TROPICAL LIFE SCIENCES RESEARCH

Effects of Oil-Coated Pellets and Feeding Frequency on The Growth and Body Composition of Tilapia

Authors:

Chee Ping Tan, Wen Jye Mok, Mohamad Jalilah, Jeremiah Olanipekun Jimoh, Leong-Seng Lim and Hon Jung Liew*

***Correspondence:** honjung@umt.edu.my

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Highlights:

- Best growth with 7% fish oil and 4 times daily feeding.
- No increase in muscle fat despite oil coating.
- Higher fat storage in the liver with 5% and 7% oil.
- Minimal changes in fatty acid composition.
- Better protein retention with 2 times daily feeding.

EARLY VIEW

Effects of Oil-Coated Pellets and Feeding Frequency on The Growth and Body Composition of Tilapia

[1,2]Chee Ping Tan, [1]Wen Jye Mok, [3]Mohamad Jalilah,[3,4] Jeremiah Olanipekun Jimoh, [5]Leong-Seng Lim and [3,6]Hon Jung Liew*

[1]Institute of Climate Adaptation and Marine Biotechnology, Universiti Malaysia Terengganu, 21030, Kuala Terengganu, Terengganu, Malaysia
[2]Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030, Kuala Terengganu, Terengganu, Malaysia
[3]Higher Institution Centre of Excellence, Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, 21030, Kuala Terengganu, Terengganu, Malaysia
[4] Department of Fisheries and Aquaculture, Federal University Oye Ekiti PMB 373, KM 3, Are/Afao Road, Oye Ekiti, Ekiti State, Nigeria
[5] Higher Institution Centre of Excellence, Borneo Marine Research Institute, Jalan UMS, 88400 Kota Kinabalu Sabah, Malaysia
[6] Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Harbin, China

***Corresponding author:** honjung@umt.edu.my; ORCID: https://orcid.org/0000-0002-7002-8338

Running head: Oil-Coated Pellets and Feeding in Tilapia

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Abstract: Fish oil with its proven health benefits is widely used for pharmaceutical purposes including aquaculture industry, nevertheless, excess intake may result in health alteration. This study investigated the effect of fish oil supplementation on growth and body composition of juvenile tilapia. The control group received only grain pellets containing 3% crude lipid, while the

treatment groups received fish oil supplementation at 5 and 7% of crude lipid. Each treatment was fed at a frequency of 2 and 4 times per day for five weeks. Results showed that 7% lipid supplementation shown the best relative weight gain under the 4 times feeding frequency, while the relative weight gain across the various lipid supplementation in the 2 times feeding frequency were not significantly different. There was no observed significant difference in the lipid composition of the muscle across the treatments indicated that there was no lipid deposition in the muscle despite the increase in the oil coating. Furthermore, the lipid levels in the livers of all tilapia fed 5 and 7% dietary lipid were significantly higher than those of the control groups. Fatty acids composition did not show many remarkable differences between control and treatment groups. The outcomes of this study suggested that both additional fish oil supplementation and feeding frequency had positive impacts on tilapia growth, tissue energy storage and fatty acid profile. Although, indices like ammonia excretion and muscle protein deposition favored two times feeding frequency, additional oil notwithstanding that tilapia juveniles will perform well with elevated oil coated diet.

Keywords: Aquaculture, Fatty Acids, Feeding, Feed Additive, Physiology, Tilapia

Abstrak: Minyak ikan dengan manfaat kesihatannya yang terbukti luas digunakan untuk tujuan farmaseutikal termasuk dalam industri akuakultur. Namun begitu, pengambilan berlebihan boleh menyebabkan perubahan kesihatan. Kajian ini menyelidik kesan penambahan minyak ikan terhadap pertumbuhan dan komposisi badan juvenil tilapia. Kumpulan kawalan hanya menerima palet bijirin yang mengandungi 3% lipid kasar, manakala kumpulan rawatan menerima suplemen minyak ikan pada kadar 5% dan 7% lipid kasar. Setiap rawatan diberi makan pada kekerapan 2 dan 4 kali sehari selama lima minggu. Keputusan menunjukkan bahawa penambahan 7% lipid menghasilkan pertambahan berat relatif terbaik pada kekerapan pemberian makanan 4 kali sehari, manakala pertambahan berat relatif pada pelbagai tahap suplemen lipid pada kekerapan 2 kali sehari tidak menunjukkan perbezaan yang ketara. Tiada perbezaan signifikan diperhatikan dalam komposisi lipid otot di antara rawatan, menunjukkan tiada pemendapan lipid dalam otot walaupun dengan peningkatan salutan minyak. Selain itu, tahap lipid dalam hati semua tilapia yang diberi makan diet lipid 5% dan 7% adalah lebih tinggi secara signifikan berbanding kumpulan kawalan. Komposisi asid lemak tidak menunjukkan banyak perbezaan ketara antara kumpulan kawalan dan rawatan. Hasil kajian ini mencadangkan bahawa penambahan minyak ikan dan kekerapan pemberian makanan memberi kesan positif terhadap pertumbuhan tilapia, penyimpanan tenaga dalam tisu, dan profil asid lemak. Walaupun begitu, indeks seperti

perkumuhan ammonia dan pemendapan protein otot lebih baik pada kekerapan pemberian makanan 2 kali sehari, namun tilapia juvenil tetap menunjukkan prestasi baik dengan diet bersalut minyak yang tinggi.

Kata kunci: Akuakultur, Asid Lemak, Pemakanan, Aditif Makanan, Fisiologi, Tilapia

INTRODUCTION

Tilapia is an important aquaculture species worldwide due to its high demand, ability to tolerate harsh conditions and fast growth (FAO 2019; Herawati *et al.* 2015). Nevertheless, tilapia farming is experiencing challenges in unsatisfactory growth performances probably due to poor feed optimization, emerging diseases, and unstable climatic conditions (Lebel *et al.* 2015; Tattiyapong *et al.* 2017; Watanabe *et al.* 2002). Therefore, in order to improve growth efficiency, the feeding regime and dietary improvement should be of utmost concern (Oliva-Teles 2012).

Along with protein, lipid is a major important nutrient for growth enhancement as it serves as a major source of metabolic energy for fish (Rahmah *et al.* 2021; Thalib *et al.* 2021). Being one of the main sources of metabolic energy, lipid is essential for growth, development (Liew *et al.* 2023), reproduction (Arts & Kohler 2009), locomotion (Ibrahim *et al.* 2023), and maintaining the integrity of cellular membranes while supporting the development of the brain (Leaver *et al.* 2008; Tocher 2003) and nervous systems in fishes (Xu *et al.* 2020). This energy is generally produced from adenosine triphosphate (ATP) via the β-oxidation from fatty acids in mitochondria that generate acetyl-Co-A through the Krebs cycle to create energy source in fish body (Henderson 1996). The ATP produced is used to supports vital basic functions such as growth, breeding and swimming (Tocher 2003). Studies have shown that by increasing dietary lipid level by as much as 2% improved growth by up to 13% in juvenile yellow drum (*Nibea albiflora*) (Wang *et al.* 2016). Similarly, it was also found that the growth of Manchurian trout (*Brachymystax lenok*) was improved by the dietary lipid levels supplemented up to 19% (Chang *et al.* 2018).

Similarly, feeding frequency has been frequently investigated by researchers since it has major impact on the growth performance of fishes (Oh *et al.* 2018; Okomoda *et al.* 2019; Thongprajukaew *et al.* 2017, Sousa *et al.* 2019). The common perception noted that higher feeding frequency would contribute to higher growth rate (Dwyer *et al.* 2002). However, optimum feeding frequency is species-specific (Alemayehu & Getahun, 2017) and exceeding the optimal feeding frequency limit may lead to growth deterioration due to digestion inefficiency (Dwyer *et al.* 2002; Okomoda *et al.* 2019). Previous studies have demonstrated that juvenile tilapia fed two

times a day at 12 hours interval and three times a day with 6 hours interval showed a better growth rate than those fed once a day (Dwyer *et al.* 2002; Thongprajukaew *et al.* 2017). There are also other findings that opined that tilapia could regain appetite and effectively digest feed given in as short as 4 hours post-feeding, while re-feeding in 2-3 hours post-feeding caused overloading of the gastric and resulted in poor growth performance due to inefficient absorption (Riche *et al.* 2004).

With all this background information, both dietary lipid level and feeding frequency have shown some levels of inconsistencies as far as their effects on growth performances of fish is concerned. While much works has been carried out on the impact of each of these factors on fish growth, little has been done about their combined effects on important aquaculture species such as tilapia. It will therefore be interesting to know what the response would be when both factors are integrated in one study. This study was thus aimed at investigating the combined effects of feeding frequency and dietary lipid levels on the growth, physiology of tilapia.

MATERIALS AND METHODS

Fish Management

Juvenile tilapia (n = 150; average weight = 4.5 ± 1.3 g; average total length = 6.3 ± 0.7 cm) were obtained from the hatchery of the Institute of Tropical Aquaculture and Fisheries, Universiti Malaysia Terengganu. All fish were cultured in a 1-tonne round fiberglass tank and hand-fed twice a day at 08:00h and 16:00h with commercial tilapia pellet for a week prior to experimentation. All uneaten food was removed after 15 minutes of feeding and 30% of water was refreshed daily. After one week of acclimatization, all fish were randomly selected and stocked in 6 rounded polyethylene (PE) tanks (120 L each) that were set up in a recirculating aquaculture system (RAS) at a stocking density of 25 fish per tank. Each RAS consisted of filter sponges placed in a porous polypropylene (PP) bucket, K2 Bioball media contained in a nylon mesh, bio-balls and ceramic balls. The water temperature was maintained at 28.6±0.5 °C, dissolved oxygen at about 6.5±0.5 mg/l and ammonia at less than 1.24±0.21 mg/l.

Experimental Design

The experiment was designed with two factorials involved 2 feeding frequencies (2 or 4 times daily) versus 3 different dietary lipid supplementation levels (3, 5 and 7%). The feeding regime for

2-folds feeding frequencies were set at 8:00h and 16:00h, meanwhile 4-folds feeding frequencies were set at 8:00h, 12:00h, 16:00h and 20:00h, respectively. All The fish were all hand-fed at a total daily ration of 3% of their body weight for 35 days.

Feed Preparation

Different dietary lipid levels were performed by coating commercial tilapia pellets with additional fish oil (VBA, Taiwan) to increase the initial 3% dietary lipid level to 5 and 7%, following the method described by Evans (1998). For each preparation, 100 g of pellet were laid in a single layer on a food tray and pre-heated in an oven at 50℃ for 30 minutes. Thereafter, 50 ml of fish oil was loaded into the home-use sprayer to spray on to the pellets. At a distance of 25 cm between the pellet and the sprayer, the oil was sprayed homogenously from top to bottom and from left to right, this process was repeated three times equally. Followed by shaking of the tray in a circular motion for 30 seconds to achieve a homogenous coating. According to the amount of fish oil required, the tray was rotated 90° clockwise, and the same spraying technique was repeated to achieve high lipid level. As is known, the commercial pellet contains 3% lipid, therefore, the additional application of 2 g and 4 g of fish oil per 100 g of feed would increase the lipid content to approximately 5% and 7%, respectively. After coating, the pellet was allowed to rest at room temperature for 30 minutes, until the visible layer of oil on the surface of the feed was absorbed into the feed. After 30 minutes, the coated feed was packed and stored in an air-tight container until use. To minimize oxidation of fish oil in feed during storage, the feed was prepared freshly every week.

Proximate Analysis

The proximate composition of the commercial tilapia pellet was analyzed according to the standard protocols (AOAC, 2000). All samples were analyzed in triplication before and after coating. Moisture was determined as 8.7±0.02 % through oven heating at 105℃ for 24 hours. Crude protein of 30.45±0.13% was determined via Kjeldahl method with a protein factor of 6.25. Crude lipid of 3.25±0.02% was extracted following total petroleum-ether extraction. Crude fiber of 3.61±0.02% was determined by using a Fibertherm® FT12 fiber analyzer (Gerhardt, Germany) and ash content of 8.64±0.03% was measured by putting sample in a muffle furnace and burned at 600℃ for 3 hours. The remaining 45.35% from the analysis was expressed as the non-nitrogenous portion of the commercial pellet. Analysis with a C2000 bomb calorimeter (IKA, China)

was used to measure the gross energy level of 16.26 MJ/kg. Additional lipid coating feed of 5% lipid was measured at 4.82±0.28% of lipid and the 7% feed contained lipid range of 6.74±0.27%.

Sampling Procedure and Growth Performances Assessment

Weekly, 10 fish from each tank were sampled for their biometric measurement and growth performance assessment to adjust feeding ratio as described by Swanepoel & Goose (2018). On day 35 of the experiment, 10 fish from each tank was randomly selected for osmorespiration assay. After the assay, the fish were euthanized in overdose of tricaine mesylate (300mg/L; Matthews & Varga 2012), thereafter liver and muscle samples for bioenergy and fatty acids analysis.

i. Relative Weight Gain (RWR, g/g)

ii. $\mathrm{RWR} = \frac{(\mathrm{W}_f - W_i)}{W_i}$

Where W_f is the final weight (g) and W_i is the initial weight (g)

iii. Specific Growth Rate, SGR (% day^{-1})

$$SGR = 100\ x\ \frac{InW_t - \mathrm{In}W_0}{t}$$

Where *Wt* is the final weight (g) at day 35 and W_0 is initial weight (g) at day 0 while *t* represents the difference in days between final and initial weights

iv. Feed Conversion Ratio (FCR)

$$FCR = \frac{F_G}{G_{BW}}$$

Where F_G is the weight of feed given (g) and G_{BW} is the body weight gained (g)

v. K-factor (CF)

$$CF = 100\ x \frac{W}{L^3}$$

Where *W* is the body weight of fish (g), L^3 is the total length (cm) in cube, and 100 is a constant to bring the value close to 1 (Ricker, 1975).

vi. Hepatosomatic Index (HSI)

$$HSI = \frac{W_L}{W_{BW}}\ x\ 100$$

Where W_L is weight of liver in gram and W_{BW} is body weight of fish (g)

Osmorespiration Assay

For osmorespiration assay, each fish was introduced into the osmorespiration chamber individually and fish was allowed to acclimatize to chamber condition for 2 h prior the assay. During acclimatization, aeration and water circulation were supplied continuously. After 2 h, the initial dissolved oxygen (DO) level was measured with a HI 9142 DO meter (Hanna Instrument, US) and 3 ml of water sample was taken for initial ammonia determination. Then, aeration and water circulation were removed and chambers were sealed tightly for 1 hour incubation. After 1 hour, the chambers were unsealed and the final DO readings were measured and water sample were collected for final ammonia determination (Liew *et al.* 2012; Rahmah *et al.* 2020; Thalid *et al.* 2021). The metabolic oxygen consumption rate was calculated based on proportion dissolved oxygen ratio (Liew *et al.* 2012). As for the ammonia contents in the water were all determined with the modified phenol-hypochlorite method (Weatherburn 1967). The ammonia excreted was then calculated according to concentration ratio (Liew *et al.* 2013):

i. Metabolic Oxygen Consumption Rate (MO_2, µmol/g/h)

$$MO_2 = \left(DO_f - DO_i\right) x\, V\, x\, 1000\, x\, \frac{1}{MW\ of\ O_2} x \frac{1}{\mathrm{BW}}\, x\, \frac{1}{\mathrm{T}}$$

ii. Ammonia Excretion (T_{amm}, µmol/g/h)

$$T_{amm} = \left(NH_{3f} - NH_{3i}\right) x\, V\, x\, 1000\, x\, \frac{1}{MW\ of\ NH_3} x \frac{1}{\mathrm{BW}}\, x\, \frac{1}{\mathrm{T}}$$

Where DO and NH_3 represents dissolved oxygen and ammonia contents respectively, initial (DO_i & NH_{3i}) and final (DO_f & NH_{3f}) incubation, both measured in mg/L; V is the volume of water in osmorespiration chamber in liter; MW is molecular weight; BW is the body weight of fish (g); and T is the incubation time in hour.

Bioenergy Analysis

Approximately 1 g of wet muscle and liver tissues from each fish were separately weighted and introduced into different 5 ml polypropylene tubes. About 4 ml of deionized water was added into the samples for homogenization. The homogenates were kept in ice throughout the whole process to prevent analyte degradation. Then the necessary aliquot was dispensed from the tubes for

each of the biochemical assay as described by Thalid *et al.* (2021) using microplate reader (Multiskan™ FC; Thermo Scientific, US). Bradford (1976) method was used for total protein assay with bovine serum albumin (BSA) as a standard and read at 595 nm. Glycogen was extracted by Anthrone method and read at 630 nm (Roe *et al.* 1966). Lipid was extracted with 1:1 methanol and chloroform mixture using tri-palmitine as standard and read at 405 nm (Bligh & Dyer, 1959). All measurements were expressed as mg/g.

Fatty Acids Analysis

The single-step extraction and esterification of fatty acids in muscle tissues were performed according to Abdulkadir *et al.* (2008). Approximately 200 mg of finely-crushed freeze-dried muscle tissue was weighted and mixed with 5 ml of hexane and 2 ml of 14% BF_3 in methanol in a screw-capped glass test tube. The samples were incubated in 100℃ water bath for 120 min and stirred constantly. Thereafter, the samples were left to cool at room temperature and another 1 ml hexane was added followed by 2 ml of water. The mixture was vortexed vigorously for 1 minute and centrifuged at 650 × g for 3 min. After centrifugation, the upper hexane layer containing the fatty acids methyl ester (FAME) sample was pipetted and filtered with 0.22 µm polytetrafluorethylene (PTFE) syringe filter prior analysis.

The samples were analyzed with a gas chromatography with flame ionization detection (GC Plus 2010; Shimadzu, Japan) using an Omegawax 320 (30 m × 0.32 mm × 0.25µm) fused silica capillary column with polyethylene glycol stationary phase. Exactly, 1 µl of sample was injected each time by an autosampler into the injector port with temperature set at 250℃ at a split ratio of 1:10. The oven was programmed to hold at 50℃ for 2 min, then ramped up to 210℃ at rate of 4℃ per min, and finally hold for 30 minutes. Detector temperature was set at 260℃ with complete resolution in the 37 FAME-mix standard (Supelco, Sigma).

The identification and concentration of each FAME in the unknown samples were determined by comparison against the retention times and three-point calibration curves were made from the 37 FAME-mix standard. Results were reported as milligram of fatty acids per gram of dry muscle. Ratios among polyunsaturated (PUFA), monounsaturated (MUFA) and saturated fatty acids (SFA) were calculated.

Lipids nutritional quality indices

In order to assess the nutritional quality of lipids in tilapia, the fatty acids composition data obtained were evaluated with three nutritional quality indexes: Index of Atherogenicity (IA) and Index of Thrombogenicity (IT), according to Ulbricht & Southgate (1991), and Hypocholesterolaemic/Hypercholesterolaemic (HH) ratio, according to Santos-Silva et al. (2002).

The IA demonstrates the relationship between pro-atherogenic fatty acids (C14:0 and C16:0) and anti-atherogenic fatty acids (unsaturated fatty acids). The formula is as follows:

i. Index of Atherogenicity (IA)

$$IA = \frac{\{(4\ x\ C14{:}\,0) + C16{:}\,0\}}{\sum UFA}$$

Notes: UFA stands for Unsaturated Fatty Acids, which include both MUFA (monounsaturated) and PUFA (polyunsaturated).

The IT measures myristic acid, palmitic acid and stearic acid that are highly correlated with thrombus formation against the anti-thrombogenic mono-unsaturated fatty acids (MUFA), and polyunsaturated ω-3 and ω-6 fatty acids. The formula is as below:

ii. Index of Thrombogenicity (IT)

$$IT = \frac{(C14{:}\,0 + C16{:}\,0 + C18{:}\,0)}{[(0.5\ x\ \sum MUFA) + (0.5\ x\ \sum \omega - 6\mathrm{PUFA}) + (3\ \mathrm{x} \sum \omega - 3\mathrm{PUFA})]} + \frac{\omega - 3}{\omega - 6}$$

Lastly, the HH ratio estimates the influence of hypocholesterolaemic fatty acids (oleic acid and PUFA) and hypercholesterolaemic fatty acids (C14:0, and C16:0) towards LDL cholesterol level. The formula is as below:

iii. HH Ratio

$$H{:}H = \frac{(cis - C18{:}\,1 + \sum \mathrm{PUFA})}{(C14{:}\,0 + C16{:}\,0)}$$

Statistical Analysis

All data observed included metabolic oxygen consumption, bioenergy, HSI, growth and fatty acids composition at different lipid coated ratio (3, 5 & 7%) within feeding frequency were analyzed by One-way ANOVA. Prior to analysis, all data were checked for normality distribution by Shapiro-wilk test, only data that fulfill normality requirements were proceeded with One-way ANOVA followed by Tukey post-hoc test. If the data being analyzed did not fulfill distribution requirements,

non-parametric analysis was performed by Kruskal Wallis test. While for significant difference between feeding frequencies at the same dietary lipid coated level were analyzed by using student t-test for parametric analysis, otherwise, Mann-Whitney U test was used as the non-parametric. Significant level was set at 95% confident limit ($P<0.05$). All statistical tests were performed in SPSS ver. 21 (IBM Corp, Armonk, NY).

RESULTS

Growth Performances

The relative weight gain (RWG), specific growth rate (SGR), feed conversion ratio (FCR), K-factor, and hepatosomatic index (HSI) of all treatments were summarized in Table 1. The growth performances were maintained consistently without influence by different dietary lipid levels with 2 times frequencies ($P>0.05$; Table 1). In contrast, the effect of different dietary lipid levels were recorded in fish fed 4 times frequencies ($P<0.05$; Table 1), where highest RWG, SGR, and K-factor were observed in fish fed with 7% dietary lipid supplement ($P<0.05$; Table 1).

Table 1: Relative weight gain (RWG), specific growth rate (SGR), feed conversion ratio (FCR), K-factor, and hepatosomatic index (HSI) of juvenile tilapia fed with different lipid content feed between 2- and 4-times feeding frequencies.

Parameter	2 times feeding			4 times feeding		
	3% (control)	5%	7%	3% (control)	5%	7%
RWG (g/g)	3.64±0.29	3.07±0.20	3.45±0.25	3.88±0.19^{ab}	2.98±0.23^{a}	4.21±0.26^{b*}
SGR (%/day)	4.36±0.19	3.98±0.14	4.22±0.18	4.52±0.11^{ab}	3.91±0.16^{a}	4.84±0.06^{b*}
FCR	0.88±0.08	0.89±0.05	0.85±0.09	0.77±0.04	0.95±0.07	0.82±0.08
K-factor	1.96±0.04	1.98±0.04	2.05±0.05	1.93±0.03^{a}	1.95±0.04^{a}	2.06±0.04^{b}
HSI	2.86±0.24	2.94±0.14	2.50±0.21	2.79±0.25	2.70±0.55	3.03±0.34

Superscript letter indicates significant difference among dietary lipid levels within feeding frequencies ($P<0.05$). Asterisk (*) designates significant difference between similar dietary lipid levels at different feeding frequencies ($P<0.05$).

Tissue Energy

The muscle and liver energy contents in form of protein, glycogen, and lipid in the fish fed with either 2 times and 4 times feeding frequencies were shown in Figure 1 and 2. Feeding frequency had an impact on protein mobilization with 2 times feeding frequency contained relatively high muscle protein (125 – 192 mg/g) compared to 4 times feeding frequency (13 – 60 mg/g) ($P<0.05$; Fig. 1.A. & 1.B). Relatively, fish that fed with 7% of dietary lipid level exhibited higher protein contents than fish fed 5 and 3% dietary lipid levels in fish either fed at 2 times or 4 times frequencies ($P<0.05$; Fig. 1.B). Where, fish fed with 5% and 7% dietary lipid levels contained significantly higher protein in muscle than fish fed with 3% dietary lipid level ($P<0.05$; Fig. 1.B). Muscle glycogen of fish under 2 times feeding frequency decreased significantly with an increase dietary lipid level ($P<0.05$; Fig. 1.C), but no effect was noticed in fish under 4 times feeding frequencies ($P>0.05$; Fig. 1.D). Nevertheless, muscle glycogen contained in fish fed with 3% and 5% dietary lipid levels under 2 times feeding frequencies were significantly higher than in the fish fed with 4 times feeding frequency ($P<0.05$; Fig. 1.C and 1.D). However, the fish fed 7% dietary lipid level under 2 times feeding frequency had lower muscle glycogen content than fish under 4 times feeding frequency ($P<0.05$; Fig. 1.C and 1.D). As for muscle lipid, the total lipid accumulated under the two feeding frequencies were insignificantly different irrespective of the dietary lipid levels ($P>0.05$; Fig. 1.E and 1.F). Nevertheless, fish that fed with 3% and 5% dietary lipid levels under 2 times feeding frequencies had higher muscle lipid levels than under 4 times feeding frequency ($P<0.05$; Fig. 1.E and 1.F).

In liver, fish fed with 7% dietary lipid level under 2 times feeding frequency exhibited higher liver protein level than other groups ($P<0.05$; Fig. 1.A), whereas higher liver protein was found in the fish fed with 5% dietary lipid level ($P<0.05$; Fig. 1.C). Nevertheless, liver glycogen for the fish under 2 times feeding frequencies was relatively stable ($P>0.05$; Fig. 2.C). In contrast, the 4 times feeding frequency produced fish with highest liver glycogen was in 5% dietary lipid level ($P<0.05$; Fig. 2.D). A similar increasing trend in liver lipid levels were noticed with increasing dietary lipid levels in both feeding frequencies ($P<0.05$; Fig. 2.E and 2.F).

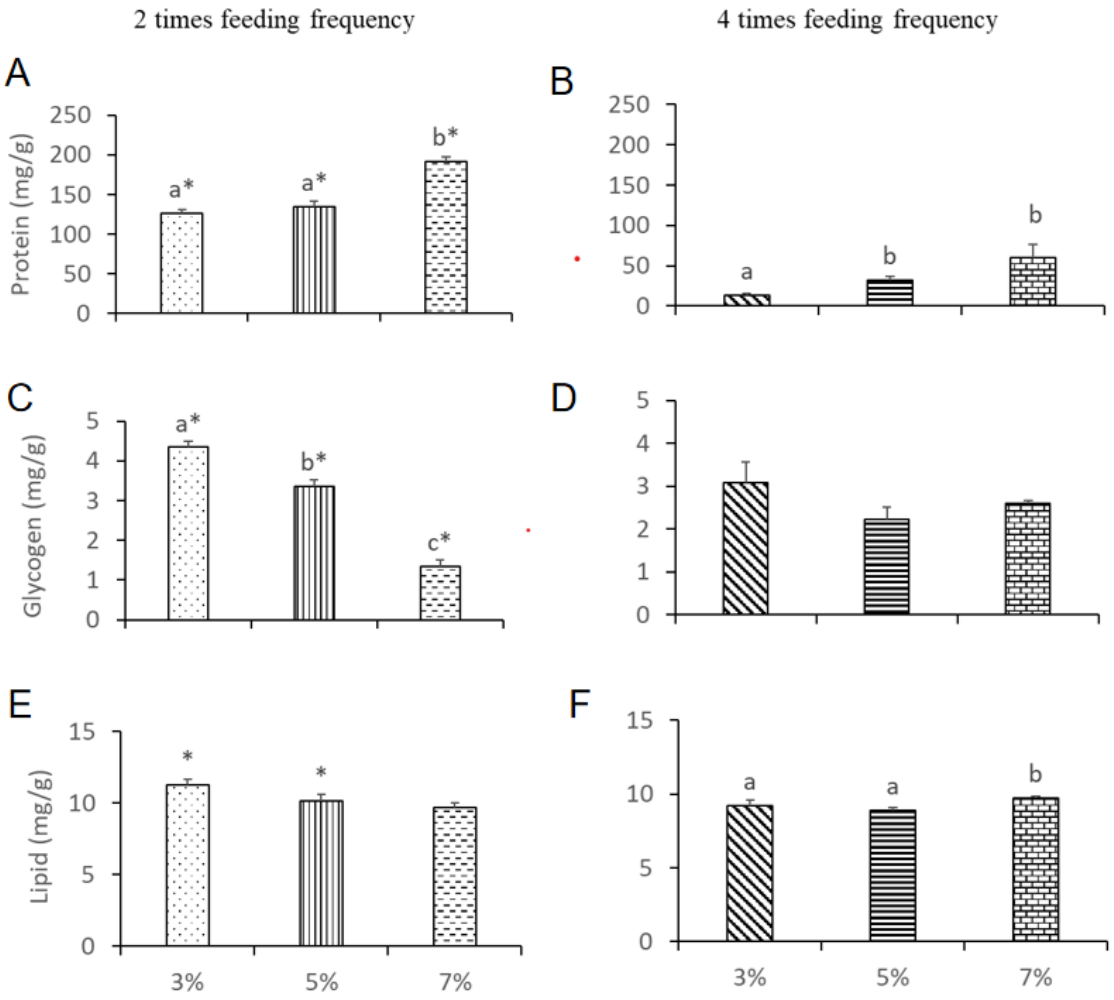

Figure 1. Muscle protein, glycogen and lipid contents (mg/g) on muscle of fresh tilapia juveniles fed with 3 different levels of lipid (3, 5, and 7%) containing diet at 2 times (Figure A, C, and E) and 4 times (Figure B, D, and F). Values were presented as mean standard error of mean (SEM). Different letters above each bar indicates significant differences among different dietary lipid levels ($p<0.05$). Asterisk (*) indicates significantly different between different feeding frequency of the same dietary lipid levels ($p<0.05$).

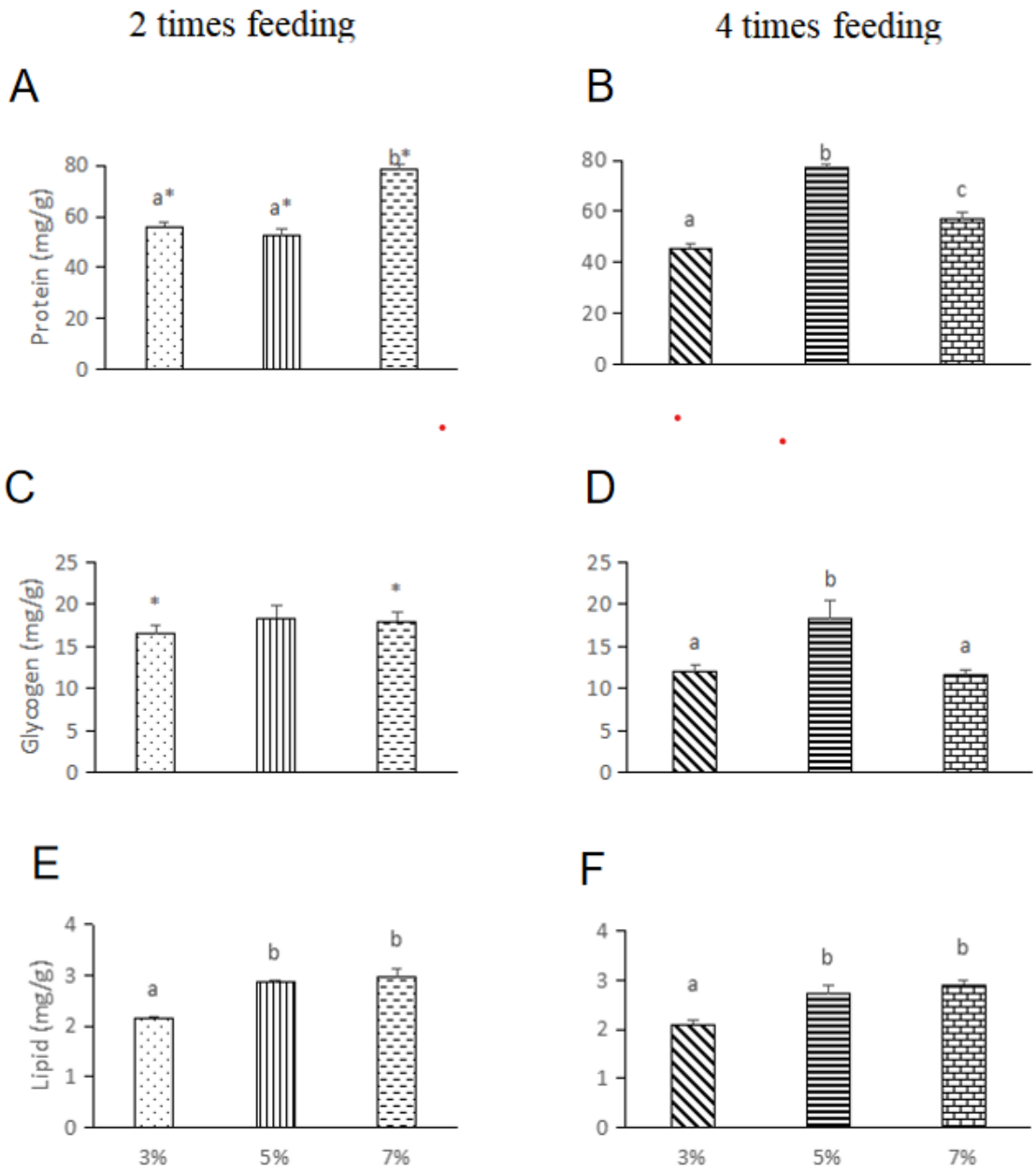

Figure 2. Liver protein, glycogen and lipid contents (mg/g) on muscle of fresh tilapia juveniles fed with 3 different levels of lipid (3, 5, and 7%) containing diet at 2 times (Figure A, C, and E) and 4 times (Figure B, D, and F). Values were presented as mean with standard error of mean (SEM). Superscript letters indicate significant differences among different dietary lipid levels ($p<0.05$). Asterisk (*) indicates significantly different between different feeding frequency of the same dietary lipid levels ($p<0.05$).

Osmorespiration

Under 2 times feeding frequency, fish fed 5% and 7% dietary lipid levels consumed twice significantly higher metabolic oxygen consumption at 24.4±3.4 and 24.0±5.7 μmol/g/h compared to fish fed with 3% dietary lipid level at 12.1±3.6 μmol/g/h, respectively ($P<0.05$, Fig. 3.A). In contrast, the fish fed 4 times feeding frequency showed different pattern with fish fed 3% dietary level exhibiting highest metabolic oxygen intake as compared to those fed 5% and 7% dietary lipid levels ($P<0.05$; Fig. 3.B). Overall, fish fed 3% dietary lipid under 4 times feeding frequency recorded almost 4 times higher metabolic oxygen consumption (44.7±9.5 μmol/g/h) than their counterparts in 2 times feeding frequency. In addition, fish under 4 times feeding frequency fed with 5% and 7% dietary lipid level shown significantly lower metabolic oxygen consumption (12.9±4.2 and 19.0±4.3 μmol/g/h) ($P<0.05$; Fig. 3.B).

On the other hand, the ammonia excretion rate for the fish fed with 3%, 5%, and 7% dietary lipid levels under 2 times feeding frequency were not significantly different from one another ($P>0.05$; Fig. 3.C). Whereas under 4 times feeding frequency, the ammonia excretion for the fish that received 3% and 7% dietary lipid levels were found significantly higher at 0.23±0.05 μmol/g/h and 0.18±0.02 μmol/g/h respectively, than the fish fed with 5% dietary lipid level (0.07±0.01 μmol/g/h) ($P<0.05$; Fig. 3.D). In comparison between feeding frequencies, fish fed with 4 times feeding frequency exhibited higher ammonia excretion rates as compared to fish fed 2 times feeding frequency under 3% and 7% dietary lipid levels ($P<0.05$; Fig. 3).

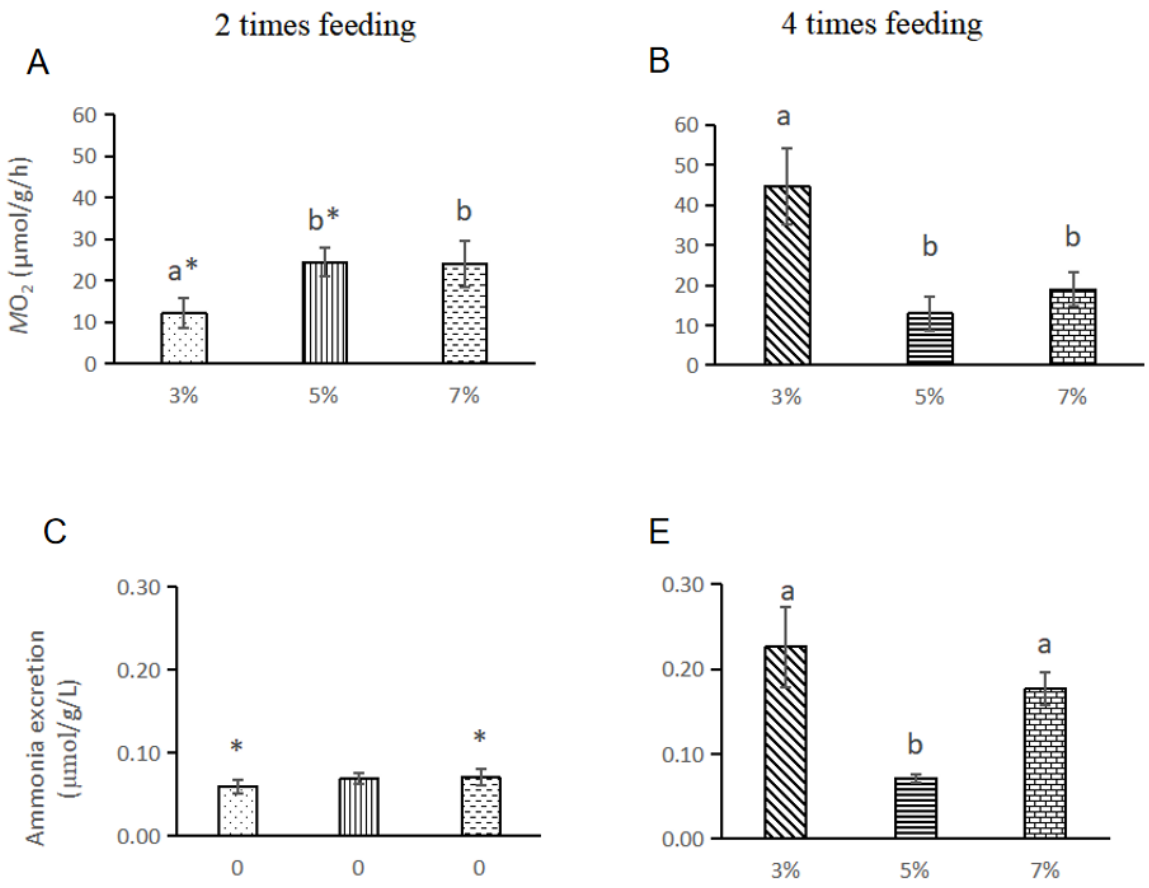

Figure 3. Metabolic oxygen (MO_2) consumption and ammonia excretion (T_{amm}) of juvenile tilapia fed with 3 different level lipid (3, 5, and 7%) containing diet at 2 times (Figure A & B) and 4 times (Figure C & D). Values were presented as mean with standard error of mean (SEM). Superscript letters indicate significant differences among different dietary lipid levels ($p<0.05$). Asterisk (*) indicates significantly different between different feeding frequency of the same dietary lipid levels ($p<0.05$).

Fatty Acids

Across all the treatments, polyunsaturated fatty acids (PUFA) accounted for 50-51% of the total fatty acids in the experimental fish, followed by saturated fatty acids (SFA) at 33-34%, and monounsaturated fatty acids (MUFA) at 15-16%. The most dominant form of PUFA was the docosahexaenoic acid (DHA), ranging from 5.80-7.69 mg/g ($P<0.05$; Table 2). While for SFA, palmitic acid (C16:0) was the most abundant fatty acids, with a recorded concentration of 4.18-

5.04 mg/g (P<0.05; Table 2). The third most accumulated under MUFA was the oleic acid (octadecenoic acid) (C18:1n9) at 3.15-3.76 mg/g (P<0.05; Table 2).

Comparing between the two feeding frequencies, tilapia fed 5% dietary lipid had significantly lowered amount of EPA (1.03±0.06 mg/g) and DHA (5.80±0.17 mg/g) than tilapia fed with 3% and 7% dietary lipid at the same feeding frequencies (P<0.05; Table 2). The same was also observed in palmitic acid where fish fed with 5% dietary lipid 4 times a day had significantly lowered palmitic acid (4.18±0.40 mg/g) than those fed with 3% and 7% dietary lipid under the same feeding frequency (P<0.05; Table 2). Notably in tilapia fed with 7% dietary lipid level, 4 times a day had significantly highest C18:1, C18:2n6 and C18:3n3 than other groups of fish (P<0.05; Table 2). The ω-6 to ω-3 ratios for tilapia fed 2 times and 4 times feeding frequencies in control were 0.21 and 0.20, respectively. As for 5% and 7% lipid levels were found relatively stable at a range of 0.25, regardless of 2 times and 4 times feeding per day, respectively. The differences in IA and IT across control and all treatment groups were miniscule, ranging at 0.60-0.63 and 0.73-0.76, respectively. The HH ratios were slightly lower in the 2 times feeding group (2.42-2.50) compared to the 4 times feeding group (2.54-2.57).

Table 2: Effects of different feeding frequency per day and the lipid contents of the fish feed on individual fatty acids composition on tilapia juvenile muscle. Results are given as mg/g of dried muscle ± standard error of mean (SEM).

Fatty Acids	2 times feeding frequencies			4 times feeding frequencies		
	Control	5 % Lipid	7 % Lipid	Control	5 % Lipid	7 % Lipid
Saturated						
14:0	1.43±0.02	1.46±0.04	1.42±0.02	1.49±0.02^{bc}	1.39±0.02^{ac}	1.51±0.02^{c}
15:0	0.66±0	0.67±0.02	0.66±0	0.66±0	0.65±0.01	0.66±0
16:0	4.89±0.25	4.34±0.23	4.58±0.21	4.84±0.11^{ab}	4.18±0.40^{a}	5.04±0.08^{b}
17:0	0.58±0	0.57±0.02	0.57±0.01	0.58±0.01	0.54±0.02	0.58±0
18:0	2.46±0.11	2.34±0.09	2.51±0.07	2.39±0.05	2.28±0.17	2.54±0.04
21:0	0.94±0.02	0.99±0.03	0.95±0.01	0.91±0.01	0.95±0.02	0.98±0.01
Subtotal	10.96	10.37	10.69	10.87	9.99	11.31
Monounsaturated						
16:1n7	0.90±0.03	0.87±0.04	0.80±0.03	0.95±0.03	0.82±0.04	0.98±0.03
18:1n9	3.25±0.10	3.25±0.14	3.48±0.13	3.35±0.08^{a}	3.15±0.23^{a}	3.76±0.09^{b}
20:1n9	0.71±0.01	0.73±0.02	0.73±0.01	0.72±0.01	0.71±0.01	0.82±0.09
Subtotal	4.86	4.85	5.01	5.02	4.68	5.56

Polyunsaturated						
18:2n6	1.43±0.06[a]	1.57±0.09[a]	1.81±0.08[b]	1.50±0.08[a]	1.48±0.13[a]	1.97±0.07[b]
18:3n3	0.67±0	0.72±0.02	0.74±0.01	0.69±0.02	0.68±0.02	0.76±0.01
20:2n6	0.68±0.01	0.70±0.02	0.72±0.01	0.57±0.12	0.68±0.01	0.65±0.04
20:3n3	1.10±0.08	0.94±0.05	1.01±0.03	1.00±0.04	0.91±0.07	1.05±0.03
20:5n3 (EPA)	1.16±0.05	1.02±0.02	1.12±0.03	1.21±0.02	1.03±0.06	1.12±0.04
22:6n3 (DHA)	7.00±0.83	6.30±0.27	7.23±0.38	7.69±0.29[a]	5.80± 0.17[b]	7.27±0.18[a]
Subtotal	16.40	15.25	16.75	16.94	15.19	17.41
∑PUFA/SFA	1.50	1.47	1.57	1.56	1.52	1.54
∑MUFA/SFA	0.44	0.47	0.47	0.46	0.47	0.49
∑PUFA/MUAF	3.37	3.14	3.34	3.37	3.25	3.13
∑PUFA + MUFA/SFA	1.94	1.94	2.04	2.02	1.99	2.03
∑PUFA/SFA	0.51	0.50	0.52	0.52	0.51	0.51
ω6/ω3	0.21	0.25	0.25	0.20	0.26	0.26
ω3/ω6	4.71	3.96	3.99	5.12	3.90	3.90

PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acid; SFA: saturated fatty acids.

Results are reported as mean ± standard error of mean in the unit of mg/g.

Difference superscript of letters indicate significant differences among different dietary lipid levels frequencies at $P<0.05$.

DISCUSSION

Growth Performances

In general, the relative weight gain, specific growth rate and feed conversion ratio of all groups that received 4 times feeding frequencies were slightly higher than all fish that received 2 times feeding frequencies. This could be linked to the fact that increase in feeding frequency has the tendency to improve feed digestibility and nutrient utilization (Elesho *et al.* 2021). However this result might have been fish size dependent in agreement with the opinion of Lee *et al.* (2000). This finding was consistent with previous research that found higher feeding frequencies contributed to a better growth performance in hybrid striped bass *Morone saxatilis* × *M. chrysops* (Liu & Liao 1999), juvenile Bluegills, *Lepomis macrochirus* (Zhang *et al.* 2018) and fry and fingerlings of African catfish, *Clarias gariepinus* (Okomoda *et al.* 2019). Nevertheless, this study

found that the hepatosomatic index (HSI) of the fish was not influenced by neither the feeding frequencies nor different dietary lipid levels. This shows that energy supplied from feed intake were sufficient for growth and daily metabolism needs as well as deposited in liver as energy. This could be indicating that more addition of oil did not interfere with its capacity to act as growth promoter in fish as stated by Sutili et al. (2018). He *et al.* (2015) reported similar finding where the HSI of Nile tilapia (*Oreochromis niloticus*) fed either 1, 7, or 13% dietary lipid levels were not affected the growth and HSI values. The K-factor in this study increased marginally with the dietary lipid levels in both 2 times and 4 times feeding frequencies. Datta et al. (2013) and Shahabuddin et al. (2015) opined that fish with K-factor of more than 1 was in a good health indication that the experimental setting and or nutritional condition were favorable to the fish in exhibiting isometric growth. Since our K-factor (ranging from 1.93 to 2.06) fell within the range of the reported K-factor, this may be indicating that additional oil coating on the feeds in this study did not affect the fitness status of the fish. The values that we obtained in this study was in paralleled with Anani & Nunoo (2016) who reported that the K-factor of Nile tilapia fed with farm-made feeds at different lipid levels were ranging between 1.39 to 2.01 respectively. However, it is worth noting that a fish's sex, gonad maturity, environment, and even nutritional status can all have an impact on the measured K-factor (Le Cren 1951; Heincke 1980, Nash *et al.* 2006; Liew *et al.* 2022). The tilapia used in this study were randomly selected regardless of sex, although not specifically reported in this study but at the end of study some fish were observed with developed distinguishable gonads. Nevertheless, the good growth rate and acceptable K-factor recorded in this study here were clear that the effects of sex and gonad maturity on the measured K-factor are negligible.

In addition to feeding frequency and dietary lipid levels, it is important to acknowledge that environmental and management factors play a major role in fish health, growth performance and product quality. For instance, stocking density has repeatedly been shown to influence growth (Refaey *et al.* 2018; Lie *et al.* 2017), stress responses (Li *et al*. 2012; Ding *et al.* 2023), nutrient utilization (Ezhilmathi *et al.* 2022; Zaki *et al.* 2023), and ultimately flesh quality (Refaey *et al.* 2018; Zhao *et al.* 2019): higher densities may lead to impaired growth rate, poorer feed conversion, elevated stress biomarkers and greater susceptibility to disease. Likewise, farm (or culture-system) size and infrastructure (e.g., pond/tank volume, water exchange capacity, flow rates) determine the capacity to maintain optimal water quality, reduce waste accumulation, and provide an environment conducive for efficient feeding and metabolism (Zhao *et al.* 2023; Kamalam & Pandey 2023; Goda *et al.* 2024). In the context of our study on Nile tilapia, even though we manipulated feeding frequency and lipid levels, the actual expression of these

nutritional interventions may have been moderated by our culture environment – for example, if stocking density or system size imposed limitations on water quality or fish behaviour, this could attenuate or amplify the effects we observed. Therefore, when interpreting the good K-factor, acceptable hepatosomatic indices, and favourable fatty acid nutritional quality indices, it is prudent to note that these outcomes reflect the combined effects of nutrition + feeding regime + culture environment. Future work should therefore consider a factorial design including stocking density/farm size as independent variables to isolate how feeding strategy effectiveness may vary under different production-scale settings.

Tissue Energy

Overall, our results showed that both different dietary lipid levels and feeding frequencies had an effect on the energy deposition pattern of tilapia. Regardless of the feeding frequencies, tilapia fed 5% and 7% of dietary lipid had higher liver lipid content than those fed 3% dietary lipid. This is because the liver is the main lipid deposition site in response to high lipid diets act as main organ for energy storage (Zou and Wang, 2023). As noted in this study, the muscle lipid of tilapia on the other hand was maintained relatively constant in all treatments without influenced by different levels of oil coating and feeding frequencies. As He *et al.* (2015) and McClelland *et al.* (1995) who showed that the Nile tilapia able to cope with a high lipid diet by depositing lipid in the adipocytes with increasing the number of adipocytes in the visceral adipose tissue. Nevertheless, other species such as salmonid (Zhao *et al.* 1995), pangasius (Sokamte *et al.* 2020), and Jade perch (Elhag *et al.* 2022) might prefer to deposit lipid in muscle.

In terms of protein composition, it was found that the muscle of Nile tilapia fed at 2 times the feeding frequency consistently had significantly higher levels of protein across all three levels of dietary lipids in the diet than all fish fed at 4 times the feeding frequency. This is clearly contrast with many studies on Korean rockfish, *Sebastes schlegeli* (Mizanur & Bai, 2014), Dark-banded Rockfish, *Sebastes inermis* (Oh *et al.* 2018), Nile Tilapia, *Oreochromis niloticus* (Gaber & Hanafy 2008; Huang *et al.* 2015; Thongprajukaew *et al.* 2017), and African catfish, *Clarias gariepinus* (Okomoda *et al.* 2019) in which manipulation of feeding frequency caused slightly to no statistically significant deviation in muscle protein content of fish. A possible explanation is that fish fed a large feed (2 times the feeding frequency) must expend more aerobic metabolic capacity on food digestion than those fed a smaller feed meal (4 times the feeding frequency), and therefore reserve more energy from swimming and maintain their muscle protein storage (Norin & Clark, 2017). In addition, fish that are fed more frequently (4 times the feeding frequency) may

also affect their muscle protein levels as the fish tend to be more aggressive, cannibalistic and prone to mortality as they become more impatient and competitive compared to the next shorter feeding cycle are up to twice the feeding frequency (Al-Khafaji *et al.* 2017; Muntaziana *et al.* 2017).

Osmorespiration

Osmorespiration consisted of the measure of metabolic oxygen consumption rate and metabolic ammonia excretion rate of a fish in a closed-system under a specific duration (Herrera-Castillo *et al.* 2024). At 2-fold feeding frequency, the 2-fold significant ($p<0.05$) increase in metabolic oxygen consumption in fish fed 5 and 7% dietary lipid compared to the control (3% dietary lipid) is consistent with the results of D'Cruz & Wood (1998) in which it was suggested that diets with higher energy content (in this case an increase in lipid content) contributed to a higher metabolic oxygen consumption rate in fish. They further explained that the elevated metabolic oxygen requirement is caused by a physiological response known as specific dynamic action (SDA). SDA refers to the energy expanded in a fish for all metabolic processes involved during digestion, nutrient uptake, synthesis of nutrients for storage in tissues, as well as the excretion of metabolic wastes (D'Cruz & Wood 1998; Jobling 1994; Secor 2008). In contrast, previous research on the SDA of tilapia has shown that when fed separately with different protein (5–41%) and lipid levels (1–18%) of formulated diets, tilapia live about 12.3–16.5 hours and 12.0 to 16.0 hours to return their metabolic oxygen consumption rate to rest (Ross *et al.* 1992). In addition, Ross *et al.* (1992) also reported that different lipid levels in formulated diet had no significant ($p > 0.05$) effect on the SDA response of tilapia.

Because the osmorespiration test was performed at least 15 to 17 hours after the last fish meal of the previous day, SDA may not fully account for the observed osmorespiration rate. Therefore, this suggests that a different feeding frequencies and lipid contents had direct and lasting effects on the metabolic oxygen consumption of juvenile tilapia. On the other hand, factors such as water temperature, fish body size, fish physical activity, accessibility to dissolved oxygen in the water, and nutritional status may also have effects on the oxygen requirement of fish (Holeton 1980; Jobling 1982; Thalib *et al.* 2021).

For the 4-times feeding frequency, the fish were expected to show the same trend in metabolic oxygen consumption rate as their 2- times feeding frequency counterpart. However, the fish group with less than 3% dietary lipid at 4 times the feeding frequency showed 3.7 times higher metabolic oxygen consumption than their counterpart with the same dietary lipid at 2 times the feeding frequency. This clearly depicted a deviation from the resting state requirement, and is

probably attributed to operator error during the osmorespiration assay, particularly related with fish handling, acclimatization period before commencement of assay, and the incubation period until oxygen reading was taken may have inevitably given stress to fish and increased its metabolic oxygen consumption (Jobling 1982; Svendsen *et al.* 2016).

Overall, the significantly ($p<0.05$) higher metabolic oxygen requirement of fish fed 5 and 7% dietary lipid in 2 times feeding frequency than the same dietary lipid group under 4 times feeding frequency could possibly be explained by the higher energy required in 2 times feeding frequency group to digest a larger portion of feed than 4 times feeding frequency group where the fixed daily 3% ration was split into 4 equal parts. This could be related to what Cook (2000) demonstrated under long term 8 weeks starvation studies on Atlantic salmon (*Salmo salar*) in which the fish exhibited lower metabolic oxygen consumption than control fed to satiation 3 times per day. The practices of 4 times feeding frequency somehow resemble starvation can be true in where it was shown earlier that fish under 4 times feeding frequency had severely lower muscle protein content than those fed twice daily. It is possible for fish to use and break down protein as an energy source during starvation (Cook 2000). The ammonia excretion rate of fish fed 5% fat diet was not affected by the feeding frequencies studied. In the 3 and 7% diet groups, ammonia excretion was significantly ($p<0.05$) higher in fish fed four times a day than in fish fed the same dietary lipid but fed twice a day. This could be related to the poorer protein efficiency (He 2015), which was consistent with the low muscle protein content in all fish (3, 5 and 7% dietary lipid) at 4-fold feeding frequency.

Fatty Acids

The tilapia being studied here had very high amount of DHA compared to previous studies (Abelti 2017, Stoneham *et al.* 2018, Young 2009). The high DHA contents of the tilapia in this study was similar to the Yellowstripe scad found in Straits of Malacca which reported an average DHA content of 7.82 mg/g (Abd Aziz *et al.* 2013), as well as the wild tilapia found in natural lake of Ethiopian highland lakes with up to 9.72 ± 2.05 mg/g (Tadesse, 2010). Such similar observations indicated that high levels of DHA in fish may be rare, but not entirely impossible. There are many factors that can affect near-fish composition. A study by Tadesse (2010) found that wild tilapia living in different lakes have markedly different fatty acid compositions, which are controlled by the availability and diversity of phyto- and zooplankton in the water. Before the start of the experiment, the tilapia used in this experiment grew and fed on commercial feed and natural phytoplankton in the culture water. The green water used to grow tilapia in the past may be

responsible for its high DHA content. Research on tilapia fed seaweed meal showed a positive linear relationship between muscle DHA content and dietary intake of seaweed meal (Stoneham *et al.* 2018). Apart from that, Abelti (2017) also pointed out that internal factors such as genetic composition, developmental stage, sex and body size have a large influence on the approximate composition of a fish (Huss, 1995) than external factors such as temperature, salinity, pH Value and concentration of dissolved oxygen. The high ω3 contents from DHA, the PUFA/SFA ratio exceeded 0.5 is a good indicator that consumption of the fish can have beneficial effects to lowering of blood cholesterol level (Gurr 1984). The tilapia studied here had high amount of ω-3 than ω-6 which is a positive indicator of healthy food to consume since the recommended ω-6 to ω-3 should be 4:1 or even less. Consumption of fish with high DHA is beneficial to cardiovascular health as it lower blood lipid and reduce risk of heart diseases (Medeiros *et al.* 2007).

Lipids nutritional quality indices

The IA and IT measure the nutritional quality of fatty acids in foods in terms of their atherogenic and thrombogenic potential for human, respectively (Ulbricht & Southgate 1991). The lower the indices, the healthier the foods are for human. Due to the high amount of PUFA recorded here, the IA and IT obtained are lower than previous studies of *O. niloticus* which reported IA of 0.60-0.68 and IT of 0.78-1.0 (Duarte *et al.* 2021; Mekonnen *et al.* 2020; Tonial *et al.* 2014). This showed that eating tilapia in this study had low chance of triggering blood vessels clotting incidence due to lipid deposition or thrombus formation within the blood vessels (Chen & Liu, 2020). This reduction can possibly be explained by Calder (2015) who stated that intake of higher proportion of mono- and poly-unsaturated fatty acids in diets concurrently reduced consumption of harmful saturated fatty acids, leading to reduce LDL cholesterol level, lower blood pressure, better diabetes management, and decrease cardiovascular risk (Nhan *et al.* 2019). In fact, on average, the amounts of total unsaturated fatty acids of tilapia in this study made up approximately 61% of the total fatty acids found in all groups.

On the other hand, a diet of high HH ratio characterize by higher amount of hypocholesterolaemic oleic acid and PUFA over the hypercholesterolaemic myristic acid and palmitic acid has been found to be associated with lowered LDL cholesterol level and reduced cardiovascular events in human (Attia *et al.* 2015; Calder, 2015; Joris & Mensink, 2016). Comparing with previous researches in similar species, the tilapia in this study have significantly higher HH ratios, and are more comparable to some marine fishes. For examples, in the works of tilapia, Mekonnen *et al.* (2020) found a HH ratio of only 1.02 while Tonial *et al.* (2014) at 1.56. On

the contrary, in the cases of the marine fishes, the HH ratios of *Rachycentron canadum*, *Hyporhamphus unifasciatus* and *Hemiramphus brasiliensis* were 2.46, 2.43 and 2.46, respectively (Fernandes *et al.* 2014; Gonçalves *et al.* 2021). Although a recommended value for HH ratio is not yet established, Gonçalves *et al.* (2021) agree with Santos-Silva *et al.* (2002) that HH ratio above 2.0 is most ideal for a healthy diet. Overall, the presence of high PUFA over SFA contents, low IA and IT coupled with high HH ratios all suggested that the tilapia in this study are highly beneficial for human in terms of promoting good cardiovascular health (Calder 2015; Chen & Liu, 2020), regardless of the modified dietary lipid level and feeding frequency.

CONCLUSION

Based the set of results in this study, it can be concluded that feeding juvenile tilapia 4 times per day on a 7% elevated dietary oil was sufficient to make a significant impact on their growth performance, tissue energy storage and fatty acid profile. Nevertheless, the elevated dietary lipid level notwithstanding, other indices such as muscle protein deposition and ammonia excretion were slightly better in 2 times a day feeding frequency when compared with the 4 times a day feeding frequency. It is therefore recommended that tilapia juveniles could be fed with diets containing elevated dietary oil on four times a day feeding frequency.

ETHICAL STATEMENT

Experimental procedures, animal handling and dissection method used in this experiment followed the Animal Ethic Guidelines approved by the Committees of Ethic Animal Care, Universiti Malaysia Terengganu (UMT/JKEPHMK/2022/71).

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DECLARATION OF COMPETING INTEREST

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

DATA AVAILABILITY

Data will be made available on request.

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AUTHORS' CONTRIBUTIONS

Chee Ping Tan: Writing – original draft, investigation, formal analysis.
Wen Jye Mok – review & editing, supervision, formal analysis.
Mohamad Siti Jalilah: Software, data curation.
Jimoh Jeremiah Olanipekun: Visualization, methodology.
Leong-Seng Lim: Writing – review & editing, validation.
Hon Jung Liew: Writing – review & editing, supervision, project administration, conceptualization.

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