta4 fads	0.016	0.056	0.973	0.80^{b}	1.34^{a}	1.26	0.84
fads6	0.491	0.142	0.884	0.85	0.98	1.05	0.77
eovl4a	0.055	0.870	0.104	0.78	1.25	1.04	1.00
elovl5	0.019	0.912	0.210	0.85^{b}	1.40^{a}	1.13	1.11
elovl6	0.081	0.147	0.096	0.98	1.22	1.01	1.20
elovl8a	0.846	0.038	0.477	0.77	0.73	0.93^{x}	0.57^{y}



Item	*P*-value			Means of main effect			
	Oil sources	DHA inclusion	Interaction	LO	CO	D–	D+
Δ6 Fads	0.484	0.768	0.437	5.83	6.51	6.31	6.03
Δ5 Fads	0.577	0.015	0.978	31.26	33.28	36.81^{x}	27.73^{y}
Δ4 Fads	0.109	0.001	0.445	13.16	15.83	17.31^{x}	11.68^{y}

Figure 4-2. Impact of different lipid sources on liver LC-PUFA biosynthesis of largemouth bass. (A) Relative mRNA expression of fatty acyl desaturase *(fads)* and elongase (*elovl*) genes (mean ± SEM, n=8); (B) Protein abundances of liver fatty acyl desaturase (Fads) content (Δ6, Δ5, and Δ4 Fads) (mean ± SEM, n = 12). Letters and asterisks are as described in Figure 4-1.

4. Discussion

4.1 Dietary control of LC-PUFA biosynthesis in largemouth bass

LO supplied the highest dietary ALA level but did not enhance liver LC-PUFA biosynthesis gene expression. By contrast, CO-fed fish received substantially lower dietary ALA inputs, while maintaining relatively higher *delta4 fads* expression compared with LO-fed fish. This combination of low precursor supply but maintained gene expression is consistent with the "substrate–limited but not gene–suppressed" pattern previously described in Atlantic salmon and rainbow trout (Gregory et al., 2016; Panserat et al., 2009). This compensatory mechanism, however, failed to offset the limited substrate availability, resulting in suboptimal DHA biosynthesis (Liu et al., 2025).

From a pathway perspective, although desaturases catalyse rate-limiting steps early in the n-3 LC-PUFA biosynthetic pathway, elongases operate downstream and display broad, overlapping substrate specificities (e.g., C18–C24 PUFAs) (Xie et al., 2021). In this study, *elovl5* expression was highest in the CO groups. This gene-expression responses mirrored the dietary ALA/DHA ratios—LOD (7.01) and COD (0.07), suggesting that a high precursor-to-product ratio may be associated with substrate-related regulatory effects. Similar effects have been reported in teleosts, where ALA-rich diets to reduced desaturase and elongase expression, likely reflecting end-product feedback (Li et al., 2025; Tocher et al., 2002b).

In this study, dietary DHA inclusion consistently downregulated the liver expression of desaturase genes *elovl8a*, accompanied by reduced protein abundance of Δ5 and Δ4 Fads. When dietary DHA is provided in adequate amounts, fish generally exhibit a downregulation of critical genes associated with the endogenous synthesis of LC-PUFAs, a trend consistent with the well-established negative feedback response to exogenous LC-PUFAs in teleosts (Glencross et al., 2015; Gregory et al., 2016; Panserat et al., 2009; Tocher et al., 2002b). Similar DHA-mediated repression of desaturase activity has been observed in salmonids and marine species, whereby DHA suppresses endogenous synthesis through post-transcriptional and enzymatic control (Glencross et al., 2015; Gonzalez-Soto & Mutch, 2021; Gregory et al., 2016; Panserat et al., 2009; Tocher et al., 2002b). These results establish a coherent regulatory model in which largemouth bass attempts transcriptional compensation under DHA scarcity, while DHA suppresses pathway activity to maintain lipid homeostasis. Notably, the protein abundance of Δ6 Fads remained unaffected by either oil sources or DHA inclusion. Previous studies have suggested that Δ6 Fads functions at an early step of the n-3 PUFA biosynthetic pathway, and may exhibit relatively conserved regulation across dietary conditions (Nakamura & Nara, 2004; Tocher, 2010).

4.2 Evidence for LC-PUFA biosynthesis from ALA based on tissue fatty acid composition

In this study, LO and CO, each contributed distinct fatty acid profiles, with LO enriching the contents of ALA and total n-3 PUFA, CO elevating the contents of LA and n-6 PUFA, which were also reflected in both muscle and liver tissues (Chatelier et al., 2006; Yu et al., 2019b). As expected, fish fed LO, which contained high ALA content (54.24%), exhibited higher tissue contents of n-3 PUFAs, including EPA and DHA, compared with those fed CO. This observation is consistent with a greater availability of n-3 precursors and may reflect a combination of dietary deposition and metabolic transformation (Li et al., 2025; Yadav, 2020; Zhang et al., 2019a). Although the present study reinforces this metabolic capability, it does not quantify absolute conversion flux (Yuan et al., 2024b), which will require further work integrating isotopic tracing or metabolic rate analyses.

High dietary DHA availability is known to promote its preferential retention in tissues, often reducing the relative abundance of other fatty acids, even when their absolute intake remains unchanged (Glencross, 2009; Liao et al., 2022; Tocher, 2003; Turchini et al., 2009). In this study, tissue DHA content significantly increased in the LOD group following DHA inclusion, whereas the observed decline EPA content may be attributed to their initially higher baseline concentrations in this group. Notably, although the SCM inclusion had a high DHA-to-EPA ratio (15:1), tissue EPA content did not increase significantly. Similar findings have been reported in teleosts, where DHA tends to accumulate more readily than EPA, whereas EPA often shows greater metabolic turnover or utilization across physiological pathways (Glencross, 2009; Liao et al., 2022; Sargent et al., 2003; Tocher, 2010; Turchini et al., 2009; Wu et al., 2021).

4.3 Contrasting effects of CO and DHA inclusion on lipid metabolism

Circulating levels of TG, TC, HDL-c, and LDL-c reflect lipid metabolism in the bloodstream in fish (Gong et al., 2024; Yun et al., 2012; Zhang et al., 2022). Among them, HDL-c and LDL-c function oppositely and are widely applied as lipid-transport markers to evaluate lipid metabolism (Caballero et al., 2006; Guo et al., 2019; Xu et al., 2024). Previous studies indicate that the HDL-c/LDL-c ratio reflects the direction of cholesterol transport toward the liver in fish (Huang et al., 2022; Ma et al., 2020). Molecularly, ACC1, FASN, and MGL are key regulators involved in lipid synthesis, storage, and mobilization.

Under non-DHA conditions, the CO group exhibited lower plasma LDL-c levels and a higher HDL-c/LDL-c ratio. CO-fed fish also exhibited lower liver TG levels, accompanied by significant upregulation of liver *fasn*, and *mgl* expression compared with LO, especially under DHA inclusion conditions. These changes indicate an altered liver lipid metabolic state associated with CO feeding. Despite the activation of genes involved in lipid mobilization, CO-fed fish still exhibited poor growth. This result may be related to the low ALA content or imbalanced fatty acid composition of CO diets, which could limit lipid availability or metabolic efficiency and be reflect in reduced liver lipid deposition (Malinska et al., 2015).

In contrast, DHA inclusion improved plasma HDL-c and reduced liver TG levels. Notably, fish in the LOD group exhibited a significantly higher HDL-c/LDL-c ratio compared with LO group. Consistent with previous findings that the HDL-c/LDL-c ratio reflects cholesterol transport direction in fish. These changes suggest that DHA inclusion may alter lipoprotein distribution and promote cholesterol transport toward liver metabolism. Generally, insufficient dietary DHA leads to lipid accumulation, particularly elevated liver TG levels, whereas DHA inclusion helps modulate lipid storage and circulation, consistent with previous observations in teleosts (Glencross et al., 2014; Jin et al., 2017a; Lei et al., 2016). Consistently, compositional analysis revealed lower liver crude lipid content in fish receiving DHA, reflecting reduced overall lipid deposition at the tissue level.

4.4 Dietary ALA and DHA benefit growth performance

ALA not only exerts intrinsic effects on growth (Glencross, 2009; Tocher, 2003; Yu et al., 2023) but also serves as a metabolic precursor for n-3 LC-PUFA, thereby influencing growth performance (Song et al., 2024a; Tocher, 2010; Turchini et al., 2022; Xie et al., 2021). Consistent with previous studies and the results of the present work, largemouth bass can convert dietary ALA into n-3 LC-PUFAs to meet growth and physiological demands, supporting growth and DHA biosynthesis under the experimental conditions (Sargent et al., 2003).

In this study, those fed the CO diet showed significantly reduced growth, regardless of DHA inclusion. Dietary inclusion of LO has been investigated for its potential to promote body growth (Turchini & Francis, 2009), as well as stimulate endogenous n-3 LC-PUFA biosynthesis in aquatic species. However, these effects vary with species and replacing the proportion of FO (Li et al., 2016; Menoyo et al., 2005; Shi et al., 2019).

Whereas CO-fed fish exhibited the poorest growth, consistent with its minimal ALA content (0.14%), this limitation could not be compensated despite the marked upregulation of genes and increased protein abundance of key enzymes involved in n-3 LC-PUFA biosynthesis (Liu et al., 2025). Previous studies show that CO can maintain growth in some species but consistently alters tissue fatty acid composition (Hou et al., 2024), whereas excessive CO in the dietary of rainbow trout, gilthead seabream, and largemouth bass (Eroldoğan et al., 2012; Güler & Yildiz, 2011; Liu et al., 2025; Wassef et al., 2015) impair growth and influence lipid metabolic markers and liver function. In addition to insufficient n-3 PUFA, the high dietary LA in CO may also contribute to reduced growth, as suggested by similar observations in large yellow croaker under FO-SO substitution (Duan et al., 2014). Collectively, these findings indicate that the poor growth of CO-fed fish may result from the combined effects of extremely low ALA and excessive LA, leading to an imbalanced n-6/n-3 PUFA ratio that constrains growth.

Although the COD group still exhibited markedly lower performance compared with the LOD, DHA inclusion resulted in a significant relative improvement in growth, as observed in COD-fed fish. This suggests that incorporating exogenous EPA and DHA into the diet can help mitigate the nutritional deficiencies of oils low in ALA (Dernekbaşı et al., 2021; Song et al., 2024a).

Moreover, in this study, supplementation with SCM, a DHA-rich microbial source, was used to increase dietary n-3 LC-PUFAs levels and was associated with changes in growth performance, feed intake, and whole-body lipids in largemouth bass. This agrees with studies attributing increased palatability and energy retention to DHA-rich microbial ingredients (Santigosa et al., 2020; Shields & Lupatsch, 2012). A comparable trend was seen in juvenile black seabream, where moderate elevation of the DHA/EPA ratio (0.65 → 1.16) raised whole-body lipids, whereas a further increase reversed the effect (Jin et al., 2017b). In contrast, a study on largemouth bass reported that dietary inclusion of n-3 LC-PUFAs had no notable impact on feed intake or whole-body composition (Yadav, 2020). Such divergence in findings likely reflects differences in fish developmental stage, basal diet composition, or the form and level of n-3 PUFA provided, and the underlying mechanisms warrant further investigation (Norambuena et al., 2015).

5. Conclusion

In largemouth bass, adequate dietary ALA supported normal growth and functioned as a key essential fatty acid precursor for endogenous DHA biosynthesis, highlighting its foundational role in sustaining LC-PUFA metabolic requirements. In contrast, severe ALA deficiency impaired growth despite upregulated desaturase gene expression, accompanied by reduced tissue DHA levels, indicating that transcriptional activation alone cannot compensate for substrate limitation. Conversely, dietary DHA effectively restored growth performance under low ALA conditions and simultaneously downregulated desaturase gene expression and key enzyme abundance, reflecting feedback regulation of endogenous LC-PUFA biosynthesis. Collectively, these results demonstrate a complementary nutritional relationship between ALA and DHA, in which ALA primarily supports endogenous biosynthetic capacity, whereas dietary DHA ensures physiological performance by compensating for precursor deficiency and regulating metabolic activity, providing a mechanistic basis for optimizing n-3 LC-PUFA balance in aquafeed formulation.

5

Chapter V Effects of dietary n-3 PUFA-enriched black soldier fly oil on growth and lipid metabolism in largemouth bass

Abstract

The present chapter aimed to enrich long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFAs) in black soldier fly *Hermetia illucens* (BSF) larvae through dietary fish processing by-products (FPBs) supplementation, and to evaluate the potential application of n-3 LC-PUFA-enriched BSF oil (BSFO) in largemouth bass *Micropterus salmoides* through a docosahexaenoic acid (DHA, C22:6n-3)-based dietary simulation. In the first enrichment trial, feeding BSF larvae substrates with varying levels of FPBs did not significantly affect growth performance, as indicated by individual body weight ($P > 0.05$). However, crude lipid content was significantly higher in the 40% FPBs group compared to the 0% group ($P < 0.05$), while crude protein content remained unchanged among treatments ($P > 0.05$). Dietary FPBs significantly increased larval α-linolenic acid (ALA, C18:3n-3) and enabled the accumulation of eicosapentaenoic acid (EPA, C20:5n-3) and DHA, which were absent in the control group ($P < 0.05$), with no significant differences between the 24% and 40% inclusion levels ($P > 0.05$). In the second feeding trial, 150 largemouth bass (17.99 ± 0.01g, mean ± SEM) were assigned to two dietary treatments with three replicates per treatment: a BSFO-based control diet and the same diet supplemented with 3% *Schizochytrium sp.* meal (SCM; 43.27% DHA). Fish fed the DHA inclusion diet exhibited better growth performance than those fed the control BSFO diet ($P < 0.05$). DHA inclusion markedly increased DHA, total n-3 PUFA, and the n-3/n-6 PUFA ratio in muscle and liver, while reducing n-6 PUFA accumulation ($P < 0.05$). In addition, plasma lipid profiles were improved, as evidenced by higher high-density lipoprotein cholesterol (HDL-c) and lower low-density lipoprotein cholesterol (LDL-c) levels ($P < 0.05$). Furthermore, the protein abundances of Δ6, Δ5, and Δ4 Fads were significantly decreased by dietary DHA ($P < 0.05$), suggesting a feedback inhibition of hepatic n-3 LC-PUFA biosynthesis. These results demonstrate that 24% FPBs effectively enriched n-3 PUFAs in BSFO without impairing larval growth. A DHA-based simulation further indicated that EPA- and DHA-enriched BSFO can improve growth, optimize tissue fatty acid profiles, and regulate lipid metabolism in largemouth bass, highlighting its potential as a sustainable alternative lipid source in aquafeeds. Overall, n-3 PUFA-enriched BSFO shows strong potential as a sustainable alternative lipid source in aquafeeds.

Keywords: black soldier fly, fish processing by-products, n-3 LC-PUFA, largemouth bass, DHA inclusion

1. Introduction

Adequate dietary α-linolenic acid (C18:3n-3, ALA) can support normal growth, whereas dietary DHA can compensate for ALA deficiency, highlighting their complementary nutritional roles. However, this also indicates that the endogenous conversion of ALA to long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFAs) is insufficient to fully meet physiological demands, particularly under diets low in preformed docosahexaenoic acid (DHA, C22:6n-3) and eicosapentaenoic acid (EPA, C20:5n-3). These limitations underscore the need for sustainable alternative lipid sources capable of ensuring an adequate supply of n-3 LC-PUFAs in carnivorous fish (Monroig et al., 2018; Sargent et al., 2003; Tocher, 2015).

Insect-derived oils, particularly black soldier fly oil (BSFO), represent a promising alternative owing to their high productivity and compatibility with circular bioeconomy systems. From a nutritional standpoint, BSFO has recently emerged as an ecologically sustainable option for carnivorous fish (Fawole et al., 2021; Moutinho et al., 2025; Yuan et al., 2024a). Compared with conventional vegetable oils (VO) rich in C18 PUFA, BSFO is characterized by a high proportion of lauric acid (C12:0). Lauric acid is rapidly oxidized via mitochondrial β-oxidation, thereby providing readily available metabolic energy and limiting excessive lipid deposition (Dabbou et al., 2021). Nevertheless, the intrinsic deficiency of n-3 LC-PUFA in conventional BSFO remains a major nutritional constraint for its application in aquafeeds (Spranghers et al., 2017; St-Hilaire et al., 2007). Such a fatty acid profile is poorly aligned with the lipid requirements of carnivorous fish and may dilute the cardioprotective n-3/n-6 ratio that underpins both fish health and the nutritional value of farmed seafood for human consumers (Naylor et al., 2009; Sargent et al., 1999).

A promising strategy to overcome this limitation is to re-engineer the insect lipid profile through substrate-mediated nutritional modulation. Insects possess a remarkable capacity for dietary fatty acid assimilation, and previous work has repeatedly demonstrated that altering the feedstock can re-profile the whole-body fatty acid signature of BSF larvae (Liland et al., 2017; St-Hilaire et al., 2007). Meanwhile, fish processing by-products (FPBs)—including offal, trimmings, and frames—are abundantly generated by the seafood industry and are naturally rich in n-3 PUFAs (Ghaly et al., 2013; St-Hilaire et al., 2007). Recycling these resources as substrates for BSF rearing could simultaneously side streams and enhance the nutritional quality of the resulting larval oil through enrichment with physiologically relevant n-3 PUFAs (St-Hilaire et al., 2007). Nevertheless, systematic evidence regarding the efficiency of such enrichment strategies, particularly under different inclusion levels, and the subsequent nutritional value of enriched BSFO for carnivorous fish remains limited (Barragan-Fonseca et al., 2017).

Therefore, the present study aimed to evaluate the feasibility of enriching n-3 PUFAs in black soldier fly larvae using dietary FPBs, and to assess the application potential of n-3 LC-PUFA-enriched BSFO in largemouth bass. Specifically, a dose–response enrichment trial was conducted to determine the effectiveness of FPBs inclusion in modulating larval fatty acid composition, followed by a DHA-based dietary simulation to evaluate the impacts of n-3 PUFA-enriched BSFO on growth performance, tissue fatty acid composition, lipid metabolism, and hepatic n-3 LC-PUFA biosynthesis of largemouth bass.

2.Materials and methods

2.1 Enrichment of n-3 PUFA in black soldier fly larvae by dietary fish processing by-products

2.1.1 Diet preparation

The salmon-derived FPBs obtained from a seafood processing facility in Gembloux, Belgium were used as the experimental ingredient, and dried chicken feed (Chicken Pellet, AVEVE, Belgium) was used as the basal diet. Six dietary treatments were initially formulated by replacing rice with graded levels of FPBs at 0, 8, 16, 24, 32 and 40% (dry matter basis). Based on preliminary growth performance, four representative levels (0, 8, 24 and 40%) were selected for subsequent analyses of nutritional composition and fatty acid profiles.

All dry ingredients were ground to pass through a 0.75 mm sieve and subsequently dried at 60 °C for 24 h. Diets were prepared to a final moisture content of 60%, mixed thoroughly, and divided equally among replicates.The ingredient composition and nutrient and fatty acid composition of experimental diets are presented in Table 5-1 and Table 5-2.

2.1.2 Larvae management

The BSF larvae used to conduct this experiment came from an experimental rearing in the Functional and Evolutionary Entomology laboratory from Gembloux Agro-Bio Tech (ULiege, Belgium). BSF larvae were reared in a controlled laboratory room (6 m²) at 27 ± 1 °C, 70 ± 5% relative humidity, under a 12 h light: 12 h dark photoperiod. During the rearing period, temperature and humidity were monitored daily.

Uniform 4-day-old larvae were selected and starved for 24 h prior to the initiation of the experiment. At the end of starvation, 20 larvae from each replicate were randomly sampled and weighed to determine the initial mean individual body weight. Larvae were stocked at a density of one larva per gram of feed. Each replicate consisted of 150 larvae, with four replicates assigned to each dietary treatment.

Larvae were reared for approximately 10 days, and the total ration for each replicate was provided at the beginning of the experiment without additional feeding thereafter. At two-day intervals, 20 larvae per replicate were sampled and weighed to monitor changes in mean body weight over time.

Table 5-1. Formulation of experimental diets with different fish processing by-products inclusion levels.

Ingredients	0%	8%	24%	40%
Dried Chicken food (%)	32	32	32	32
Rice (%)	68	60	44	28
fish processing by-products (%)	0	8	24	40
Proximate composition				
Dry matter (%)	56.72	57.02	59.41	62.98
Crude protein (%, DM)	13.59	14.49	17.90	20.91
Crude lipid (%, DM)	2.27	6.09	15.43	22.07
Crude ash (%, DM)	4.98	5.37	6.56	5.27

Table 5-2. Fatty acid composition of experimental diets with different fish processing by-products inclusion levels (%TFA).

Items	0%	8%	24%	40%
C4:0	87.86	84.41	73.13	73.47
C12:0	0.09	0.11	0.09	0.06
C14:0	0.06	0.29	0.62	0.64
C16:0	1.86	1.98	3.18	3.10
C18:0	0.36	0.42	0.74	0.76
C24:0	-		0.31	0.30
C16:1n-7	-	0.20	0.70	0.73
C18:1n-9	3.42	5.78	10.53	10.79
C22:1n-9	-		0.39	0.45
C18:2n-6	6.12	5.06	6.30	5.45
C18:3n-3	0.25	0.73	1.16	1.21
C20:5n-3	-	0.21	0.78	0.80
C22:6n-3+C24:1n-9	-	0.48	0.91	1.00
ΣSFA	90.22	87.21	78.07	78.31
ΣMUFA	3.42	5.98	11.62	11.97
Σn-3PUFA	0.25	1.42	2.85	3.00
ΣPUFA	6.36	6.48	9.15	8.45

2.1.3 Final sampling and chemical analyses

The rearing period ended when prepupae first appeared. The prepupae/larvae were harvested within 24 h, dried with a paper towel, weighted to determine the final average individual weight and final total larval mass. All samples were stored at −20 °C, freeze-dried and ground for proximate composition and fatty acid composition analysis following previously described methods (Hoc et al., 2021a).

2.1.4 Statistical analyses

All data are presented as means ± standard error of the mean (SEM). Statistical analyses and figure generation were performed using GraphPad Prism 8. Data were tested for normality and homogeneity of variances. For data meeting the assumptions of normality and equal variances, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests was used to evaluate significant differences among groups.

When data did not meet these assumptions, non-parametric Kruskal-Wallis tests followed by Dunn's multiple comparisons were applied. *P* values of less than 0.05 were considered statistically significant.

2.2 Application of n-3 PUFA-enriched black soldier fly oil in largemouth bass

2.2.1 Diet preparation, rearing conditions, sampling and analytical procedures

Diet preparation. Two isonitrogenous and isoenergetic experimental diets were formulated using BSFO as the sole lipid source. The BSFO (Guangzhou Konfu Biotechnology Co., Ltd., China) was extracted from larvae reared for 8−9 days on residues derived from kitchen waste oil−water separation, followed by drying (75−85 °C) and mechanical pressing. The two diets differed only in DHA supplementation and were designated as BSFO (control) and BSFOD (DHA-supplemented). DHA supplementation was achieved by incorporating 3% *Schizochytrium sp.* meal (SCM). The remaining procedures followed those in in Section 2.1 of Chapter IV. The feed formulation and proximate composition of the experimental diets are presented in Table 5-3, while the fatty acid compositions of the oil sources and experimental diets are shown in Table 5-4.

Rearing conditions. Before the trial, 150 healthy fish were randomly selected and assigned to six tanks (0.26 m^3 each) at a density of 25 fish per tank, with three replicate tanks per treatment. The initial body weight was 17.99 ± 0.01g (mean ± SEM, n=6). Fish were reared for 8 weeks under the same conditions described in Section 2.2 of Chapter IV.

Sampling. Based on the procedures described in Section 2.3 of Chapter IV, muscle samples were further collected from the same four fish per tank. From each fish, four cubes (3 cm × 3 cm × 2 cm) were immediately excised for fillet quality analysis, while the remaining portions were pooled within each tank and stored at −20 °C for subsequent lipid content and fatty acid composition analysis.

Analytical procedures. Growth performance parameters, including survival rate (SR), final body weight (FBW), weight gain (WG), specific growth rate (SGR), feeding rate (FR), feed conversion ratio (FCR), carcass weight (CW), carcass ratio (CR), condition factor (CF), visceral adipose index (VAI), hepatopancreas somatic index (HSI), and viscerosomatic index (VSI), were calculated according to the procedures described in Section 2.4.1 of Chapter III. The analytical methods for proximate and fatty acid composition, biochemical parameters, hepatic histology examination, and gene expression analysis were performed as described in Sections 2.4.2−2.4.6 of Chapter III. Biochemical parameters included plasma triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), and liver total protein (TP), TG, and TC contents. Fillet quality analysis was conducted according to Section 2.4.3 of Chapter III, with additional evaluation of taste-related compounds using an electronic tongue system (SA402B, Insent Inc., Japan) following the method of Shi et al. (Shi et al., 2018a). Briefly, 5 g of cooked fillet from each tank was homogenized with 120 mL distilled water and stirred for 5 min, followed by centrifugation at 5000 rpm for 10 min. Two 60-mL aliquots of the supernatant were subjected to electronic tongue analysis.

2.2.2 Statistical analyses

All data are presented as mean ± SEM. Statistical analyses and figure generation were performed using GraphPad Prism 8. Before statistical analysis, normality was assessed using the Shapiro-Wilk test, and equality of variances was assessed using an F test. For comparisons between two groups, statistical differences were evaluated using an unpaired two-tailed Student's t-test when variances were equal; when variances were unequal (F test $P < 0.05$), Welch's t-test was applied. A value of $P < 0.05$ was considered statistically significant. A value of $P < 0.05$ was considered statistically significant.

Table 5-3. Feed formulation and proximate composition of the experimental diets.

Ingredients (%, as-is basis)	BSFO[1]	BSFOD[1]
Yeast extract[2]	5.50	5.50
Cottonseed protein concentrate	35.00	35.00
Defatted superworm meal	5.00	5.00
Wheat gluten	4.00	3.50
Clostridium autoethanogenum protein	15.00	15.00
Wheat flour	8.00	8.00
Cassava tuber meal	6.00	6.00
Black soldier fly oil	11.60	10.00
Schizochytrium sp. meal, 43.27% DHA[3]	-	3.00
Permix[4]	1.10	1.10
DL-Met	0.50	0.50
L-Lys HCl	1.00	1.00
$Ca(H_2PO_4)_2H_2O$	2.45	2.45
Taurine	0.50	0.50
Choline chloride (60% choline)	0.40	0.40
Microcrystalline cellulose	3.95	3.05
Total	100	100
Proximate composition (DM basis)		
Dry matter (%)	94.54	94.12
Crude protein (%)	51.49	52.10
Crude lipid (%)	12.75	11.90
Ash (%)	7.50	7.52
Gross energy (MJ/Kg)	21.98	22.07
E/P (kJ/g protein)[5]	42.69	42.36

1. BSFO, control diet without DHA supplementation; BSFOD, diet supplemented with DHA.
2. Yeast extract (FA28, protein content > 68.7%), was obtained from Angel Yeast CO., Ltd.
3. *Schizochytrium sp.* meal (SCM, ALGAMAC-3050), protein content >17.6%, Oil content >56.2%,, was obtained from Aquafauna Bio-Marine, Inc.
4. The premix provides the following per kg of diets: vitamin premix: VA 20 mg, VD3 10 mg, VE 400 mg, VB1 10 mg, VB2 15 mg, VB6 15 mg, niacin 100 mg, VC ester 1000 mg, calcium pantothenate 40 mg, VK3 20 mg, biotin 2 mg, VB12 8 mg, folic acid 10 mg, corn gluten powder 150 mg; inositol 200 mg; mineral premix: $MgSO_4$ 2040 mg, $FeSO_4·H_2O$ 300 mg, $ZnSO_4·H_2O$ 200 mg, $MnSO_4·H_2O$ 100 mg, Na_2SeO_3 10 mg, $CoCl_2·6H_2O$ 5 mg, KI 80 mg, zeolite powder 705 mg; mould inhibitor 3400 mg; tert-butylhydroquinone (TBHQ) 200 mg.
5. E/P(kJ/g protein), the ratio of diet gross energy(MJ/Kg) / diet crude protein (%)×100.

Table 5-4. Fatty acid composition of oil source and experimental diets (%TFA).

Items	Oil source	Experimental diets	
	BSFO	BSFO	BSFOD
C10:0	0.73	1.22	1.12
C12:0	21.04	23.45	21.09
C14:0	3.14	4.21	5.13
C16:0	16.76	24.76	27.03
C17:0	0.18	0.59	0.58
C18:0	2.86	4.57	4.44
C20:0	0.05	0.12	0.16
C16:1n-7	1.54	2.59	2.35
C18:1n-9	24.43	28.22	25.72
C18:2n-6	25.93	31.58	29.30
C20:4n-6	0.29	0.47	0.50
C18:3n-3	2.28	3.73	2.76
C20:5n-3	0.29	0.44	0.71
C22:6n-3	ND	ND	7.06
∑SFA	44.88	66.67	67.89
∑MUFA	26.15	31.21	28.44
SFA/MUFA	1.72	2.14	2.39
∑n-3PUFA	2.57	4.17	10.77
∑n-6PUFA	26.40	32.05	29.80
n-3/n-6PUFA	0.10	0.13	0.36
∑PUFA	28.98	36.22	40.58

Note: ND means the fatty acid were not detected.

Abbservation: BSFO, black soldier fly oil; BSFO diet, control diet without DHA supplementation; BSFOD diet, diet supplemented with DHA; SFA, Saturated FAs; MUFA, Monounsaturated FAs; PUFA, Polyunsaturated FAs.

3. Results

3.1 Enrichment of n-3 PUFA in black soldier fly larvae by dietary fish processing by-products

The final average body weight of BSF larvae did not differ significantly among the different dietary FPBs inclusion levels ($P > 0.05$). In contrast, the final total larval biomass in the 24% group was significantly lower than that in the 0% group ($P < 0.05$), whereas no significant differences were observed among the other treatments (Figure 5-1).

No significant differences were detected in crude protein content among treatments ($P > 0.05$). Crude lipid content was significantly higher in the 40% FPBs inclusion group than in the 0% group ($P < 0.05$). However, dry matter and crude ash contents in the 40% group were significantly lower than those in the 8% group ($P < 0.05$, Table 5-5).

Regarding fatty acid composition, the proportion of ALA in larvae from the 24% and 40% groups was significantly higher than that in the 0% group ($P < 0.05$) (Table 5-6). C20:5n-3 and C22:6n-3 + C24:1n-9 were not detected in the 0% group, while their levels in the 8% group were significantly lower than those in the 24% and 40% groups ($P < 0.05$). The total n-3 PUFA content did not differ significantly between the 24% and 40% groups, but was significantly higher than that in the 8% and 0% groups ($P < 0.05$).

A **B**

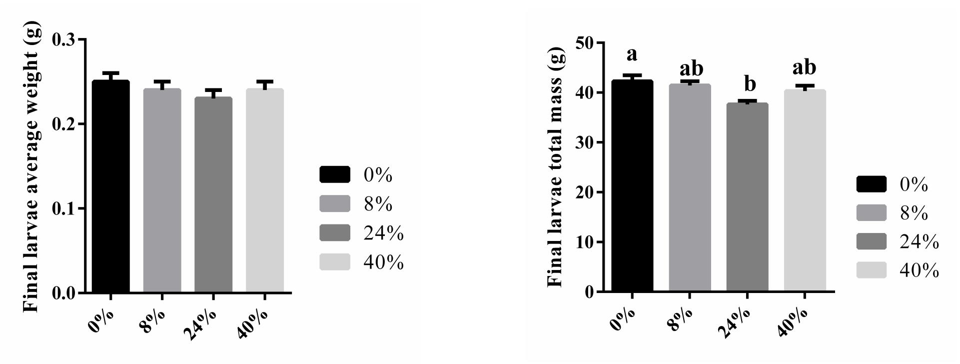


Figure 5-1. Growth of larvae from different test group (wet basis, n=4). (A) Final larvae average weight; (B) Final larvae total mass (wet basis, n=4).

Table 5-5. Nutrient composition of BSF larvae meal from different fish processing by-products inclusion groups (mean ± SEM, n=4).

Larvae meal	0%	8%	24%	40%	*P*-values
Dry matter (%)	38.6±0.67^{ab}	41.21±1.51^{a}	38.20±0.51^{ab}	36.68±0.36^{b}	0.013
Crude protein (%, DM)	23.58±0.09	24.93±0.08	30.88±0.06	34.66±0.05	0.083
Crude lipid (%, DM)	20.01±2.09^{b}	28.77±3.49^{ab}	30.88±2.17^{ab}	34.66±0.9^{a}	0.013
Crude ash (%, DM)	9.95±0.32^{a}	9.78±0.51^{a}	9.95±0.51^{a}	8.40±0.14^{b}	0.032

Table 5-6. Fatty acid composition of black soldier fly oil from different fish processing by-products inclusion groups (% TFA, mean ± SEM, n=4).

Items	0%	8%	24%	40%	*P*-value
C10:0	0.64±0.11	0.39±0.05	0.36±0.05	0.35±0.03	0.172
C12:0	23.37±4.55	16.50±1.59	16.42±2.03	18.45±0.64	0.590
C14:0	3.53±0.73	2.65±0.30	2.85±0.40	3.21±0.11	0.568
C16:0	5.27±1.17	4.34±0.52	3.9±0.73	5.50±0.18	0.372
C18:0	1.10±0.24	0.83±0.09	1.01±0.20	1.05±0.07	0.557
C16:1n-7	1.56±0.29	1.60±0.19	1.11±0.11	1.24±0.05	0.384
C18:1n-9	6.65±0.98	6.77±0.83	5.31±0.54	6.40±0.22	0.466
C18:2n-6	4.10±0.66	3.36±0.42	2.87±0.28	3.56±0.10	0.345
C18:3n-3	0.22±0.09^{c}	0.48±0.05^{bc}	0.6±0.07^{ab}	0.76±0.04^{a}	<0.001
C20:5n-3	ND	0.94±0.13^{b}	1.41±0.14^{a}	1.70±0.09^{a}	0.001
C22:6n-3+C24:1n-9	ND	0.24±0.02^{b}	0.37±0.03^{a}	0.44±0.03^{a}	<0.001
ΣSFA	87.59±1.94	86.64±1.64	88.40±1.17	85.92±0.46	0.586
ΣMUFA	8.21±1.27	8.37±1.02	6.42±0.64	7.64±0.24	0.461
Σn-3PUFA	0.22±0.09^{c}	1.63±0.21^{b}	2.31±0.26^{a}	2.89±0.14^{a}	<0.001
ΣPUFA	4.21±0.69	4.99±0.62	5.19±0.54	6.45±0.23	0.088

Note: ND means the fatty acid were not detected.

Abbservation: SFA, Saturated FAs; MUFA, Monounsaturated FAs; PUFA, Polyunsaturated FAs.

3.2 Application of n-3 PUFA-enriched black soldier fly oil in largemouth bass

3.2.1 Growth performance and proximate composition

Growth performance and morphometric parameters are presented in Table 5-7. No significant difference in SR was observed between the BSFO and BSFOD groups ($P > 0.05$). FBW and WG in the BSFOD group were significantly higher than those in the BSFO group, whereas FCR was significantly lower in the BSFOD group ($P < 0.05$, Table 5-7). CR was significantly higher in the BSFOD group than in the BSFO group, while CF and VSI exhibited the opposite trend ($P < 0.05$).

No significant differences were observed between the two groups in the remaining growth and proximate composition parameters ($P > 0.05$, Table 5-8).

3.2.2 Fatty acid composition of muscle and liver tissue

Muscle fatty acid composition. No significant differences were observed in individual SFAs or in ∑SFA content between the two groups ($P > 0.05$, Table 5-9). The contents of C17:1n-9, LA, C20:4n-6, EPA, and ∑n-6 PUFA were significantly higher in the BSFO group than in the BSFOD group ($P < 0.05$). In contrast, the contents of DHA, DHA+EPA, ∑n-3 PUFA, and ∑PUFA were significantly lower in the BSFO group compared with the BSFOD group ($P < 0.05$).

Liver fatty acid composition. Except for C11:0, individual SFAs, MUFAs, and n-6 PUFAs, as well as ∑SFA, ∑MUFA, and ∑n-6 PUFA, showed significantly higher levels in the BSFO group than in the BSFOD group ($P < 0.05$, Table 5-10). The content of EPA in the BSFO group was significantly higher than that in the BSFOD group ($P < 0.05$), which contrasted with the trends observed for DHA, DHA+EPA, ∑n-3 PUFA, and the n-3/n-6 PUFA ratio, all of which were significantly lower in the BSFO group ($P < 0.05$).

Table 5-7. Growth performance and morphometric parameters of largemouth bass under different dietary treatments (mean ± SEM).

Items	BSFO	BSFOD	*P* value
Growth performance (n=3)			
SR (%)	100.00±0.00	97.33±1.33	0.116
FBW (g)	52.79±0.06^{b}	58.76±0.30^{a}	< 0.001
WG (%)	193.49±0.43^{b}	222.26±3.12^{a}	0.012
SGR (%·d^{-1})	1.96±0	2.15±0.01	0.107
FR (%)	2.20±0.02	2.20±0.02	1.000
FCR	1.23±0.01^{a}	1.15±0.01^{b}	0.003
Morphometric parameters (n=12)			
CW (g)	40.49±0.89	43.67±0.88	0.132
CR (%)	63.36±0.41^{b}	64.62±0.21^{a}	0.035
CF (g/cm^{3})	1.78±0.01^{a}	1.71±0.02^{b}	0.035
VAI (%)	3.65±0.08	3.49±0.24	0.447
HSI (%)	2.22±0.03	2.13±0.08	0.502
VSI (%)	10.32±0.11^{a}	9.68±0.34^{b}	0.046

Table 5-8. Proximate composition of largemouth bass under different dietary treatments (%, wet basis) (mean ± SEM, n=3).

Items		BSFO	BSFOD	*P* value
Whole-body	Dry matter	28.59±0.82	26.84±0.54	0.149
	Crude protein	16.86±0.22	17.43±0.20	0.125
	Crude lipid	10.99±0.29	12.46±0.93	0.206
	Ash	4.95±0.26	5.05±0.30	0.831
Muscle	Crude lipid	0.97±0.19	0.77±0.09	0.410
Liver	Crude lipid	3.54±0.45^{a}	2.11±0.19^{b}	0.042

Table 5-9. Muscle fatty acid composition of largemouth bass under different dietary treatments (mg/g, wet basis) (mean ± SEM, n=3).

Items	BSFO	BSFOD	*P* value
C11:0	1.85±0.08	1.92±0.07	0.544
C12:0	0.77± 0.17	0.44 ± 0.08	0.150
C14:0	0.57±0.10	0.35±0.04	0.132
C16:0	3.27±0.45	2.57±0.16	0.214
C17:0	0.07±0.01	0.07±0.01	1.000
C18:0	1.01±0.10	0.93±0.02	0.473
C16:1n-7	0.31±0.05	0.16±0.02	0.067
C17:1n-9	0.08±0.00^a	0.06±0.00^b	0.047
C18:1n-9	3.31±0.48	2.01±0.19	0.064
C20:1n-9	0.16±0.02	0.09±0.01	0.050
C18:2n-6	3.47±0.39^a	2.16±0.17^b	0.037
C20:2n-6	0.14±0.02	0.11±0.01	0.134
C20:4n-6	0.20±0.01^a	0.15±0.01^b	0.007
C18:3n-3	0.21±0.03	0.15±0.01	0.148
C20:5n-3	0.10±0.01^a	0.07±0.00^b	0.018
C22:6n-3	0.67±0.03^b	1.47±0.07^a	< 0.001
C20:5n-3+C22:6n-3	0.77±0.03^b	1.53±0.08^a	< 0.001
∑SFA	7.54±0.87	6.29±0.22	0.235
∑MUFA	3.85±0.55	2.32±0.22	0.061
∑n-3PUFA	0.98±0.06^b	1.69±0.08^a	0.002
∑n-6PUFA	3.95±0.42^a	2.47±0.19^b	0.034
n-3/n-6PUFA	0.25±0.01^b	0.68±0.02^a	< 0.001
∑PUFA	4.92±0.48	4.16±0.27	0.242

Abbservation: SFA, Saturated FAs; MUFA, Monounsaturated FAs; PUFA, Polyunsaturated FAs.

Table 5-10. Liver fatty acid composition of largemouth bass under different dietary treatments (mg/g, wet basis) (mean ± SEM, n=3).

Items	BSFO	BSFOD	*P* value
C11:0	2.28±0.11	2.19±0.08	0.498
C12:0	0.94 ± 0.20	0.74 ± 0.21	0.530
C14:0	1.60±0.22^a	0.81±0.04^b	0.025
C16:0	7.05±0.70^a	4.57±0.20^b	0.027
C17:0	0.20±0.02^a	0.13±0.01^b	0.027
C18:0	3.45±0.19^a	2.11±0.15^b	0.005
C20:0	0.12±0.01^a	0.07±0.00^b	0.004
C16:1n-7	0.94±0.08^a	0.44±0.03^b	0.005
C17:1n-9	0.09±0.01^a	0.06±0.00^b	0.019
C18:1n-9	10.14±0.86^a	4.77±0.23^b	0.004
C20:1n-9	0.79±0.03^a	0.30±0.03^b	< 0.001
C18:2n-6	7.63±0.89^a	4.21±0.17^b	0.019
C20:2n-6	0.45±0.01^a	0.29±0.02^b	0.006
C20:3n-6	0.66±0.01^a	0.21±0.02^b	< 0.001
C20:4n-6	0.83±0.03^a	0.44±0.02^b	< 0.001
C18:3n-3	0.26±0.04	0.24±0.03	0.797
C20:5n-3	0.11±0.01^a	0.06±0.01^b	0.042
C22:6n-3	1.40±0.05^b	2.96±0.30^a	0.007
C20:5n-3+C22:6n-3	1.51±0.05^b	3.02±0.31^a	0.009
∑SFA	15.64±1.22^a	10.62±0.55^b	0.02
∑MUFA	11.95±0.98^a	5.57±0.26^b	0.003
∑n-3PUFA	1.77±0.07^b	3.27±0.33^a	0.012
∑n-6PUFA	9.57±0.95^a	5.15±0.15^b	0.01

Items	BSFO	BSFOD	*P* value
n-3/n-6PUFA	0.19±0.02^{b}	0.63±0.06^{a}	0.002
∑PUFA	11.33±1.00	8.42±0.42	0.055

Abbservation: SFA, Saturated FAs; MCFA; MUFA, Monounsaturated FAs; PUFA, Polyunsaturated FAs.

3.3.3 Fillet quality

Dietary DHA inclusion significantly increased fillet adhesiveness and richness, while significantly reduced the a* value (redness) ($P < 0.05$, Table 5-11). No significant differences were observed in the other fillet quality parameters between the two groups ($P > 0.05$).

Table 5-11. Fillet quality of largemouth bass under different dietary treatments (mean ± SEM, n=12).

Item	BSFO	BSFOD	*P* value
Drip loss (%)	13.01±2.13	15.54±1.45	0.336
Cooking loss (%)	18.74±2.54	17.29±1.05	0.605
L	44.78±0.30	45.28±0.57	0.450
a	5.77±0.29^{a}	3.67±0.17^{b}	< 0.001
b	-0.83±0.20	-1.09±0.17	0.336
Whiteness	44.46±0.31	45.14±0.57	0.309
Texture of fresh fillet			
Hardness (N)	0.77±0.13	0.64±0.09	0.446
Adhesiveness (mJ)	0.03±0.00^{b}	0.04±0.00^{a}	0.020
Cohesiveness (Ratio)	0.55±0.02	0.51±0.01	0.118
Springiness (mm)	0.41±0.03	0.43±0.02	0.595
Electronic tongue of cooked fillet (n=3)			
Bitterness	8.58±0.17	7.98±0.17	0.070
Umami	9.65±0.37	9.73±0.49	0.899
Saltiness	-2.45±0.40	-3.29±0.48	0.247
Richness	0.68±0.00^{b}	3.16±0.05^{a}	< 0.001

3.3.4 Plasma and liver lipid metabolism

Plasma HDL-c concentration and the HDL-c/LDL-c ratio were significantly higher in the BSFOD group than in the BSFO group, whereas LDL-c concentration exhibited the opposite trend ($P < 0.05$, Table 5-12). The liver triglyceride (TG) level in the BSFO group was significantly higher than that in the BSFOD group ($P < 0.05$).

Histopathological examination of the liver revealed no obvious hepatocellular swelling, nuclear displacement, or severe vacuolization in either group (Figure 5-2).

Table 5-12. Biochemical indicators in the plasma and liver of largemouth bass under different dietary treatments (mean ± SEM, n=12).

Items		BSFO	BSFOD	*P* value
Plasma	TG (mmol/L)	4.60±0.66	6.26±1.12	0.073
	TC (mmol/L)	5.88±0.34	6.10±0.59	0.65
	HDL-c (mmol/L)	6.51±0.0.76^{b}	10.83±0.25^{a}	< 0.001
	LDL-c (mmol/L)	0.96±0.11^{a}	0.76±0.03^{b}	0.042
	HDL-c/LDL-c	7.54±1.49^{b}	14.83±0.93^{a}	< 0.001
Liver	TG (mmol/g prot)	0.18±0.03^{a}	0.11±0.01^{b}	0.014
	TC (mmol/g prot)	0.06±0.00	0.04±0.01	0.059

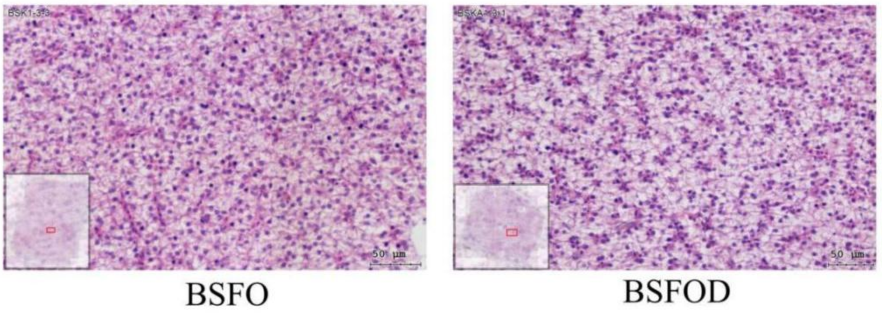


Figure 5-2. Representative H&E-stained liver sections of largemouth bass under different dietary treatments (n = 12). No marked hepatocellular swelling, nuclear displacement, or severe vacuolization observed.

3.3.5 Liver LC-PUFA biosynthesis

The mRNA expression level of *fads2* did not differ significantly between the BSFO and BSFOD groups ($P > 0.05$, Figure 5-3A). In contrast, the expression levels of *delta4 fads*, *fads6*, *elovl4a*, and *elovl5* were significantly higher in the BSFO group than in the BSFOD group ($P < 0.05$), whereas *elovl8a* exhibited the opposite trend ($P < 0.05$). Moreover, dietary DHA inclusion significantly decreased the protein abundances of Δ6, Δ5, and Δ4 Fads ($P <$ 0.05, Figure 5-3B).

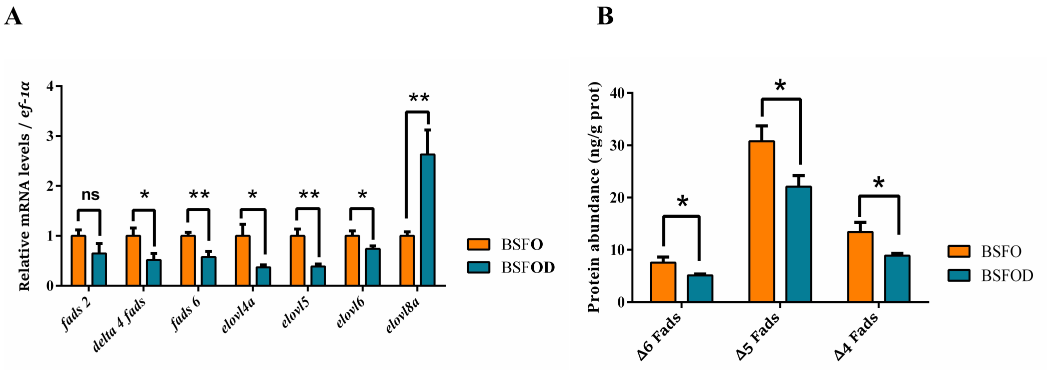


Figure 5-3. Genes expression and protein abundances of desaturases of in the liver of largemouth under different dietary treatments. (A) Relative mRNA expression of fatty acyl desaturase *(fads)* and elongase (*elovl*) genes (mean ± SEM, n=8); (B) Protein abundance of liver Fads (Δ6, Δ5, Δ4 Fads) (mean ± SEM, n = 12).

4. Discussion

4.1 Enrichment efficiency of n-3 PUFA in black soldier fly larvae

This study demonstrates that dietary FPBs can effectively enrich n-3 PUFAs in BSF larvae without compromising larval growth performance. Although total biomass was lower in the 24% group, individual body weight was not affected, indicating that dietary FPBs inclusion did not impair larval growth performance. The difference in total biomass may reflect variation in overall rearing efficiency, as survival could not be individually quantified in the present study. Although the final individual body weight was not affected by increasing FPBs inclusion, the progressive increase in crude lipid content and the marked changes in fatty acid composition indicate that substrate composition primarily modulated lipid deposition. These findings are consistent with previous reports showing that dietary lipid sources exert a stronger influence on insect lipid profiles (Liland et al., 2017; St‐Hilaire et al., 2007). The significant enrichment of ALA, as well as the appearance of EPA and DHA in larvae reared on higher FPBs levels, confirms that BSF larvae are capable of directly assimilating dietary n-3 PUFA from their substrate (Hoc et al., 2021a). Given the limited endogenous capacity of insects to elongate and desaturate C18 precursors into LC-PUFA, the presence of EPA and DHA in enriched larvae most likely reflects direct dietary transfer rather than *de novo* biosynthesis (Hoc et al., 2020). In crickets, mealworms, and BSF, dietary inclusion of linseed oil resulted in a dose-dependent enrichment of ALA, leading to a 10–20-fold increase in total n-3 PUFA and a reduction of the n-6/n-3 ratio from 18–36 to below 5, a range considered optimal for human health (Oonincx et al., 2020). Combined with earlier studies demonstrating minimal LC-PUFA biosynthesis activity in insects and a strong dependence on dietary supply (Spranghers et al., 2017; St‐Hilaire et al., 2007), the present findings further support this interpretation.

Compared with previous enrichment strategies based on VO or microalgae, the use of FPBs as a substrate offers a distinct advantage by providing EPA and DHA, thereby bypassing the metabolic limitations of the insect (Georgescu et al., 2022; Tirtawijaya & Choi, 2022). The present dose–response data further indicate that moderate to high inclusion levels (24–40%) are required to achieve meaningful enrichment, highlighting a threshold effect in substrate-mediated lipid modulation (Erbland et al., 2020). Collectively, these results demonstrate the feasibility of producing n-3 PUFA-enriched BSF larvae through simple dietary manipulation and provide a practical foundation for generating nutritionally upgraded insect oils for aquafeed applications.

4.2 Regulation of LC-PUFA biosynthesis by dietary DHA

The regulatory responses of the hepatic LC-PUFA biosynthetic pathway provide important mechanistic insights into the nutritional control of fatty acid metabolism in largemouth bass. While the mRNA expression of *fads2* was unaffected, several key genes, including *delta4 fads*, *fads6*, *elovl4a* and *elovl5*, were upregulated in the BSFO group, whereas their protein abundances were significantly reduced by dietary DHA inclusion. Such an apparent dissociation between transcriptional activation and translational suppression is widely recognized as a characteristic feature of feedback regulation in fish LC-PUFA biosynthetic pathways, where dietary supply exerts strong control beyond the transcriptional level (Morais et al., 2012; Tocher et al., 2006).

Under DHA-deficient conditions, the endogenous biosynthetic pathway is transcriptionally induced as a compensatory response to limited dietary availability, a phenomenon commonly reported in both marine and freshwater fish species (Tocher, 2010; Zheng et al., 2005). Conversely, when preformed DHA is provided, the pathway is suppressed at the post-transcriptional level, leading to reduced desaturase protein abundance and attenuated endogenous synthesis (Hastings et al., 2001; Monroig et al., 2022).

These findings are fully consistent with our previous stable isotope tracing and functional characterization studies, which demonstrated that dietary DHA exerts strong negative feedback on Δ6, Δ5 and Δ4 desaturase activities (Castro et al., 2016; Leaver et al., 2008). Together, these data establish a coherent regulatory model in which dietary DHA governs LC-PUFA biosynthesis through both transcriptional compensation and post-transcriptional inhibition. This dual-level regulation underscores the central role of nutritional signals in controlling endogenous LC-PUFA metabolism in carnivorous fish.

4.3 Tissue fatty acid deposition and nutritional quality

One of the most notable outcomes of this study is the marked improvement in tissue fatty acid composition in fish fed with DHA inclusion diet. Both muscle and liver tissues exhibited significantly higher DHA, total n-3 PUFA and n-3/n-6 ratios in the BSFOD group, accompanied by a reduction in excessive n-6 PUFA deposition. The preferential deposition of DHA in muscle tissue indicates efficient dietary transfer and retention, which is essential for maintaining the nutritional value of farmed fillets (Ruyter et al., 2022). Moreover, the reduction in n-6 PUFA accumulation suggests that dietary DHA can effectively counterbalance the n-6-rich background typical of insect oils. This rebalancing of the n-3/n-6 ratio is particularly important for maintaining membrane function and inflammatory homeostasis in carnivorous fish (Stillwell & Wassall, 2003). Collectively, these findings highlight that substrate-enriched insect oil is not only a sustainable lipid source, but also a nutritionally competent substitute capable of safeguarding fillet quality.

4.4 Lipid metabolism and hepatic lipid accumulation

Dietary DHA inclusion markedly improved systemic and hepatic lipid metabolism, as evidenced by higher plasma HDL-c levels, lower LDL-c concentrations, and reduced hepatic triglyceride accumulation in the BSFOD group, indicating a more favorable lipoprotein profile and enhanced lipid transport efficiency (Tocher, 2015; Turchini & Francis, 2009). The reduction in hepatic TG content suggests that dietary DHA alleviated lipid deposition in the liver and lowered the risk of excessive steatosis, a common metabolic disorder under high-energy feeding conditions in intensive aquaculture systems (Glencross, 2009; Wang et al., 2020). Notably, the absence of overt histopathological alterations in both groups implies that these metabolic benefits occurred prior to the onset of structural liver damage, consistent with the concept that alterations in lipid metabolism precede visible histological impairment (Glencross, 2009). Mechanistically, DHA has been shown to enhance mitochondrial β-oxidation, suppress hepatic lipogenesis, and promote very-low-density lipoprotein (VLDL) assembly and export through conserved regulatory pathways involving PPARα and SREBP-1c in both fish and mammals (Tocher, 2015). Collectively, the present results support the conclusion that DHA inclusion improves systemic lipid transport and reduces hepatic lipid burden, thereby contributing to the enhanced growth performance and feed efficiency observed in the BSFOD group.

4.5 Fillet quality and textural properties

Dietary DHA inclusion exerted selective but meaningful effects on fillet quality attributes. In the present study, fish fed the DHA-inclusion diet showed significantly increased adhesiveness in fresh fillets and enhanced richness in cooked fillets, whereas the a* value (redness) was significantly reduced, with no significant changes observed in the other texture and color parameters. These results indicate that DHA enrichment modified specific sensory-related properties without compromising the overall physical integrity of the fillet, consistent with previous observations that dietary lipid quality primarily affects subtle textural and sensory traits rather than gross muscle structure (Johnston et al., 2006).

The increase in adhesiveness and richness may be associated with alterations in muscle lipid composition and intramuscular fat distribution. DHA is preferentially incorporated into membrane phospholipids and neutral lipid fractions, which can influence water-holding capacity, inter-fiber cohesion and mouthfeel (He et al., 2022; Wang et al., 2024a). Previous studies have demonstrated that dietary fatty acid composition, plays a decisive role in shaping fillet texture and sensory perception in carnivorous fish (Johnston et al., 2006). In this context, the present findings suggest that DHA-enriched insect oil can subtly improve palatability-related attributes without inducing excessive fat deposition.

The reduction in fillet redness (a* value) may reflect changes in muscle pigment stability or lipid oxidation status. Muscle color is closely associated with myoglobin content, oxidative stability and membrane integrity, all of which can be influenced by dietary PUFA composition (Keinänen et al., 2022; Li & Zhu, 2024). Diets rich in n-6 PUFA have been linked to increased susceptibility of muscle pigments to oxidative discoloration, whereas n-3 LC-PUFA, particularly DHA, may stabilize membrane structure and attenuate oxidative stress in muscle tissue (Torstensen et al., 2005). Although the biological significance of the observed decrease in a* value remains limited, the absence of adverse changes in lightness (L*) or yellowness (b*) indicates that dietary DHA did not negatively affect the visual quality of the fillet.

Importantly, the lack of significant differences in most texture and color parameters demonstrates that a DHA-inclusion diet preserves fillet technological quality. From an industrial perspective, this is advantageous, as it suggests that sustainability-driven lipid substitution may be achieved without compromising processing performance or consumer acceptance. Taken together, these results indicate that DHA-enriched insect oil maintains, and in some aspects improves, fillet quality, thereby reinforcing its suitability as a functional FO alternative.

4.6 Growth performance and feed utilization in largemouth bass

The DHA-inclusion diet significantly improved growth performance and feed utilization in largemouth bass, as indicated by higher FBW and WG, together with a lower FCR in the BSFOD group. These results clearly indicate that adequate DHA supply enhances feed efficiency and growth under the present experimental conditions. The growth-promoting effect of dietary DHA is consistent with its established roles in maintaining membrane integrity, mitochondrial function and energy metabolism in carnivorous fish (Castro et al., 2016; Tocher, 2010). In species with limited endogenous LC-PUFA biosynthetic capacity, insufficient dietary DHA often constrains growth by impairing lipid digestion and utilization efficiency. The present findings therefore support the concept that DHA is not merely a structural fatty acid, but also a functional modulator of feed efficiency (Liu et al., 2023; Zhang et al., 2025). Importantly, these results align closely with our previous work demonstrating interactive effects between dietary ALA and DHA on growth and LC-PUFA metabolism in largemouth bass. Together, these studies reinforce the conclusion that adequate DHA supply is indispensable for maximizing growth performance when alternative lipid sources are used to replace FO.

5. Conclusion

This study demonstrates a practical circular strategy that integrates seafood by product valorisation with insect based aquafeed development. Dietary inclusion of 24% FPBs in BSF larval diets effectively enriched BSFO with n-3 PUFAs, enabling the accumulation of EPA and DHA without adversely affecting larval growth. Subsequent feeding trials in largemouth bass showed that a DHA supplemented BSFO diet—simulating BSFO produced from FPBs significantly improved growth performance and tissue DHA deposition, while also enhancing the n-3/n-6 PUFA balance and systemic lipid metabolism without compromising overall fillet quality. These findings demonstrate that n-3 PUFA enriched BSFO can function as a biologically effective alternative to conventional marine lipids under reduced FO conditions. From an applied perspective, this insect enrichment fish feeding framework provides a practical approach for converting food processing by products into high value aquafeed ingredients, thereby reducing reliance on marine derived resources while maintaining fish health and product nutritional quality.

6

Chapter VI General discussion, conclusion and perspectives

General discussion

The progressive phase-out of fish oil (FO) from aquafeeds, driven by limited marine resources and the expanding demand from global aquaculture (FAO, 2026), has created an urgent need to identify species-specific capacities for endogenous long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis and to develop effective lipid replacement strategies (Turchini et al., 2010). Largemouth bass represents a particularly important model species in this transition. As one of the most widely farmed freshwater carnivorous species in China, its nutritional physiology serves as a critical reference point for understanding how carnivorous teleosts can adapt to reduced marine lipid dependency within the context of the FAO's “Blue Transformation” roadmap, which has driven concrete action worldwide (FAO, 2026). Moreover, it has a clear nutritional dependence on n-3 LC-PUFA (An et al., 2023; Yadav, 2020), and some studies have suggested that it may possess a certain capacity for endogenous LC-PUFA biosynthesis; however, robust supporting evidence remains limited (Song et al., 2024a; Yadav, 2020; Zhang et al., 2019a). This unique combination makes it an ideal system for evaluating both the physiological constraints and the opportunities for reducing marine lipid dependency in carnivorous teleosts. Notably, the herbivorous marine rabbitfish represents a striking exception to the freshwater/marine dichotomy, possessing all required enzymatic activities for DHA biosynthesis from C18 PUFA despite its marine habitat—a trait attributed to its low trophic level and benthic algal diet (Li et al., 2010a). This exception underscores that trophic ecology, rather than salinity, may be the primary evolutionary driver of LC-PUFA biosynthetic capacity, further validating the relevance of largemouth bass as a carnivorous model.

Prior to this study, we hypothesized that largemouth bass would exhibit a limited capacity to convert C18 PUFAs into LC-PUFAs, based on its carnivorous feeding ecology and presumed dependence on preformed docosahexaenoic acid (DHA, C22:6n-3). The present results both confirm and refine this hypothesis. However, accurately evaluating such biosynthetic capacity remains challenging, as previous studies have largely relied on single-level approaches—such as gene expression analysis or feeding trials—which are insufficient to link molecular regulation with actual metabolic flux (Castro et al., 2016; Tocher, 2015; Xie et al., 2021). To address this limitation, a multi-level framework was established, integrating heterologous expression, hepatocyte assays, stable isotope tracing, and feeding trials to systematically evaluate LC-PUFA biosynthesis from molecular to organismal levels.

Functional characterization revealed Δ6, Δ5, and Δ4 desaturase activities, indicating the theoretical capacity for DHA biosynthesis from dietary α-linolenic acid (ALA, 18:3n-3). This enzymatic system aligns with some freshwater species such as Nile tilapia (Δ6Δ5 Fads, Δ4 Fads) and rainbow trout (Δ6 Fads, Δ5 Fads), which possesses a complete set of biosynthetic enzymes (Xie et al., 2021). However, evidence from cellular incubation and *in vivo* isotope tracing indicates that, despite the presence of Δ6 Fads and Δ5Δ4 Fads, the primary metabolic bottleneck resides in the later stages of the biosynthetic pathway, particularly during the conversion of C20 intermediates to DHA. Moreover, that the accumulation of C20 intermediates alongside limited DHA production has been widely observed in teleosts, indicating that pathway completeness does not necessarily translate into metabolic efficiency (Tocher, 2015). Taken together, these findings demonstrate that largemouth bass possesses a functional and physiologically active pathway for endogenous n-3 LC-PUFA biosynthesis, but that this pathway is intrinsically inefficient. This constrained biosynthetic capacity is consistent with the metabolic characteristics of carnivorous fish, such as salmonids, which are characterized by a strong dependence on dietary DHA and a limited ability for endogenous LC-PUFA biosynthesis (Kuah et al., 2015). Compared with herbivorous and omnivorous freshwater species such as carp and tilapia, which exhibit relatively efficient LC-PUFA biosynthesis, largemouth bass shows a markedly reduced conversion efficiency (Galindo et al., 2021).

Further feeding trials were conducted to evaluate the contribution of endogenous ALA conversion to tissue LC-PUFA deposition and growth. The two trials served complementary purposes: the commercial diet trial reflected practical farming conditions, whereas the fishmeal (FM)- and FO-free trial minimized confounding LC-PUFA inputs to better assess endogenous biosynthetic capacity. Under commercial conditions, increased dietary ALA and desaturase expression did not elevate tissue n-3 LC-PUFA levels, likely because the persistence of FM-derived preformed LC-PUFAs in commercial formulations created a “nutritional masking effect,” similar to that reported in Atlantic salmon fed high-FM diets, where endogenous LC-PUFA biosynthesis is suppressed despite elevated fads expression. (Zhang et al., 2024b). Moreover, compared with cottonseed oil (CO), soybean oil (SO) contains a smaller difference in ALA content, which may be insufficient to elicit significant changes in tissue EPA and DHA levels. Nevertheless, this experiment also illustrated that when dietary DHA adequately meets growth requirements, further endogenous conversion is suppressed, as the biosynthetic process entails significant energetic costs (Metherel et al., 2024; Tocher, 2015). Consequently, it was necessary to explore this question further through FM- and FO-free diet feeding trials, using lipid sources with greater differences in ALA content.

In the FM- and FO-free trial, when dietary ALA was provided at sufficient levels over an 8-week feeding period in the absence of marine ingredients, the limited conversion capacity resulted in significant elevations of tissue EPA and DHA, and supported growth rates indistinguishable from fish receiving both sufficient ALA and DHA. This conditional sufficiency contrasts with marine carnivorous species such as golden pompano *Trachinotus ovatus*, which exhibit limited endogenous LC-PUFA biosynthetic capacity and a stronger dependence on dietary LC-PUFA despite possessing the key elongase and desaturase genes involved in LC-PUFA biosynthesis (Zhu et al., 2018; Zhu et al., 2020), and aligns more closely with the metabolic plasticity observed in rainbow trout (Thanuthong et al., 2011), which can utilize ALA-enriched alternative lipids when DHA is absent (Xie et al., 2021).

Feeding trials further confirmed that adequate ALA in linseed oil (LO) diets supported normal growth of largemouth bass and promoted the endogenous biosynthesis of EPA and DHA, indicating that ALA can function as a key essential fatty acid for this species under appropriate dietary conditions (Thanuthong et al., 2011; Tocher, 2010). Integrating the tissue fatty acid composition results from Chapter IV (Section 3.2) and Chapter V (Section 3.2.2) (Table 6-1), fish fed the LO-based diet, which provided the highest level of ALA, exhibited higher muscle EPA and DHA levels than fish fed the other lipid sources in the absence of dietary DHA, indicating a greater contribution of endogenous LC-PUFA biosynthesis. Interestingly, dietary DHA supplementation eliminated the differences in muscle DHA among lipid sources, whereas the higher muscle EPA levels associated with ALA-rich diets were maintained. Furthermore, fish fed ALA-rich diets consistently exhibited elevated hepatic EPA and DHA concentrations regardless of DHA supplementation. These findings suggest that tissue LC-PUFA status in largemouth bass is determined by the combined effects of dietary precursor availability, direct DHA supply, and endogenous fatty acid metabolic regulation.

Moreover, dietary DHA compensated for insufficient ALA in CO diets, highlighting the complementary nutritional roles of precursor fatty acids and preformed LC-PUFAs in aquafeed formulation. The physiological importance of DHA further suggests that its requirement is conditional, depending on dietary composition and the metabolic capacity for endogenous LC-PUFA biosynthesis (Glencross, 2009; Tocher, 2015). These findings suggest that largemouth bass is a metabolically constrained but functionally responsive species, where low-efficiency pathways can still be nutritionally relevant under specific dietary conditions. The initial hypothesis should therefore be revised, as limited biosynthetic capacity does not equate to nutritional irrelevance, but rather reflects a low-efficiency yet conditionally sufficient trait with implications for feed formulation.

From an aquaculture perspective, at a global scale, improving the utilization efficiency of C18 precursors such as ALA represents a critical pathway toward reducing dependence on finite marine-derived resources (Tocher, 2015). This is particularly relevant for rapidly expanding freshwater aquaculture sectors globally, where cost-effective and scalable feed solutions are urgently needed (FAO, 2026). Consistent with the tissue LC-PUFA deposition patterns discussed above, an integrated analysis of the growth performance results from Chapters IV (Section 3.1) and Chapter V (Section 3.2.1) revealed that fish fed ALA-rich LO diets exhibited significantly higher FBW, WG, and SGR than those fed CO-based diets, while growth performance was comparable between fish fed LO- and black soldier fly oil (BSFO)-based diets regardless of DHA inclusion (Table 6-2). These findings indicate that enhancing dietary n-3 precursor availability can support both tissue LC-PUFA status and growth performance in largemouth bass. Consequently, the use of LO and other ALA-rich lipid sources in FO replacement formulations, including partial or complete substitution strategies, represents a viable approach for aquafeed development. Nevertheless, economic feasibility and resource sustainability must also be considered. In particular, balancing precursor supply (e.g., ALA) with direct DHA provision appears essential for maintaining optimal growth performance, tissue LC-PUFA status, and metabolic health (Turchini et al., 2009).

Beyond conventional vegetable oils, sustainable alternative lipid resources are also receiving increasing attention in aquafeed development. Black soldier fly *Hermetia illucens* (BSF) larvae have attracted increasing attention as sustainable bioconversion platforms capable of converting fish processing by-products (FPBs) into n-3 PUFA-enriched biomass (Hoc et al., 2021a; St‐Hilaire et al., 2007). This approach represents a shift from linear resource utilization to circular nutrient recycling, in which low-value organic waste is converted into high-value LC-PUFA-enriched biomass through biological processes. Notably, fish fed BSFO-based diets achieved growth performance comparable to those fed LO-based diets, while DHA supplementation further improved growth and tissue fatty acid composition. These findings demonstrate that BSFO can effectively support the nutritional requirements of largemouth bass when appropriately formulated, consistent with previous studies in mirror carp and rainbow trout (Hoc et al., 2021b; Moutinho et al., 2024; Xu et al., 2021). Rather than serving as a complete replacement for FO, DHA-enriched BSFO may be better positioned as a strategic partial substitute within optimized feed formulations, contributing to both nutritional adequacy and circular bioeconomy development (Bélanger et al., 2021; Trevi et al., 2023). Although its application in largemouth bass diets appears promising (Arena et al., 2023; Hoc et al., 2021a), large-scale adoption will depend on improvements in raw material standardization, cost efficiency, and feed processing compatibility. Furthermore, the limited availability of standardized production data currently restricts a robust assessment of the global supply potential of BSFO relative to fish oil and vegetable oils.

Despite these promising findings, several limitations should be acknowledged. Although the feeding trials were conducted over a relatively short period, previous studies in teleosts have demonstrated that dietary lipid substitution induces time-dependent remodeling of tissue fatty acid profiles and metabolic regulation. In Atlantic salmon, for example, the transition from freshwater to seawater stages involves significant upregulation of LC-PUFA biosynthesis in hepatocytes during parr–smolt transformation (Sprague et al., 2019), while in tilapia, ontogenetic shifts in *fads2* and *elovl5* expression peak at sub-adult stages (Stage III) before declining during reproductive maturation (Chen et al., 2025). These developmental and environmental modulations suggest that the observed responses in largemouth bass may represent early-stage adaptive processes within a longer-term trajectory. Future investigations should therefore adopt multi-stage, life-cycle approaches to fully elucidate the persistence and physiological consequences of these effects.

Table 6-1 Muscle and liver fatty acid composition of largemouth bass fed different lipid sources (mg/g, wet basis) (mean ± SEM, n=3).

Item	Muscle		Liver	
	C20:5n-3	C22:6n-3	C20:5n-3	C22:6n-3
Individual treatment means				
LO	0.20±0.01^{A*}	0.88±0.05^{A}	0.78±0.06^{A*}	2.46±0.17
CO	0.09±0.00^{B*}	0.65±0.02^{B}	0.13±0.02^{B}	0.81±0.31
BSFO	0.10±0.01^{B*}	0.67±0.03^{B}	0.11±0.01^{B}	1.40±0.05
LOD	0.11±0.00^{X}	1.37±0.03*	0.36±0.05^{X}	3.31±0.36
COD	0.06±0.00^{Y}	1.45±0.07*	0.04±0.01^{Y}	2.80±0.22
BSFOD	0.07±0.00^{Y}	1.47±0.07*	0.06±0.01^{Y}	2.96±0.30
Means of main effect				
LO				2.89^{a}
CO				1.81^{b}
BSFO				2.18^{ab}
D–				1.56^{y}
D+				3.02^{x}
Two-way ANOVA: *P*-value				
Oil sources				0.008
DHA inclusion				<0.001
Interaction	<0.001	0.013	<0.001	0.446

Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (LO, CO, BSFO) and DHA inclusion (D−, D+), respectively ($P < 0.05$). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant DHA inclusion effect within each oil source.

Table 6-2 Growth performance of largemouth bass fed with different lipid sources (mean ± SEM, n=3).

Item	FBW (g)	WG (%)	SGR ($\%\cdot d^{-1}$)	FR (%)	FCR
Individual treatment means					
LO	54.30 ± 0.88^{A}	198.20 ± 4.23^{A}	2.01 ± 0.03^{A}	2.14 ± 0.01	1.18 ± 0.01^{B}
CO	38.99 ± 1.90^{B}	116.24 ± 9.12^{B}	1.34 ± 0.07^{B}	2.00 ± 0.02	$1.71\pm0.06^{A*}$
BSFO	51.32 ± 1.09^{A}	185.47 ± 5.24^{A}	1.91 ± 0.04^{A}	2.20 ± 0.02	1.23 ± 0.01^{B}
LOD	55.26 ± 1.11^{X}	205.76 ± 5.35^{X}	2.04 ± 0.04^{X}	2.21 ± 0.03	1.17 ± 0.00
COD	$50.69\pm0.93^{Y*}$	$179.27\pm4.47^{Y*}$	$1.87\pm0.04^{Y*}$	2.12 ± 0.03	1.25 ± 0.02
BSFOD	$57.29\pm1.09^{X*}$	$214.25\pm5.24^{X*}$	$2.10\pm0.04^{X*}$	2.20 ± 0.02	1.15 ± 0.01
Means of main effect					
LO				2.18^{a}	
CO				2.06^{b}	
BSFO				2.20^{a}	
D–				2.12^{y}	
D+				2.17^{x}	
Two-way ANOVA: *P*-value					
Oil sources				< 0.001	
DHA inclusion				0.006	
Interaction	<0.001	<0.001	<0.001	0.056	<0.001

Lowercase letters (a, b; x, y) indicate significant differences for main effects of oil source (LO, CO, BSFO) and DHA inclusion (D−, D+), respectively ($P < 0.05$). Uppercase letters (A, B; X, Y) indicate differences among treatment combinations when a significant interaction is detected, and an asterisk (*) denotes a significant DHA inclusion effect within each oil source.

General conclusion

Largemouth bass possesses genes encoding key desaturases (Δ6, Δ5, and Δ4) and exhibits a low-efficiency but conditionally sufficient endogenous pathway for n-3 LC-PUFA biosynthesis. Within this metabolic framework, ALA functions as a key essential fatty acid, capable of supporting growth when supplied at adequate levels. However, dietary lipid composition, particularly the balance between ALA and dietary n-3 LC-PUFA, plays a critical role in determining growth performance and tissue fatty acid composition. While ALA can sustain physiological requirements through endogenous conversion, dietary DHA can effectively compensate for insufficient precursor availability, highlighting the complementary nutritional roles of precursor fatty acids and preformed LC-PUFAs. This metabolic flexibility appears to be regulated through feedback mechanisms that maintain fatty acid homeostasis.

The comparable growth performance achieved with LO- and BSFO-based diets, together with their ability to maintain adequate tissue n-3 LC-PUFA levels, highlights the practical value of combining endogenous biosynthetic capacity with sustainable lipid resources in fish oil (FO)-free aquafeed formulations. From an applied perspective, these findings demonstrate that several lipid sources, including SO, LO, moderately supplemented CO, and n-3 LC-PUFA-enriched BSFO, can serve as promising alternatives to FO in largemouth bass diets. Furthermore, the distinct tissue DHA deposition patterns observed before and after DHA supplementation indicate that DHA accumulation is regulated not only by dietary supply but also by endogenous metabolic processes. Collectively, these results emphasize the importance of balancing precursor availability and direct LC-PUFA provision while accounting for species-specific metabolic constraints, thereby facilitating the development of nutritionally effective, sustainable, and economically viable aquafeeds.

Overall, by integrating molecular characterization, stable isotope tracing, controlled feeding trials, and insect-mediated lipid enrichment, this study bridges fundamental understanding of LC-PUFA metabolism with practical feed innovation. The findings advance current knowledge of lipid nutrition in carnivorous fish and provide a scientific basis for the development of sustainable aquafeed strategies under increasing global resource constraints.

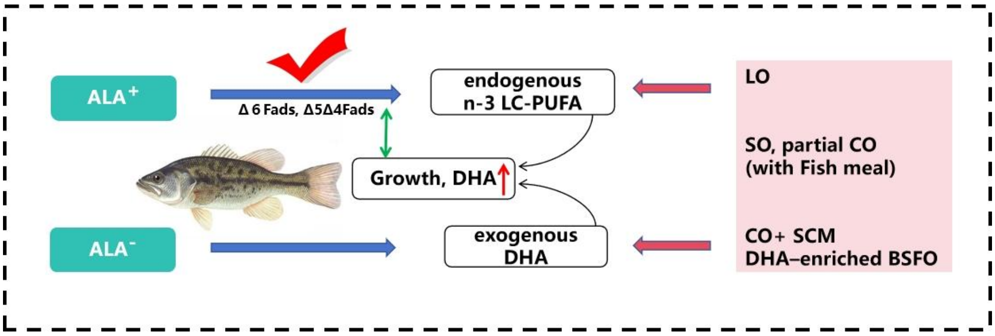


Figure 6-1. Schematic representation of the metabolic and nutritional interactions between dietary ALA and DHA in largemouth bass. Endogenous LC-PUFA biosynthesis is functionally present but efficiency-limited, and is regulated by substrate availability and feedback inhibition from dietary DHA. Alternative lipid sources, including linseed oil (LO), soybean oil (SO), cottonseed oil (CO), black soldier fly oil (BSFO) enriched in DHA, and *Schizochytrium sp.* meal (SCM), can modulate this system to support growth and fatty acid homeostasis.

Innovations

This thesis provides new insights into the endogenous n-3 LC-PUFA biosynthesis phenotype and its nutritional regulation in largemouth bass, integrating molecular, physiological, and applied perspectives.

A multi-level analytical framework to uncover physiological constraints on endogenous biosynthesis of largemouth bass. To address the limitations of traditional single-level approaches, which are insufficient to fully resolve the relationship between enzymatic potential and actual metabolic flux, a multi-level analytical framework was established in this study. By integrating heterologous expression, hepatocyte assays, stable isotope tracing, and feeding trials, this approach enables a comprehensive evaluation of LC-PUFA biosynthesis from molecular to organismal levels.

Sustainable DHA production via substrate-driven insect bioconversion for aquaculture feed. A directed bioconversion system utilizing BSF larvae was established to produce DHA-enriched functional lipids. Feeding trials confirmed the high bioavailability and metabolic efficacy of this lipid source in largemouth bass, providing a practical pathway to reduce reliance on FO and support the development of alternative aquafeed strategies.

Future research priorities

This study has identified key desaturase genes, which catalyze the conversion of C18 precursors into long-chain products such as EPA and DHA. However, the actual contribution of these enzymes to tissue DHA levels appears limited. Even when precursors like ALA are available, gene expression does not necessarily translate into high metabolic flux. These findings highlight both progress and remaining gaps in our understanding of LC-PUFA metabolism in this species. Future research should examine the relationship between gene expression and actual metabolic flux. For example, the link between relative gene expression and actual n-3 LC-PUFA conversion should be quantified using isotopic tracing or metabolic flux analysis. Addressing these gaps will clarify how dietary inputs regulate LC-PUFA biosynthesis and help refine nutritional strategies at the molecular level.

The present study suggests that endogenous LC-PUFA biosynthesis in largemouth bass is condition-dependent and strongly influenced by the balance between precursor availability and direct LC-PUFA supply. Therefore, optimizing the dietary ALA:DHA ratio should be a key focus of future research. Systematic evaluation of different ALA and DHA combinations may help define species-specific nutritional requirements and further clarify the mechanisms regulating LC-PUFA deposition and metabolic partitioning. In addition, an “ALA-based plus LC-PUFA−complemented” blended oil strategy deserves further exploration. Integrating plant oils (e.g., LO or SO), microbial oils, and insect-derived lipids such as BSFO may enable partial replacement of fish oil while maintaining adequate n-3 LC-PUFA supply through both endogenous biosynthesis and direct dietary provision. Such a strategy aligns with the metabolic characteristics of largemouth bass and may contribute to reducing dependence on marine-derived resources and improving the sustainability of aquafeeds.

7

Chapter VII References and appendix

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Appendix

Publications

[1] **Liu Y**, Megido R C, Francis F, et al. Functional characterization of fatty acid desaturases and dietary lipid sources modulation of fatty acid conversion in largemouth bass (*Micropterus salmoides*)[J]. Aquaculture, 2026: 743641.

[2] **Liu Y**, Xue M, Francis F, et al. Growth, fatty acid composition, and fillet quality of largemouth bass (*Micropterus salmoides*) fed soybean oil and cottonseed oil diets[J]. Aquaculture Reports, 2025, 43: 102938.

[3] Wang J, Xie X, **Liu Y**, et al. Growth, antioxidant capacity, and liver health in largemouth bass (*Micropterus salmoides*) fed multi-strain yeast-based paraprobiotic: a lab-to-pilot scale evaluation[J]. Antioxidants, 2024, 13(7):792.

[4] Carpentier J, Abenaim L, Luttenschlager H, Dessauvages K, **Liu Y**, Samoah P, Francis F. & Caparros Megido R. Microorganism contribution to mass-reared edible insects: Opportunities and challenges[J]. Insects, 2024, 15(8): 611.