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P. Pasomboon, S. Kumkhong, Stéphane Panserat, S. Boonanuntanasarn. Linseed oil injection into the yolk reserve strongly modulates lipid gene expression and fatty acid composition in juvenile Nile tilapia. Animal, 2025, 19 (10), pp.101647. ⟨10.1016/j.animal.2025.101647⟩. ⟨hal-05284113⟩

HAL Id: hal-05284113

https://hal.science/hal-05284113v1

Submitted on 26 Sep 2025

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Animal 19 (2025) 101647

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Linseed oil injection into the yolk reserve strongly modulates lipid gene expression and fatty acid composition in juvenile Nile tilapia

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ARTICLE INFO

Article history:
Received 7 August 2024
Revised 25 August 2025
Accepted 29 August 2025
Available online 4 September 2025

Keywords:
Egg microinjection
Lipid catabolism
Long-chain polyunsaturated fatty acid
Nutritional programming
Oreochromis niloticus

ABSTRACT

Nutritional programming (**NP**) is an efficient tool for altering metabolic responses to specific nutrients. We aimed to investigate the effects of n-3 polyunsaturated fatty acid (**PUFA**)-based NP on lipid metabolic responses in Nile tilapia, an economically important freshwater fish species. To increase n-3 PUFA levels during early development, n-3 PUFA-based NP was conducted by injecting linseed oil into the yolk reserve, while the control group received a normal saline injection. Early injection of linseed oil altered the fatty acid profile of fish fry, suggesting the potential for n-3 PUFA stimulation in larvae. The long-term effects of the linseed oil injection were then assessed in juvenile fish. Early linseed oil injection improved feed efficiency throughout the juvenile stage ($P < 0.05$) and increased n-3 long chain-PUFA (LC-PUFA) levels in the liver and muscles ($P < 0.05$). Compared to fish injected with saline, those with a history of early linseed oil injection showed reduced hepatic fat content and an upregulation of the expression of several genes related to fatty acid oxidation in the liver (*cpt1cb, acox1, pparα,* and *acadl*) and muscles (*acox1* and *acadl*; $P < 0.05$). Early linseed oil injection also modulated the expression of genes related to lipogenesis, fatty acid desaturation and elongation, hepatic cholesterol synthesis, fatty acid transportation, as well as interactions between carbohydrate and lipid metabolism, and eicosanoid synthesis. Moreover, the long-term impact of NP was determined when fish were challenged with a linseed oil-rich diet. In the context of the challenging diet, growth performance appeared to be similar between NaCl-injected and linseed oil-injected fish. The linseed injection history reduced the hepatic and muscular protein content, suggesting protein catabolism. With a linseed oil-rich diet challenge, linseed oil injection history led to an increase in n-3 LC-PUFA levels in the liver and muscle, as well as fatty acids and their related metabolism. Taken together, early nutritional stimuli were achieved by injecting linseed oil into the yolk reserve in Nile tilapia larvae, resulting in long-term effects on fatty acid composition, fatty acid oxidation, and LC-PUFA synthesis.
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Implications

Early omega-3 polyunsaturated fatty acid-based nutritional programming modulates lipid metabolism later in life, leading to alterations in lipid utilisation and deposition in juvenile Nile tilapia. This study demonstrated that early linseed oil injection into the yolk reserve serves as a nutritional intervention to modulate lipid metabolism, including improvements in feed utilisation, fatty acid oxidation, and long-chain polyunsaturated fatty acid synthesis. Early linseed oil injection led to increased omega-3 polyunsaturated fatty acid levels in the liver and muscle, demonstrating its potential as a beneficial strategy for producing a freshwater source of these essential fatty acids.

Introduction

Growing demand for global food has led to increased aquaculture production rather than capture fisheries (FAO, 2022), contributing to a sustainable fishery product supply. Indeed, fish provide not only high-quality protein but also micronutrients and healthy omega-3 polyunsaturated fatty acids (**n-3 PUFA**) (WorldFish, 2020). Marine fish are a key source of n-3 long-chain PUFAs (**LC-PUFAs**), which serve as functional foods for human health and provide essential fatty acids (**EFAs**) for aquaculture

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https://doi.org/10.1016/j.animal.2025.101647
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and livestock feeds (Davidson, 2006; Ruyter et al., 2006; Lee et al., 2019). Due to advances in the intensification of aquaculture production, focused research is required to understand lipid metabolism and develop farmed freshwater fish as a viable alternative source of n-3 PUFA.

In fish, dietary lipids provide an energy source and EFAs. Hence, optimal dietary lipids, in terms of both fatty acid profiles and levels, enable the efficient use of proteins for growth and confer health benefits (Glencross, 2009). Generally, dietary lipids are the main factors affecting lipid metabolism, which reflects the fatty acid composition in fish (Ruyter et al., 2006; Zhu et al., 2022). Fish oil has traditionally been incorporated or supplemented as a lipid source to confer health benefits and promote n-3 LC-PUFA deposition (Tocher, 2015). However, the fish oil supply is limited and insufficient to support the rising demand of aquafeed industries. Other sources of n-3 PUFA, such as vegetable oils, particularly linseed oil, have been used to replace fish oil. Dietary fish and linseed oils influence fatty acid deposition in fish, resulting in increased n-3 LC-PUFA and n-3 PUFA, respectively (Ruyter et al., 2006; Turchini and Francis, 2009; Tocher, 2015; Yu et al., 2019; Li et al., 2021; Zhu et al., 2022). To promote a sustainable aquaculture sector for global nutritious lipid sources, more intensive investigations of the metabolism of linseed oil, a plant source of n-3 PUFA, are needed. This is because of the high levels of the precursor alpha-linolenic acid (C18:3n-3) required for n-3 LC-PUFA biosynthesis.

After the hydrolysis of dietary lipids and their absorption into hepatocytes, lipid metabolism includes mitochondrial and peroxisomal oxidation, fatty acid biosynthesis, and triglyceride-rich lipoprotein secretion (Yan et al., 2015), and the interaction between these processes contributes to fatty acid deposition in fish tissues. Recently, the concept of nutritional programming (**NP**) has been demonstrated to permanently modulate metabolic responses in vertebrates, including fish (Panserat et al., 2019). The concept of NP is used to describe how the experience of dietary nutrients or environmental factors during early life at a critical developmental stage can influence developmental function in animals and have long-term consequences on metabolism and physiology (Lucas, 1998). In particular, the effects of NP on dietary lipids have been demonstrated in marine fish, leading to alterations in lipid utilisation and deposition. For instance, in gilthead seabream (*Sparus aurata*), the replacement of fish oil with linseed oil in a broodstock diet influences fecundity and spawn quality, and the parental diet influences the fatty acid composition of progeny eggs (Izquierdo et al., 2015). In addition, in gilthead seabream and sea bass (*Dicentrarchus labrax*), early dietary lipid stimuli influenced the fatty acid composition of larvae. While early dietary lipids with different n-3 PUFA levels did not affect the survival rate of sea bass larvae, they significantly lowered the survival of gilthead seabream (Vagner et al., 2009; Turkmen et al., 2019b). Indeed, the effects of NP by lipids would vary between different stimulus methods and fish species.

Generally, EFAs vary according to fish species, particularly in marine and freshwater fish species. For comprehensive and comparable NP effects on lipids in fish, further investigation of NP in freshwater fish is required. Nile tilapia (*Oreochromis niloticus*) is an economically important freshwater fish, with its production expected to increase continuously. For tilapia farming, dietary linolenic acid at 0.45–0.64% dry weight and linoleic acid (18:2n-6) or arachidonic acid (C20:4n-6) at 0.5% are recommended (Takeuchi et al., 1983; Lim et al., 2011; Chen et al., 2013). It is recommended to supplement the diet of Nile tilapia with 10% fish oil for 60 days to contribute to the nutritional enrichment of the fillet product with eicosapentaenoic acid (C20:5n-3), docosahexaenoic acid (C22:6n-3), and n-3 docosapentaenoic acid (C22:5n-3) (Duarte et al., 2021). Indeed, the effects of NP on carbohydrates have been extensively demonstrated (Kumkhong et al., 2020a; Kumkhong et al., 2021). Hence, Nile tilapia is a good model for exploring NP. Microinjection is an effective technique for delivering a nutrient of interest during early development in fish, enabling yolk nutrient intervention to achieve nutritional stimulus during the larval stage (Kumkhong et al., 2020a; Rocha et al., 2014). In the present study, we aimed to analyse the long-term effects of linseed stimulation via injection into the yolk reserve during the early larval stage.

Material and methods

Experimental fish, alevin collection, and microinjection

Nile tilapia broodstock (0.8–1.2 kg) were reared in an earthen pond (800 m^2) at Suranaree University of Technology Farm, Suranaree University of Technology, Nakhon Ratchasima, Thailand. The Nile tilapia broodstock was fed to satiety with a commercial diet (30% CP, 4% CF) once daily. Fertilised eggs were collected from female Nile tilapia, and their developmental stages were classified according to the process outlined by Fujimura and Okada (2007). Eggs at stages 11–12 (six females; replications) were reared in a hatching tray (20 × 30 × 5 cm^3) with flow-through freshwater at 27–29 °C. Newly hatched larvae (3–10 h after hatching; stage 17) with normal appearances were selected for microinjection as described by Kumkhong et al. (2020a). Linseed oil (Holland & Barrett; United Kingdom), which is rich in C18:3n-3 (Table S1), was used for n-3 PUFA intervention in tilapia larvae. Sixty nanolitres of either normal saline (0.85% NaCl) or linseed oil were injected into the yolk sac for normal saline-injected control or linseed oil stimuli alevins (100 larvae/batch), respectively. Both uninjected larvae and microinjected larvae were reared in a hatching tray with gentle aeration for 1 week, and the survival rate was determined 1 week postinjection (**wpi**). Only larvae injected with NaCl and linseed oil were continued in culture through the juvenile stage for the experiment.

Experimental design, fish culture, and challenging diet

To investigate the long-term effects of linseed (n-3 PUFA) stimuli on lipid metabolism throughout the juvenile stage, the experimental design was completely randomised into two treatment groups: 1) NaCl-injected larvae and 2) linseed oil-injected larvae, with six replicates (family). A schematic of the experimental design is shown in Fig. 1. All experimental larvae with separate cages (replicate; 40 × 40 × 60 cm^3) were reared in cement ponds (2 × 2 × 0.8 m^3) with aeration through eight wpi. To exclude confounding effects related to sex, all-male fish were used. To exclude confounding effects related to sex, all male fish were used. To produce an all-male fish population, swim-up fry were fed a commercial powdered feed (40% CP, 8% CF) mixed with 17 α-methyltestosterone at 60 mg/kg. This feed was administered five times daily (09.00; 11.00; 13.00; 15.00; 17.00) during two to five wpi (Boonanuntanasarn et al., 2018). Subsequently, fish were cultured in cement ponds (2 × 2 × 0.8 m^3) and fed with commercial feed (32% CP, 4% CF) to satiety twice daily (09.00 and 16.00) through 24 wpi. To determine the survival rate, dead fish were removed and recorded.

Table 1 presents the approximate composition of the commercial diets used in this study. The ingredients and proximate composition of the linseed oil (n-3 PUFA)-rich diet used as a challenge diet are shown in Table 1. The diets were analysed for moisture, CP, CF, and ash content according to the standard method of the Association of Official Analytical Chemists (AOAC, 1990) and fatty acid content (Tables 1 and 2). During 25–28 wpi, the experimental fish were fed a linseed oil-rich diet. A total of 10 fish from each replicate (n = 10/replication; six replicates) were randomly

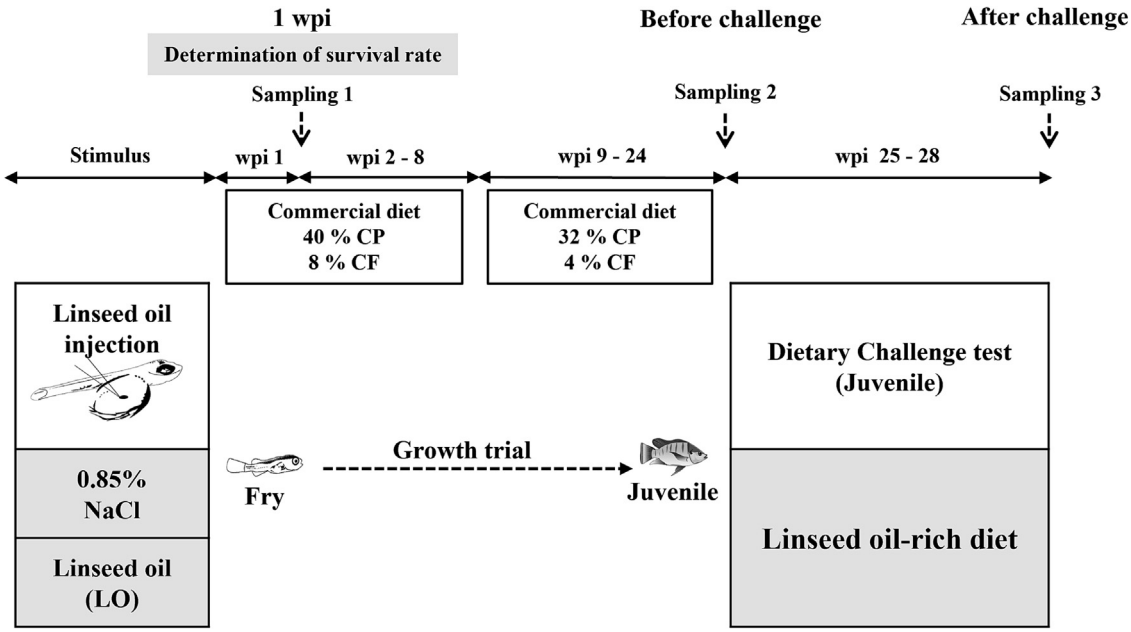

Fig. 1. Experimental design for linseed oil stimulus via injection (history) and subsequent dietary linseed oil-rich challenge. Nile tilapia alevins were injected with either saline (0.85% NaCl) or linseed oil into the yolk reserve at the newly hatched stage. Before exogenous feeding began, larvae were sampled at 1 week postinjection (1 wpi) to determine fatty acid composition. Subsequently, the experimental fish were reared for a growth trial from 2 to 24 wpi. During this period, commercial diets were administered: from 2 to 8 wpi (40% CP, 8% crude fat) and from 9 to 24 wpi (32% CP, 4% crude fat) to both NaCl-injected and linseed oil-injected groups. From 25 to 28 wpi, all fish were subjected to a dietary linseed oil-rich challenge (see Tables 1 and 2). Fish were sampled both prior to the challenge (24 wpi) and following the challenge (28 wpi). Survival rates were recorded at 1 and 28 wpi (after the challenge). Abbreviation: wpi, week postinjection.

Table 1
Ingredients and proximate composition of diets for experimental tilapia.

Ingredients (%)	Commercial 40% CP	Commercial 32% CP	Challenging diet (linseed oil-rich diet)
Fish meal			30.0
Soybean meal			26.0
Rice flour			30.0
Rice bran			12.0
Linseed oil			3.0
Fish premix[1]			1.0
Vitamin C			1.0
Proximate composition (% dry basis)			
Protein	48.6	36.9	33.0
Lipid	10.7	4.0	8.7
Fibre	3.5	6.1	4.2
Ash	15.6	10.9	11.5
NFE[2]	31.4	46.3	42.6
Gross energy (kJ g^{-1})	21.0	18.1	18.5

[1] Vitamin and trace mineral mix provided the following (IU kg−1 or g kg−1 diet): biotin, 0.25 g; folic acid, 0.003 g; inositol, 0.25 mg; niacin, 0.0215 g; pantothenic acid, 0.03 g; vitamin A, 5 000 IU; vitamin B1, 0.0025 g; vitamin B2, 0.0012 g; vitamin B6, 0.0075 g; vitamin B12, 0.00005 mg; vitamin C, 1 g; vitamin D3, 1 000 IU; vitamin E, 100 IU; vitamin K, 0.008 g; copper, 0.02 g; iron, 0.2 g; selenium, 0.3 mg; zinc, 0.32 g.
[2] Nitrogen-free extract = DM − (CP + crude lipid + crude fibre + ash).

selected, transferred to culture in a cage (90 × 80 × 110 cm^3), and fed a linseed oil-rich diet. The growth performance was also determined. Throughout the experimental period, air and water temperatures were determined daily and were 19.0–27.6 °C and 26.4–32.0 °C, respectively. Dissolved oxygen content and pH were measured once a week using a dissolved oxygen metre and a pH metre, and values were within acceptable ranges of 4.1–5.8 mg/L and 7.5–7.9, respectively.

Table 2
Fatty acid composition of commercial and challenge diets for experimental tilapia.

Fatty acids (mg/100 g lipid)	Commercial diet 32% CP	Challenging diet (linseed oil-rich diet)
C10:0	5.55	0.96
C12:0	112.20	4.48
C14:0	75.33	47.49
C14:1	0.75	0.48
C16:0	885.21	591.86
C16:1	64.67	69.42
C18:0	263.10	172.62
C18:1n-9 cis	790.12	604.66
C18:2n-6 trans	2.25	2.12
C18:2n-6 cis	520.75	495.44
C20:0	16.98	16.25
C18:3n-6	0.94	1.11
C20:1n-9	1.51	3.21
C18:3n-3	116.35	198.36
C18:4n-3	4.34	3.29
C20:2	8.41	5.31
C22:0	8.08	8.73
C20:3n-6	2.29	2.26
C22:1n-9	3.47	1.98
C20:3n-3	2.45	2.02
C20:4n-6	11.28	20.54
C20:5n-3	12.26	31.92
C24:0	7.94	16.23
C24:1	2.95	3.81
C22:6n-3	25.98	171.46
SFA	1 396.11	881.45
MUFA	863.47	683.55
PUFA	707.30	933.82
n-3-PUFA	161.38	407.05
n-6-PUFA	537.51	521.47
n-3/n-6-PUFA	0.30	0.78
n-6/n-3-PUFA	3.33	1.28

Abbreviations: SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.

Fish sampling, blood collection, and proximate composition

To determine the fatty acid composition profile in microinjected larvae before exogenous feeding began, this study sampled larvae at wpi 1. Pooled larvae (three pooled whole-body larvae/replicate tanks) were randomly selected at one wpi, immediately frozen in liquid nitrogen, and stored at −80 °C for fatty acid analysis. At wpi 24 (before the challenge) and wpi 28 (after the challenge), fish (five fish per tank) were sampled at 5 h after feeding. To determine plasma metabolites, after euthanasia, blood samples (2 fish per tank) were collected from the caudal vein using a hypodermic syringe and mixed with K_2EDTA (at 1.5 mg/mL) as an anticoagulant. The samples were stored on ice until plasma collection. Plasma was collected after centrifugation at 9 000 g for 10 min at 4 °C and stored at −80 °C for metabolite analysis. After blood sampling, the livers were collected and weighed to determine the hepatosomatic index. The liver sample (two fish per tank) was collected and immediately frozen with liquid nitrogen and maintained at −80 °C for RNA extraction. Subsequently, a portion of the liver and muscle (two fish per tank) was collected for the analysis of chemical composition (protein, fat, and ash) and fatty acids according to AOAC (1990). In addition, one fish (n = 6 fish per stimulus treatment) was randomly selected from each replication pond for the proximate composition of the whole body, according to AOAC (1990). At 28 wpi, after a challenge with a linseed-rich diet, fish were sampled as previously described (at 24 wpi).

Blood metabolite analysis

Plasma metabolites (two fish/replication; n = 12 per experimental group), including glucose, triglyceride, cholesterol, protein, blood urea nitrogen (**BUN**), serum glutamic oxaloacetic transaminase (**SGOT**), and serum glutamate-pyruvate transaminase (**SGPT**), were analysed.

Plasma glucose levels were evaluated using the GOD-PAP method with a Glucose kit (BLT00025, Erba Mannheim, Brno, Czech Republic) (Trinder, 1969). Triglyceride levels were analysed using the glycerol-3-phosphate oxidase–sodium N-ethyl-N-(3-sulfopro pyl)-m-anisidine (GPO-ESPAS) method (TG 250 kit, BLT00059, Erba Mannheim) as described by Bucolo and David (1973). Cholesterol levels were analysed using the cholesterol oxidase–phenol+amino phenazone (CHOD-PAP) method (CHOL 250 kit, BLT00036, Erba Mannheim) as described by Flegg (1973). Plasma protein levels were measured using the biuret method with the TP 250 kit (BLT00054, Erba Mannheim) (Gornall et al., 1949). Plasma BUN levels were determined using a modified indophenol colorimetric method with the UREA 250 kit (BLT00061, Erba Mannheim) (Weatherburn, 1967). SGOT and SGPT levels were evaluated using the colorimetric method with AST/GOT and ALT/GPT kits (BLT00050 and BLT00052, Erba Mannheim), respectively (Reitman and Frankel, 1957).

Fatty acid composition analysis

This study evaluated the fatty acid compositions of commercial and challenging linseed oil-rich diets. In addition, fatty acid deposition in the whole body of the larvae, liver, and muscle samples was determined as described by Tanomman et al. (2013). Briefly, samples were homogenised in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene as an antioxidant. For fatty acid methyl esterification, saponification of the extracted lipids (100 mg) was carried out with 0.5 N NaOH in 1.5 mL of methanol with heating for 7 min at 100 °C. Subsequently, esterification of the extracted lipids was performed using 14% of Boron trifluoride in methanol by heating for 5 min at 100 °C. Then, the levels of fatty acid methyl ester were quantitatively analysed using a gas chromatograph (GC2014; Shimadzu, Japan) equipped with a flame ionisation detector with a Restek Rtx®-Wax-fused silica capillary column (30 m × 0.25 mm internal diameter × 0.25 μm film thickness; Restek Corporation, Bellefonte, PA, USA), as described by Tanomman et al. (2013).

Total RNA extraction and real-time reverse transcription–polymerase chain reaction

Metabolic evaluation at the transcriptional level was demonstrated to correlate with fatty acid deposition, which was used to interpret lipid metabolism (Turkmen et al., 2019a; Tanomman et al., 2013). This study, therefore, determined lipid metabolism and its related processes at the molecular level, including fatty acid oxidation (carnitine O-palmitoyltransferase – *cpt1*; acyl-CoA oxidase 1 – *acox1*; peroxisome proliferator-activated receptor alpha –*ppar*α, and acyl-CoA dehydrogenase long chain –*acadl*), lipogenic (peroxisome proliferator-activated receptor gamma –*ppar*γ), fatty acid desaturation (fatty acid desaturase 2 – *fads2*), and fatty acid elongation (fatty acid elongase 6 – *elovl6* and fatty acid elongase 7 – *elovl7*). In addition, gene expression associated with the transport of fat (microsomal triglyceride transfer protein – *mttp*), cholesterol synthesis (cholesterol 25-hydroxylase – *ch25h*; 3-hydroxy-3-methylglutaryl-CoA synthase 1 – *hmgcs1*), the interaction between glucose and fatty acid metabolism (carbohydrate response element-binding protein – *chrebp*; acetyl CoA carboxylase alpha – *acaca*), and lipid oxidation (arachidonate 5-lipoxygenase – *alox5*) was examined. Relative gene expression was determined using quantitative real-time RT-PCR of RNA extracted from the liver and muscles (weeks 24 and 28; two fish/replication; n = 12 per experimental group). Total RNA was extracted from the liver (50 mg or 100 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. The quality was verified on 1% agarose gel, and the total RNA concentration was measured using NanoDrop (Thermo Fisher, Madison, WI, USA). Reverse transcription was performed in duplicates for each RNA sample used for cDNA synthesis (n = 12 per treatment group). Briefly, 1 μg of total RNA was used with 100 units of a SuperScript III RNAseH-Reverse transcriptase kit (Invitrogen) and 40 units of RNase OUT enzyme with random primers (Promega, Charbonnières, France).

Table S2 shows the primer sequences used in the real-time RT-PCR assays. The Roche Light Cycler 480 system (Roche Diagnostics, Neuilly-sur-Seine, France) was used for real-time RT-PCR of the transcripts. The assays were performed using a reaction mix of 6 μL per sample, each containing 2 μL of diluted cDNA template (1:40), 0.24 μL of each primer (10 μM), 3 μL of Light Cycler 480 SYBR®Green I Master mix (Roche Diagnostics), and 0.76 μL of DNAse/RNAse–free water (5 Prime GmbH, Hamburg, Germany) as described in Boonanuntanasarn et al. (2018). The PCR protocol was initiated at 95 °C for 10 min for initial denaturation of the cDNA and hot-start Taq polymerase activation, followed by 45 cycles of a three-step amplification programme (15 s at 95 °C; 10 s at 60–64 °C [according to the primer set used], 15 s at 72 °C to extend DNA). Melting curves were systematically monitored (temperature gradient at 1.1 °C/s from 65–97 °C; 5 acquisitions/°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included replicate samples (duplicates of RT and PCR amplifications) and negative controls (reverse transcriptase- and cDNA-template-free samples). For the analysis of mRNA levels, relative quantification of target gene expression was performed using the Roche Applied Science E-method (Pfaffl, 2001). The relative gene expression of *ef1*α was used for the normalisation of measured mRNA in each tissue, as its relative expression did not significantly change over the sampling process (data not shown). In all cases, PCR efficiency val-

ues were measured from the slope of a standard curve using serial dilutions of cDNA and ranged between 1.8 and 2.0.

Data analysis

Data analysis was performed using SPSS for Windows version 10 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was performed to determine the survival rate of larvae and the fatty acid profile of fry injected with linseed oil or NaCl (0.85% NaCl), and uninjected larvae. Tukey's test was used to rank the groups when significant differences were observed. An independent *t*-test was conducted to evaluate the differences between the two groups: NaCl versus linseed oil stimuli at 24 and 28 wpi (after a challenge with a linseed oil-rich diet). Throughout the experiment, the effects and differences were considered significant when *P*-values were less than 0.05 ($P < 0.05$).

Results

Effects of early linseed oil injection into the yolk reserve on survival rate and fatty acid composition in fry

Compared to uninjected larvae, the survival rates of NaCl-injected and linseed oil-injected larvae were lower ($P < 0.05$); however, there were no significant differences in fish larvae that were microinjected with NaCl and linseed oil (Fig. 2). This study continued to investigate the effects of early nutritional intervention via linseed oil injection. In addition, the survival rates of both injected fish appeared to be similar throughout the juvenile stage (week 28), which ranged from 95 to 99% (data not shown). At one wpi, the fry were sampled to determine their fatty acid profiles (Table 3). Our findings showed that the fatty acids of the control uninjected and NaCl-injected fry appeared to be similar. Upon comparing the fatty acid composition of both fry groups, we found that linseed oil-injected fry showed higher saturated fatty acid (**SFA**; C12:0, C14:0, C16:0, C22:0), monounsaturated fatty acid (**MUFA**; C16:1, C18:1n-9), PUFA (C20:2, C22:2), n-6 PUFA (C18:3n-6, C20:3n-6), and n-3 PUFA levels (C18:3n-3, C18:4n-3, C20:5n-3, C22:6n-3; $P < 0.05$). In particular, linseed oil-injected fry had a higher n-3/n-6 PUFA content ($P < 0.05$). These findings demonstrate that direct linseed oil injection into the preserved yolk altered the fatty acid profile of tilapia fry at one wpi, suggesting efficient n-3 PUFA stimulation during early larval development.

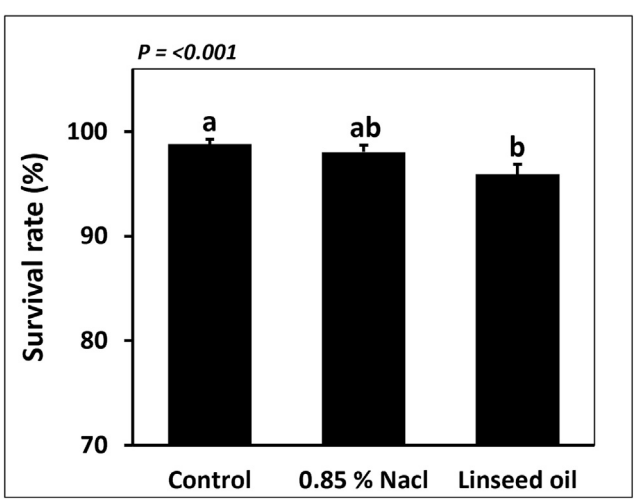

Fig. 2. The survival rate of the control group (uninjected larvae) and larvae injected with saline (0.85% NaCl) and linseed oil was assessed at 1 week postinjection (wpi; mean ± SD, n = 6). Compared to uninjected larvae (98.8 ± 0.5%), the survival rates of NaCl-injected and linseed oil-injected larvae were lower. However, no significant differences were observed between the survival rates of fish larvae microinjected with NaCl (98.0 ± 0.7%) and those microinjected with linseed oil (95.9 ± 1.0%). Different letters in the bar graph indicate significant differences ($P < 0.05$).

Effects of early linseed oil injection into the yolk reserve on growth performance throughout the juvenile stage before and after challenging with a linseed oil-rich diet

After microinjection, the NaCl- and linseed oil-injected fish were cultured and fed commercial diets. Our results showed that in the juvenile stage, despite the absence of significant differences in the final BW, average daily gain, or specific growth rate, fish injected with linseed oil showed a lower feed conversion ratio ($P < 0.05$; Table 4). In addition, upon a challenge with a linseed oil-rich diet, the growth performance of NaCl- and linseed oil-injected fish was similar ($P > 0.05$; Table 4).

Effects of early linseed oil injection into the yolk reserve on intermediary metabolism, fatty acid composition, and expression of genes related to lipid and its related metabolism in juvenile fish

Table 5 shows the hepatosomatic index and proximate composition of the liver, muscle, and whole body of juvenile fish. Among the biochemical composition parameters, only the hepatic lipid content was lower than that of the NaCl-injected fish ($P < 0.05$). Our results showed that at the juvenile stage, no significant differences were observed in plasma metabolites, including glucose, triglyceride, cholesterol, total protein, BUN, SGOT, and SGPT, between fish injected with NaCl and linseed oil before challenging with the linseed oil-rich diet (Table 6). The fatty acid compositions in the liver and muscles of the experimental juvenile fish before the challenge are shown in Tables 7 and 8, respectively. Our results showed that compared to NaCl-injected fish, early linseed oil injection led to increased SFA (C12:0, C16:0, C20:0, C24:0), MUFA (C14:1, C16:1, C22:1n9), n-6 PUFA (C18:2n-6, C20:4n-6), and n-3 PUFA (C18:3n-3, C18:4n-3, C20:3n-3, C20:5n-3, C22:6n-3) levels in the liver ($P < 0.05$; Table 7). In addition, increased levels of PUFAs, such as C20:3n-3, C20:5n-3, and C20:4n-6, were observed in the muscles of fish injected with linseed oil ($P < 0.05$; Table 8).

We investigated the effects of early linseed oil injection on the expression of genes related to lipids and their metabolic responses in the livers of juvenile fish (before challenge; Table 9). At the molecular level, compared to NaCl-injected fish, early linseed oil injection led to the upregulation of several genes related to fatty acid β-oxidation (*cpt1cb, acox1, pparα*, and *acadl*), fatty acid biosynthesis (*fads2, elovln6,* and *pparγ*), and fat transportation (*mttp*; $P < 0.05$). For cholesterol synthesis, compared with the control fish injected with NaCl, early linseed oil injection suppressed *ch25h* expression and induced *hmgcs1* expression ($P < 0.05$). In addition, for the metabolism of other lipids, upregulation of the expression of genes related to the interactions between carbohydrate and lipid metabolism (*chrebp* and *acaca*) and eicosanoid synthesis (*alox5*) was observed in fish injected with linseed oil ($P < 0.05$). Table 10 demonstrates that compared with fish injected with NaCl, upregulation of the expression of *acox1, acadl, pparγ, fads2,* and *acaca* was observed in the muscles ($P < 0.05$) of fish injected with early linseed oil. Fish injected with linseed oil had lower mRNA levels of *mttp*, *chrebp,* and *elovl7* than the control NaCl-injected fish ($P < 0.05$).

Effects of early linseed oil injection into the yolk reserve on intermediary metabolism, fatty acid composition, and expression of genes related to lipid and its metabolism in juvenile fish challenged with linseed oil-rich diet

When fish were challenged with a linseed oil-rich diet, the protein content in the liver and muscle of linseed oil-injected fish was

Table 3
Fatty acid composition of tilapia fry injected with saline (0.85% NaCl) or linseed oil at 1 week postinjection (n = 6).

Fatty acids (mg/100 g lipid)	Control[1]	0.85% NaCl History	Linseed oil History	RSE	*P*-value
C10:0	1.91	1.99	2.37	0.18	0.195
C12:0	0.92[b]	0.93[b]	1.16[a]	0.08	0.003
C14:0	2.43[b]	2.46[b]	5.63[a]	0.94	0.002
C14:1	1.55	1.55	2.13	0.25	0.115
C16:0	65.36[b]	65.99[b]	84.26[a]	5.25	0.002
C16:1	5.85[b]	5.79[b]	8.50[a]	0.81	0.001
C18:0	23.63	23.80	31.53	3.04	0.079
C18:1n-9 cis	44.84[b]	44.19[b]	53.77[a]	3.20	0.012
C18:2n-6 cis	26.02	25.61	28.51	1.23	0.085
C20:0	0.86	0.85	0.99	0.06	0.058
C18:3n-6	2.68[b]	2.66[b]	3.10[a]	0.15	0.006
C20:1n-9	2.30	2.32	3.32	0.30	0.001
C18:3n-3	27.74[b]	28.21[b]	95.78[a]	19.40	<0.001
C18:4n-3	16.98[b]	16.97[b]	28.55[a]	4.16	0.042
C20:2	58.10[b]	63.41[b]	90.33[a]	7.62	0.015
C22:0	8.50[b]	8.54[b]	10.30[a]	0.58	0.014
C20:3n-6	31.25[b]	31.90[b]	43.50[a]	3.48	0.009
C22:1n-9	29.85	29.65	30.88	0.79	0.366
C20:3n-3	23.53	24.17	26.59	1.33	0.220
C20:4n-6	2.04	2.16	2.30	0.06	0.091
C22:2	0.10[b]	0.10[b]	0.21[a]	0.03	0.001
C20:5n-3	3.99[b]	3.99[b]	6.34[a]	0.73	0.009
C24:0	0.17	0.16	0.21	0.05	0.461
C24:1	0.51	0.52	1.08	0.21	0.051
C22:6n-3	3.01[b]	3.05[b]	5.57[a]	0.72	<0.001
SFA	112.27[b]	113.39[b]	144.82[a]	8.98	0.001
MUFA	84.90[b]	84.02[b]	99.68[a]	5.00	0.004
PUFA	195.44[b]	202.22[b]	330.79[a]	35.41	<0.001
n-3-PUFA	75.24[b]	76.38[b]	162.84[a]	24.71	<0.001
n-6-PUFA	61.99[b]	62.33[b]	77.41[a]	4.58	0.007
n-3/n-6-PUFA	1.21[b]	1.23[b]	2.10[a]	0.27	<0.001
n-6/n-3-PUFA	0.82[a]	0.82[a]	0.48[b]	0.09	0.001

Abbreviations: RSE, residual standard error; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.
Different letters indicate significant differences in the mean values among experimental groups.
[1] Control = uninjected fry.

Table 4
Growth performance of juvenile tilapia injected with saline (0.85% NaCl) or linseed oil, fed a commercial diet prior to challenge (24 weeks postinjection; n = 6) and a linseed oil-rich diet after challenge (28 weeks postinjection; n = 6).

Item	0.85% NaCl history	Linseed oil history	RSE	*P*-value
Before challenge (24 wpi)				
Initial weight (g)	10.07 mg	10.42 mg	0.19	0.703
Final weight (g)	97.88	101.45	6.70	0.386
Weight gain (g)[1]	97.45	101.02	6.70	0.386
Feed intake (g day^{-1})	0.99[a]	0.84[b]	0.02	<0.001
ADG (g day^{-1})[2]	0.62	0.64	0.04	0.340
SGR (% day^{-1})[3]	3.46	3.48	0.05	0.484
FCR[4]	1.59[a]	1.32[b]	0.08	<0.001
After challenge (2 wpi)				
Initial weight (g)	101.20	101.20	0.85	0.942
Final weight (g)	160.85	163.40	5.30	0.421
Weight gain (g)[1]	59.65	62.20	5.68	0.421
Feed intake (g day^{-1})	2.52	2.51	0.06	0.887
ADG (g day^{-1})[2]	1.99	2.10	0.19	0.444
SGR (% day^{-1})[3]	1.54	1.60	0.12	0.509
FCR[4]	1.27	1.20	0.11	0.580

Abbreviations: RSE, residual standard error; wpi, week postinjection.
Different letters indicate significant differences in the mean values among experimental groups.
[1] Weight gain = final BW–initial BW.
[2] Average daily gain (ADG) = (final BW–initial BW) / experimental days.
[3] Specific growth rate (SGR) = 100 × [(ln final BW–ln initial BW) / experimental days].
[4] Feed conversion ratio (FCR) = dry feed fed / wet weight gain.

lower than that in NaCl-injected fish ($P < 0.05$; Table 5). No significant differences were observed in fat content, ash content, or hepatosomatic index between the NaCl-injected and linseed oil-injected fish (Table 5). Additionally, plasma metabolites showed no significant differences between the experimental groups (Table 6). The effects of early linseed oil injection during the larval stage on hepatic fatty acid levels were evident when the fish were challenged with a linseed oil-rich diet (Table 7). Compared to fish

Table 5
Proximate composition of the liver, muscle, and whole body of juvenile tilapia injected with saline (0.85% NaCl) or linseed oil, fed a commercial diet prior to challenge (24 weeks postinjection; n = 6) and a linseed oil-rich diet after challenge (28 weeks postinjection; n = 6).

Item	Before challenge (24 wpi)				After challenge (28 wpi)			
	0.85% NaCl history	Linseed oil history	RSE	*P*-value	0.85% NaCl history	Linseed oil history	RSE	*P*-value
Liver								
Protein	101.36	105.33	4.49	0.157	98.20[a]	90.14[b]	2.31	<0.001
Fat	28.70[a]	24.36[b]	1.56	0.001	41.80	41.41	0.50	0.203
Ash	10.54	10.26	0.29	0.123	11.88	12.03	0.18	0.199
HSI	1.79	2.03	0.34	0.209	2.73	3.34	0.58	0.090
Muscle								
Protein	184.84	180.68	6.40	0.287	205.52[a]	196.91[b]	4.98	0.013
Fat	12.44	12.33	0.43	0.666	24.17	24.57	0.67	0.255
Ash	11.17	10.83	0.51	0.173	12.16	12.26	0.09	0.537
Whole body								
Protein	148.28	148.79	4.80	0.857	153.98	155.18	1.11	0.093
Fat	18.61	18.09	1.16	0.453	44.43	44.47	0.88	0.946
Ash	21.63	21.21	1.36	0.599	42.22	42.21	0.40	0.947

Abbreviations: wpi, week postinjection; RSE, residual standard error; HSI, hepatosomatic index.
Different letters indicate significant differences in the mean values among experimental groups.

Table 6
Plasma metabolites of juvenile tilapia injected with saline (0.85% NaCl) or linseed oil, fed a commercial diet prior to challenge (24 weeks postinjection; n = 6) and a linseed oil-rich diet after challenge (28 weeks postinjection; n = 6).

Item	Before challenge (24 wpi)				After challenge (28 wpi)			
	0.85% NaCl history	Linseed oil history	RSE	*P*-value	0.85% NaCl history	Linseed oil history	RSE	*P*-value
Glucose (mM)	4.17	4.20	0.47	0.910	5.24	5.30	0.80	0.907
Triglyceride (mM)	1.63	1.35	0.45	0.915	1.77	1.95	0.63	0.682
Cholesterol (mM)	1.20	1.20	0.16	0.315	1.20	1.24	0.18	0.630
Total protein (mg/L)	31.19	32.00	2.13	0.527	32.83	30.66	3.08	0.249
BUN (mM)	1.00	0.94	0.15	0.559	0.93	0.87	0.08	0.272
SGOT (U/L)	61.26	58.42	4.20	0.836	68.43	63.25	5.49	0.681
SGPT (U/L)	33.67	33.93	3.21	0.890	37.26	39.06	8.63	0.725

Abbreviations: wpi, week postinjection; RSE, residual standard error; BUN, blood urea nitrogen; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamate-pyruvate transaminase.

injected with NaCl, early linseed oil injection led to an increase in the levels of several fatty acids, including SFA (C10:0, C12:0, C16:0, C20:0, C24:0), MUFA (C14:1, C16:1, C18:1n9, C22:1n9, C24:1), PUFA (C20:2), n-6 PUFA (C18:2n-6, C18:3n-6, C20:4n-6), and n-3 PUFA (C18:3n-3, C18:4n-3, C20:3n-3, C20:5n-3, C22:6n-3) in the liver ($P < 0.05$; Table 7). Table 8 shows that, although not all, several fatty acids elevated in the liver also showed an increase in the muscles, including SFA (C12:0, C14:0, C22:0), MUFA (C16:1, C20:1n9), n-6 PUFA (C20:4n-6), and n-3 PUFA (C20:3n-3, C20:5n-3; $P < 0.05$).

When fish were challenged with a linseed oil-rich diet, metabolic responses similar to those observed before the challenge were recorded. Compared with NaCl-injected fish, early linseed oil injection upregulated the expression of several hepatic genes related to fatty acid β-oxidation (*cpt1cb, acox1,* and *pparα*), fatty acid biosynthesis (*fads2, elovln6,* and *pparγ*), fat transportation (*mttp*), interaction between carbohydrate and lipid metabolism (*chrebp* and *acaca*), and eicosanoid synthesis (*alox5*; $P < 0.05$; Table 9). Additionally, in the muscles, upregulation of *cpt1cb, acox1, acadl, pparγ,* and *fads2* ($P < 0.05$) was observed in fish that were injected with linseed oil early. However, downregulation of the expression of *mttp* and *elovl7* was also observed ($P < 0.05$; Table 10).

Discussion

Previous studies on marine fish, such as gilthead seabream and seabass, revealed that NP effects were achieved through broodstock and early larval feeds with fish oil replaced by linseed oil (Vagner et al., 2009; Izquierdo et al., 2015; Turkmen et al., 2017; Xu et al., 2021). Epigenetic modifications, such as DNA methylation within the *fads* cluster, have been shown to influence LC-PUFA contents in mammals. In fish, however, it remains unclear whether epigenetic modifications are associated with the long-term NP effects of dietary lipid variations in gilthead sea bream broodstock (Xu et al., 2021; Rahbar et al., 2018). While not a practical method for nutritional stimulation in tilapia farming, manipulating yolk n-3 PUFA intake through microinjection provides insights into the underlying metabolic responses to NP effects of n-3 PUFA overload during early development. In this study, we analysed, for the first time, the effects of a nutritional stimulus by directly injecting linseed oil, a source of n-3 PUFA, into the yolk reserve of Nile tilapia, a freshwater fish. Our results showed that although the microinjection method lowered the overall survival rate, no significant differences were observed between NaCl-injected and linseed oil-injected larvae, suggesting that linseed oil delivered to the yolk reserve did not exert detrimental effects on larval development. This approach made it possible to study the long-term effects of linseed oil injection history on growth performance and lipid metabolism in juveniles, as well as their NP impacts when fed a linseed oil-rich challenge diet.

Injection of linseed oil into the yolk reserve did not impact growth performance in juvenile fish

Our results showed that, although the injection of linseed oil into the yolk reserves of larvae did not influence growth perfor-

Table 7
Fatty acid composition of the liver of juvenile tilapia injected with saline (0.85% NaCl) or linseed oil, fed a commercial diet prior to challenge (24 weeks postinjection; n = 6) and a linseed oil-rich diet after challenge (28 weeks postinjection; n = 6).

Item	Before challenge (24 wpi)				After challenge (28 wpi)			
	0.85% NaCl history	Linseed oil history	RSE	*P*-value	0.85% NaCl history	Linseed oil history	RSE	*P*-value
C10:0	0.41	0.43	0.03	0.169	0.56[b]	0.65 [a]	0.05	0.007
C12:0	0.45[b]	0.52 [a]	0.03	<0.001	0.63[b]	0.79 [a]	0.04	<0.001
C14:0	13.73	14.17	0.70	0.299	15.40	16.69	1.95	0.277
C14:1	0.34[b]	0.41 [a]	0.00	<0.001	0.41[b]	0.49 [a]	0.05	0.026
C16:0	73.94[b]	87.18 [a]	2.93	<0.001	106.10[b]	118.80 [a]	5.45	0.002
C16:1	14.43[b]	16.78 [a]	1.19	0.007	19.97[b]	24.18 [a]	2.05	0.005
C18:0	40.60	42.45	1.98	0.137	43.14	44.67	2.08	0.231
C18:1n-9 cis	112.54	116.97	6.83	0.287	123.37[b]	142.32 [a]	3.68	<0.001
C18:2n-6 cis	23.80[b]	25.45 [a]	0.66	0.001	28.64[b]	35.32 [a]	1.82	<0.001
C20:0	0.61[b]	0.97 [a]	0.03	<0.001	1.03[b]	1.23 [a]	0.07	<0.001
C18:3n-6	1.46	1.64	0.16	0.093	1.76[b]	1.97 [a]	0.07	<0.001
C20:1n-9	1.72	1.74	0.34	0.940	1.95	1.97	0.22	0.856
C18:3n-3	7.02[b]	9.64 [a]	0.51	<0.001	8.15[b]	10.74 [a]	0.19	<0.001
C18:4n-3	0.59[b]	0.81 [a]	0.00	<0.001	0.70[b]	1.16 [a]	0.31	0.028
C20:2	1.18	1.27	0.23	0.514	1.21[b]	1.40 [a]	0.04	<0.001
C22:0	0.53	0.57	0.24	0.822	0.85	0.92	0.22	0.617
C20:3n-6	2.31	2.79	0.35	0.059	2.74	3.12	0.29	0.050
C22:1n-9	0.13[b]	0.16 [a]	0.00	0.011	0.22[b]	0.40 [a]	0.07	0.001
C20:3n-3	0.23[b]	0.39 [a]	0.00	<0.001	0.30[b]	0.48 [a]	0.03	<0.001
C20:4n-6	6.02[b]	7.94 [a]	1.03	0.009	7.32[b]	10.44 [a]	0.69	<0.001
C22:2	0.26	0.28	0.00	0.181	0.38	0.40	0.14	0.774
C20:5n-3	2.71[b]	3.27 [a]	0.22	0.001	2.99[b]	5.39 [a]	0.32	<0.001
C24:0	0.24[b]	0.33 [a]	0.03	<0.001	0.34[b]	0.57 [a]	0.14	0.034
C24:1	2.09	2.27	0.15	0.076	3.69[b]	4.53 [a]	0.44	0.018
C22:6n-3	15.44[b]	19.64 [a]	1.78	0.002	16.44[b]	21.14 [a]	0.77	<0.001
SFA	130.52[b]	146.62 [a]	4.86	<0.001	168.04[b]	184.32 [a]	7.40	0.003
MUFA	131.26	138.32	7.77	0.146	149.61[b]	173.88 [a]	5.62	<0.001
PUFA	61.00[b]	73.11 [a]	3.74	0.001	70.63[b]	91.56 [a]	3.45	<0.001
n-3-PUFA	25.98[b]	33.75 [a]	2.20	0.001	28.58[b]	38.91 [a]	0.89	<0.001
n-6-PUFA	33.59[b]	37.82 [a]	1.64	0.001	40.46[b]	50.85 [a]	2.69	<0.001
n-3/n-6-PUFA	0.77[b]	0.89 [a]	0.04	0.006	0.71[b]	0.77 [a]	0.03	0.025
n-6/n-3-PUFA	1.30 [a]	1.12[b]	0.08	0.008	1.41 [a]	1.31[b]	0.07	0.025

Abbreviations: wpi, week postinjection; RSE, residual standard error; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids. Different letters indicate significant differences in the mean values among experimental groups.

mance, it lowered the FCR during the juvenile stage when fish were fed a commercial diet, suggesting that early n-3 PUFA stimulation may enhance dietary utilisation efficiency. Indeed, the protein-sparing effect of lipids was observed in Nile tilapia fingerlings, leading to a reduced FCR (Thirunavukkarasar et al., 2022). However, subsequent challenges with a linseed oil-rich diet for 4 weeks might not have been sufficient to observe significant differences in FCR. Early nutritional stimuli led to improved growth performance in juvenile gilthead seabream that was fed a diet containing low levels of fish oil and fish meal (Izquierdo et al., 2015). In addition, the partial replacement of fish oil with linseed oil in a broodstock diet resulted in improved growth and feed utilisation in seabream offspring up to the juvenile stage upon administering a low fishmeal/fish oil diet (Turkmen et al., 2017). Hence, although comparisons must be made with caution, our findings suggest that the different effects of early NP in marine fish and Nile tilapia might be attributed to the well-known differences in EFA requirements between fish species (Vagner et al., 2009; Turkmen et al., 2017). Nile tilapia exhibits high adaptability to utilise various dietary lipids for growth. At the optimal temperature, no significant differences were observed in the growth performance of juvenile Nile tilapia fed different sources of dietary fatty acids, such as fish, linseed, sunflower, olive, or coconut oils (Corrêa et al., 2017). The growth performance of adult Nile tilapia fed a diet containing palm oil, soybean oil, or linseed oil for 90 days appeared to be similar (Boonanuntanasarn et al., 2019). Indeed, although EFA requirements of tilapia species are not clear (Glencross, 2009), it was recommended that dietary n-3 PUFA and n-6 PUFA requirements of Nile tilapia are 0.45–0.64% dry weight of linolenic acid and 0.5% dry weight of C18:2n-6 or C20:4n-6 (Takeuchi et al., 1983; Lim et al., 2011; Chen et al., 2013). Taken together, the effects of early lipid stimulation on growth performance may vary depending on fish species and their EFA requirements.

Injection of linseed oil into the yolk reserve modified lipid metabolism in juvenile fish fed a commercial diet

Impact of the injection of linseed oil into the yolk on lipid metabolism in the liver

This study investigated the long-term effects of early linseed oil stimulation on intermediary metabolism in juveniles. The liver is the centre of intermediary metabolism; therefore, its chemical composition is influenced by nutrients absorbed from the digestive tract, nutrient catabolism, metabolite biosynthesis, and export through other organs (Roques et al., 2020). Although no significant differences were observed in hepatosomatic index among proximate compositions in the liver, the impact of linseed oil injection into the yolk reserve had a long-term impact associated with reduced hepatic lipid content compared to NaCl-injected fish. The reduction in hepatic content might be explained by lipid catabolism (fatty acid β-oxidation), which is influenced by early experience with n-3 LC-PUFA-rich linseed oil overload. Although it does not always reflect metabolic responses, metabolic evaluation at the transcriptional level has been conducted to interpret metabolic pathways. Indeed, our results showed that early linseed oil stimulus was associated with increased expression of genes related to fatty acid catabolism, including *cpt1cb, acox1, pparα,* and *acadl*. Hence, early linseed oil stimulus induced fatty acid oxidation in

Table 8
Fatty acid composition of the muscle of juvenile tilapia injected with saline (0.85% NaCl) or linseed oil, fed a commercial diet prior to challenge (24 weeks postinjection; n = 6) and a linseed oil-rich diet after challenge (28 weeks postinjection; n = 6).

Item	Before challenge (24 wpi)				After challenge (28 wpi)			
	0.85% NaCl history	Linseed oil history	RSE	*P*-value	0.85% NaCl history	Linseed oil history	RSE	*P*-value
C10:0	0.26	0.25	0.05	0.748	0.25	0.29	0.03	0.070
C12:0	0.12	0.14	0.00	0.123	0.12[b]	0.16 [a]	0.00	0.011
C14:0	1.74	1.79	0.25	0.742	1.72[b]	3.45 [a]	0.67	0.001
C14:1	0.25	0.25	0.03	0.933	0.23	0.33	0.10	0.121
C16:0	18.90	19.33	2.30	0.754	18.54	23.91	4.46	0.064
C16:1	2.54	2.61	0.35	0.732	2.27[b]	3.37 [a]	0.68	0.019
C18:0	8.62	8.51	0.73	0.806	10.91	11.18	2.99	0.878
C18:1n-9 cis	19.63	20.28	2.48	0.663	19.64	25.33	4.97	0.076
C18:2n-6 cis	8.63	8.54	0.83	0.844	9.06	11.22	2.18	0.117
C20:0	0.26	0.29	0.03	0.358	0.26	0.33	0.08	0.172
C18:3n-6	0.32	0.30	0.03	0.232	0.45	0.45	0.13	0.933
C20:1n-9	1.15	1.15	0.15	0.985	1.13[b]	1.64 [a]	0.30	0.015
C18:3n-3	0.57	0.57	0.04	0.905	0.54	0.74	0.18	0.079
C18:4n-3	0.23	0.23	0.08	0.973	0.20	0.28	0.11	0.238
C20:2	0.61	0.67	0.18	0.552	0.63	0.78	0.24	0.291
C22:0	0.19	0.18	0.03	0.562	0.15[b]	0.23 [a]	0.03	0.007
C20:3n-6	0.80	0.93	0.11	0.091	0.85	1.01	0.21	0.221
C22:1n-9	0.12	0.11	0.05	0.622	0.17	0.18	0.03	0.716
C20:3n-3	0.14[b]	0.19 [a]	0.03	0.002	0.19[b]	0.29 [a]	0.04	0.002
C20:4n-6	2.46[b]	2.98 [a]	0.35	0.026	2.76[b]	3.75 [a]	0.59	0.026
C22:2	0.06	0.04	0.04	0.602	0.18	0.31	0.15	0.162
C20:5n-3	0.47[b]	0.53 [a]	0.04	0.026	0.46[b]	0.83 [a]	0.09	<0.001
C24:0	0.09	0.10	0.03	0.400	0.13	0.15	0.03	0.172
C24:1	0.14	0.11	0.07	0.426	0.16	0.16	0.09	0.927
C22:6n-3	3.62	4.24	0.67	0.147	4.47	5.31	0.87	0.151
SFA	30.17	30.58	3.31	0.835	32.08	39.70	2.64	0.121
MUFA	23.84	24.51	3.11	0.716	23.60	31.00	5.98	0.058
PUFA	17.90	19.21	1.60	0.185	19.79[b]	24.97 [a]	3.85	0.042
n-3-PUFA	5.02	5.75	0.69	0.096	5.86[b]	7.45 [a]	1.08	0.030
n-6-PUFA	12.21	12.75	1.08	0.412	13.12	16.43	2.91	0.077
n-3/n-6-PUFA	0.41	0.45	0.05	0.204	0.45	0.46	0.05	0.558
n-6/n-3-PUFA	2.44	2.26	0.25	0.256	2.25	2.21	0.29	0.831

Abbreviations: wpi, week postinjection; RSE, residual standard error; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.
Different letters indicate significant differences in the mean values among experimental groups.

Table 9
mRNA levels of genes involved in lipid metabolism in the liver of juvenile tilapia injected with saline (0.85% NaCl) or linseed oil, fed a commercial diet prior to challenge (24 weeks postinjection; n = 6) and a linseed oil-rich diet after challenge (28 weeks postinjection; n = 6). Gene descriptions are provided in Table S2.

Item	Before challenge (24 wpi)				After challenge (28 wpi)			
	0.85% NaCl History	Linseed oil history	RSE	*P*-value	0.85% NaCl history	Linseed oil history	RSE	*P*-value
Fatty acid β-oxidation								
cpt1cb	0.45[b]	1.34[a]	0.06	<0.001	0.56[b]	1.43[a]	0.07	<0.001
acox1	0.51[b]	2.00[a]	0.22	<0.001	0.40[b]	1.60[a]	0.04	<0.001
pparα	0.15[b]	0.30[a]	0.08	<0.001	0.39[b]	0.85[a]	0.00	<0.001
acadl	0.45[b]	1.34[a]	0.05	<0.001	1.06	1.00	0.07	0.339
Fatty acid biosynthesis								
pparγ	0.34[b]	1.70[a]	0.09	<0.001	0.31[b]	1.57[a]	0.09	<0.001
fads2	0.22[b]	1.85[a]	0.04	<0.001	0.21[b]	1.87[a]	0.04	<0.001
elovl6	0.23[b]	1.95[a]	0.03	<0.001	0.24[b]	1.83[a]	0.07	<0.001
elovl7	0.99	1.02	0.29	0.861	1.18	0.87	0.30	0.100
Fat transportation								
mttp	0.86[b]	1.17[a]	0.05	<0.001	0.96[b]	1.23[a]	0.05	<0.001
Cholesterol metabolism								
ch25h	0.84[a]	0.36[b]	0.22	0.004	1.28	0.81	0.38	0.056
hmgcs1	0.70[b]	1.06[a]	0.18	0.005	0.83	0.96	0.22	0.314
Interaction between carbohydrate and lipid metabolism								
chrebp	0.56[b]	1.21[a]	0.00	<0.001	0.50[b]	1.03[a]	0.03	<0.001
acaca	0.26[b]	2.22[a]	0.12	<0.001	0.27[b]	1.76[a]	0.08	<0.001
Eicosanoid synthesis								
alox5	0.54[b]	1.37[a]	0.16	<0.001	0.65[b]	1.42[a]	0.18	<0.001

Abbreviations: wpi, week postinjection; RSE, residual standard error.
Different letters indicate significant differences in the mean values among experimental groups.

Table 10
mRNA levels of genes involved in lipid metabolism in the muscle of juvenile tilapia injected with saline (0.85% NaCl) or linseed oil, fed a commercial diet prior to challenge (24 weeks postinjection; n = 6) and a linseed oil-rich diet after challenge (28 weeks postinjection; n = 6). Gene descriptions are provided in Table S2.

Item	Before challenge (24 wpi)				After challenge (28 wpi)			
	0.85% NaCl history	Linseed oil history	RSE	*P*-value	0.85% NaCl history	Linseed oil history	RSE	*P*-value
Fatty acid β-oxidation								
cpt1cb	0.74	0.78	0.05	0.249	0.86[b]	0.97[a]	0.06	0.012
acox1	0.94[b]	1.10[a]	0.08	0.018	0.81[b]	1.04[a]	0.03	<0.001
pparα	0.25	0.24	0.05	0.536	0.89	1.00	0.05	0.095
acadl	0.72[b]	0.81[a]	0.05	0.027	0.72[b]	0.81[a]	0.08	0.027
Fatty acid biosynthesis								
pparγ	0.76[b]	0.93[a]	0.09	0.017	0.74[b]	0.97[a]	0.08	0.001
fads2	0.62[b]	0.98[a]	0.05	<0.001	0.63[b]	1.11[a]	0.06	<0.001
elovl6	0.88	0.74	0.13	0.050	0.93	0.92	0.06	0.183
elovl7	1.12[a]	0.78[b]	0.04	<0.001	1.27[a]	0.82[b]	0.08	<0.001
Fat transportation								
mttp	0.81[a]	0.70[b]	0.08	0.022	1.12[a]	0.98[b]	0.11	0.046
Cholesterol metabolism								
ch25h	0.61	0.57	0.08	0.378	0.55	0.51	0.10	0.524
hmgcs1	0.81	0.82	0.06	0.854	0.86	0.91	0.05	0.126
Interaction between carbohydrate and lipid metabolism								
chrebp	0.81[a]	0.70[b]	0.11	<0.017	1.12	1.05	0.09	0.216
acaca	0.90[b]	1.20[a]	0.09	<0.001	0.82	0.91	0.08	0.059
Eicosanoid synthesis								
alox5	0.87	0.87	0.05	0.979	0.60	0.66	0.18	0.562

Abbreviations: wpi, week postinjection; RSE, residual standard error.
Different letters indicate significant differences in the mean values among experimental groups.

both the mitochondria and peroxisome, at least at a molecular level, demonstrating the existence of NP in relation to variation of yolk fatty acid composition. However, the effects of NP of lipids on fatty acid β-oxidation in marine fish vary. Nutritional intervention with PUFA, including early feeding and parental feeding, downregulated genes related to β-oxidation in seabass and sea bream (Vagner et al., 2009; Turkmen et al., 2017; Xu et al., 2021). Even though we cannot discard the possibility that NP linked to first feeding and parental feeding is different from NP linked to egg injection, differences in the impact of NP on lipids in marine fish such as sea bass, sea bream, and tilapia—a freshwater fish—could also be due to the requirement for EFA. Taken together, these findings demonstrate that the modulation of lipid metabolism in juvenile is linked to early lipid stimulus history.

Nutritional intervention with PUFA influenced fatty acid biosynthesis pathways. In this study, early linseed oil overload in the yolk reserve resulted in modulation of the fatty acid deposition profile, particularly for high-molecular-weight SFA (C16:0, C18:0, C20:0, C24:0), MUFA (C14:1, C16:1, C22:1n9), n-6 PUFA (C18:2n-6, C20:4n-6), and n-3 PUFA (C18:4n-3, C20:3n-3, C20:5n-3, C22:6n-3), which may be related to fatty acid biosynthesis enzymes in the liver. *fads2* in Nile tilapia expresses delta 6 and 5 desaturases in the liver, which are the first limiting enzymes for n-3 LC-PUFA biosynthesis (Tanomman et al., 2013). SFA and MUFA are preferred substrates for ELOVL6 (Monroig et al., 2010). In addition, *pparγ* regulates de novo fatty acid synthesis, particularly that of MUFA (Shi et al., 2013; Xiong et al., 2022). Our results showed that early linseed stimulus induced the expression of *fads2, elovl6,* and *pparγ* as it was previously observed in gilthead seabream juveniles after first feeding or broodstock feeding with linseed oil (Vagner et al., 2009; Izquierdo et al., 2015; Turkmen et al., 2017; Xu et al., 2021). These findings suggest that n-3 PUFA injection was associated with the induction of LC-PUFA as well as LC-SFA and LC-MUFA biosynthesis in the liver. Overall, one more time, NP through lipids is clearly demonstrated in our study as previously shown in different fish species using other nutrients such as carbohydrates, minerals and vitamins (Panserat et al., 2019; Hou and Fuiman, 2020).

Impact of linseed oil injection into yolk on lipid metabolism in the muscles

An increase in fatty acid accumulation in muscles, such as C20:3n-3, C20:4n-6, and C20:5n-3, might be accompanied by the transportation of these fatty acids from the liver and/or LC-PUFA biosynthesis processes in the muscles. These findings suggest that linseed oil injection into the yolk may be associated with the modification of lipid metabolism in the muscles at later stages of life, although the extent may be lower than that observed in the liver. Indeed, at the molecular level, several genes related to lipid metabolism were modulated. For instance, the downregulation of *elovl7* was observed, which was responsible for the elongation of C16-C22 fatty acids, irrespective of the number of double bonds (Naganuma et al., 2011). Nevertheless, C16-C22 saturated fatty acids appeared to remain unchanged according to nutritional intervention history. Indeed, compared to NaCl-injected fish, the transportation of fatty acids might be related to the upregulation of hepatic *mttp* and the downregulation of muscular *mttp* in juvenile fish with early linseed oil injection. In addition, LC-PUFA biosynthesis in the muscles may be linked to the upregulation of muscular *fads2* in juvenile fish following early linseed oil injection. These findings support the hypothesis that the application of NP via linseed oil injection could enable increased LC-PUFA deposition in the carcass, potentially through pathways related to fatty acid biosynthesis and transport. The present data can be very important in the future for aquaculture demonstrating the existence of a new strategy to improve the flesh quality of fish for human food in terms of fatty acid composition (Glencross, 2009).

Impact of linseed oil injection into yolk on other metabolic pathways

In this study, we evaluated whether early linseed oil injection had long-term effects on lipid-related metabolic processes, such as carbohydrate metabolism and eicosanoid synthesis.

Carbohydrate-responsive element-binding protein (*chrebp*; also known as *mlxipl*) is a glucose-dependent inducer of the transcription of glycolytic and lipogenic genes, such as pyruvate kinase (*pk*), fatty acid synthase (*fasn*), and *acaca*. Its expression is regulated by PUFA (Ishii et al., 2004; Dentin et al., 2006). Acetyl CoA carboxylase (*acaca*) catalyses malonyl-CoA synthesis from acetyl-CoA, which is a substrate for the production of LC-fatty acids (Brownsey et al., 2006). Our results showed that early linseed stimulus led to variation in the expression of *chrebp* (upregulated in the liver but downregulated in the muscle) and *acaca* (upregulated in both the liver and muscle), which are involved in glucose metabolism linked to lipogenesis or the regulated balance between glycogen and triglyceride storage. Hence, at the molecular level, early linseed oil injection stimulus impacts the interaction of glucose and lipid metabolism in both the liver and muscles.

These findings revealed that NP with n-3 PUFA also impacts other metabolic processes linked to n-3 PUFA. In addition, arachidonate 5-lipoxygenase (*alox5*) converts C20:4n-6 into leukotrienes, which are lipid mediators associated with inflammation (Rådmark et al., 2015). Dietary LC-PUFA affects *alox5* expression in a dose-dependent manner (Wang et al., 2016; Araújo et al., 2022). Our findings showed the upregulation of hepatic *alox5* expression in linseed oil-injected juveniles, suggesting that an early history of n-3 PUFA overload in larvae would have long-term effects on eicosanoid synthesis in the liver in juvenile fish. More investigations will be needed to elucidate the consequent eicosanoid-mediated physiological processes.

Linseed oil injection into the yolk reserves influenced lipid metabolism in juvenile fish fed a challenged n-3 polyunsaturated fatty acid-rich diet

In fish, the long-term impact of NP during early developmental stages was revealed later in life when they were challenged with a similar conditional diet (Kumkhong et al., 2020b; Srisakultiew et al., 2022). Generally, commercial diets for juvenile Nile tilapia contain about 4% crude fat and 28–32% CP. To produce a revealing n-3 PUFA-rich dietary condition for the juvenile Nile tilapia, this study formulated a linseed oil-rich diet and challenged both NaCl-injected and linseed oil-injected fish to investigate how linseed oil intervention history influenced lipid metabolism and its related processes. Our results showed that with an n-3 PUFA-rich diet, compared to NaCl-injected fish, induction of fatty acid β-oxidation was observed at the molecular level (upregulation of hepatic *cpt1cb, acox1, pparα,* and muscular *cpt1cb, acox1,* and *acadl*) in linseed oil-injected fish. However, the effects seemed to be weaker than those before the challenge, because no significant changes were observed in lipid content between NaCl-injected fish and linseed oil-injected fish.

With the challenge of a linseed oil-rich diet, the effects of early linseed oil injection stimuli on the elevation of n-3 and n-6 LC-PUFA and those on the biosynthesis of LC-SFA and LC-MUFA in the liver and muscles were more conspicuous when compared to that of fish before the challenge. In addition, modulatory effects at the molecular level appeared to follow the same trend as that of fish before the challenge when compared with NaCl-injected fish, including (1) upregulation of *fads2* and *pparγ* expression in both the liver and muscles, (2) upregulation of *elovln6* and *mttp* in the liver; and (3) downregulation of muscular *mttp* and *elovl7*. Similarly, both nutritional history in broodstock and low fishmeal/-fish oil diets influence several major fatty acids in the liver of juvenile gilthead seabream offspring (Turkmen et al., 2017). However, in sea bass, early stimulus diets did not noticeably change fatty acids in juvenile fish challenged with a PUFA-restricted diet (Vagner et al., 2009). Overall, the differences in the effect of NP on lipid deposition varied with fish species, stimulus methods and dietary challenge. In Nile tilapia, early linseed oil or n-3 PUFA stimulation through the yolk reserve in the larval stage appeared to induce the deposition of higher levels of n-3 LC-PUFAs rather than the utilisation of dietary n-3 PUFA for energy. Dietary supplementation with fish oil has been shown to increase C20:5n-3 and C22:6n-3 levels while reducing n-6/n-3 PUFA ratios (Duarte et al., 2021). Our findings suggest that NP could be a valuable tool for enhancing tilapia production as a freshwater source rich in n-3 LC-PUFAs.

The impacts of linseed oil injection history on lipid-related metabolic processes and eicosanoid synthesis were also revealed when fish were challenged with an n-3 PUFA-rich diet. For instance, when compared with NaCl-injected fish, upregulation of hepatic *chrebp* and *acaca* was observed in linseed oil-injected fish. These findings suggest that early linseed oil injection influenced the interaction between glucose and fatty acid metabolism in the liver. In addition, an early history of n-3 PUFA overload led to elevated expression of hepatic *alox5*. Overall, NP via linseed oil injection into the yolk strongly modified the molecular metabolism of juveniles fed two different diets (before and after challenge), showing a strong effect of NP in tilapia irrespective of the diets. These data are promising for the adaptation of fish to new aquafeeds with different lipid sources, which is the future of fish nutrition in aquaculture (Glencross, 2009).

Conclusion

Programming through linseed oil (n-3 PUFA) injection into the yolk reserves of Nile tilapia larvae exerted long-term effects in the juvenile stage, including improved feed efficiency, induction of lipid catabolism, enhanced cholesterol and eicosanoid synthesis, and modulation of lipid transport and lipid–carbohydrate metabolic interactions. The linseed oil intervention history induced PUFA biosynthesis in the liver and muscle, leading to increased n-3 LC-PUFA deposition, which benefits Nile tilapia production as a freshwater source of n-3 LC-PUFAs. Notably, feeding a linseed oil-rich challenge diet confirmed the NP impact of early linseed oil overload. The (molecular) mechanisms of the NP by lipids in fish have to be determined in order to continue these studies, even though epigenetics is probably one of the main actors involved.

Supplementary material

Supplementary Material for this article (https://doi.org/10.1016/j.animal.2025.101647) can be found at the foot of the online page, in the Appendix section.

Ethics approval

All experimental protocols were approved by the Ethics Committee of Suranaree University of Technology, Animal Care and Use Committee (approval no. A-18/2562).

Data and model availability statement

The data that support the findings of this study are not deposited in an official repository but are available upon request.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

The authors have no conflicts of interest to declare.

Acknowledgements

We would like to thank Mr. Sunai Plymee (Suranaree University of Technology Farm) for maintaining juvenile fish.

Financial support statement

This project was funded by the National Research Council of Thailand (NRCT) and Suranaree University of Technology (N42A650324). This work was supported by (i) Suranaree University of Technology (SUT) and (ii) the NSRF via the Program Management Unit for Human Resources & Institutional Development, Research and Innovation (PMU–B) (grant number B13F660067). This study was also supported by grants from the Higher Education Research Promotion and the National Research University Project of Thailand, the Office of the Higher Education Commission, and Thailand Science Research and Innovation (TSRI).

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