Thesis for Degree of Master of fisheries science

Effects of fish meal replacement on growth performance, energy metabolism, and stress responses of juvenile olive flounder (*Paralichthys olivaceus*) under chronic and acute temperature stresses

by

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August 2023

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사료 내 어분 대체가 만성 및 급성 고수온 스트레스에 노출된 넙치(*Paralichthys olivaceus*) 치어의 성장, 에너지 대사 및 스트레스 반응에 미치는 영향

Advisors : Prof. Seunghyung Lee

by

Sooa Yoon

A thesis submitted in partial fulfillment of the requirement
for the Degree of

Master of Fisheries Science

in the Department of Fisheries Biology, Graduate School
Pukyong National University

August 2023

윤수아의 수산학석사 학위논문을 인준함

2023년 8월 18일

위 원 장 이 학 박 사 김 종 명 (인)

위 원 수산학 박 사 최 윤 희 (인)

위 원 이 학 박 사 이 승 형 (인)

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A dissertation

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August 18, 2023

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Abstract

Global warming attributed to climate change is causing rapid environmental changes including high fluctuation in water temperature, resulting in mass mortality and disease outbreaks in aquaculture practices. Fish meal production is predicted to decrease over time due to reduction in capture production of fishes for use of fish meal in association with climate change. Regardless the fact that fish meal is an important ingredient for aquafeed, there is no choice but to reduce fish meal content in aquafeed for sustainable aquaculture. Therefore, the current study was conducted to understand what relationship between manipulation of fish meal content and physiological responses under chronic and acute temperature stress exists in juvenile olive flounder (*Paralichthys olivaceus*), a commercially important aquaculture fish species in Asia countries, including Rep. of Korea, Japan, and China.

Four hundred eighty juveniles averaging 13.6 ± 0.02 g (mean ± SEM) were randomly distributed into each of the 24 rectangular tanks (20 fish per tank). Each set of 12 tanks was assigned to either constant water temperature group reared at 19.5 (named as non-stressed group: NS) or gradually increased water temperature group reared at from 19.5 to 30 (1.5 increment/week) (named as chronic temperature-stressed group: CTS) throughout the trial. Each NS and CTS group consisted of four fish meal replacement treatments including 0, 20, 40, and 60% fish meal content in diet (2 × 4 factorial arrangement; $N = 3$ per treatment). Following the 8-week trial, fish from the NS group were exposed to acute temperature stress (2-h heat shock at 30 and 2-h recovery at 19.5 , named as acute temperature-stressed group: ATS). Growth performance and stress- and immune-related measurements for stressed and non-stressed juveniles were investigated.

Results from the chronic exposure experiment showed that the CTS group had significantly lower final body weight (FBW), weight gain (WG), specific growth rate (SGR), feed efficiency (FE), condition factor, and viscerosomatic index than the NS group ($P < 0.05$). There was also a significant main effect of the fish meal replacement in both NS and CTS groups showing that FBW, WG, SGR, and FE generally decreased with increasing fish meal replacement level. There was a significant main effect of temperature stress on nutrient composition of the whole body,

showing that crude protein content of the CTS group was significantly higher than that of the NS group. A significant temperature stress main effect on plasma glucose (GLU), triglyceride (TG), total protein (TP), and glutamic oxaloacetic transaminase (GOT) levels was observed, exhibiting that those of the CTS group were significantly higher than the NS group. There was a significant main effect of fish meal replacement on plasma total cholesterol (TCHO) and TG, showing that the TCHO level was significantly increased with increasing fish meal replacement level, whereas the pattern of change in the TG level was inverse. There was no effect of temperature stress and fish meal replacement level on superoxide dismutase (SOD), immunoglobulin M (IgM), and lysozyme (LZM); however, a significant interaction between the stress and replacement level on glutathione peroxidase (GPX) activity was observed, showing that its level of the FM0 group was significantly higher than that of the FM20 and FM60 groups, whereas no effect of the temperature stress was detected. A significant main effect of the temperature stress on plasma cortisol level was observed, showing that the CTS group had a significantly higher cortisol level compared to the NS group. There was neither the temperature stress nor fish meal placement effects on relative gene expression levels of Adenosine monophosphate-activated protein kinase beta (AMPKβ), peroxidase proliferator-activated receptor gamma (PPARγ), heat shock protein 70 (HSP70), and glucose 6-phosphatase (G6pase) in liver tissue.

Results from the acute exposure experiment show that the overall pattern of changes in the plasmatic enzymes and metabolites were, to some extent, incomparable to those from the chronic exposure experiment. Plasma TCHO, TG, TP, and GOT levels of the FM0 diet were significantly higher than those of the FM60 diet, whereas plasma GLU and glutamic pyruvic transaminase (GPT) levels of the FM0 diet were significantly lower than those of the FM60 diet. There was no difference in plasma GPX, SOD, IgM, and LZM levels of the juveniles fed the experimental diets. Plasma cortisol and HSP70 levels were not significantly affected by the experimental diets. Relative expression levels of HSP70 and G6pase of the FM0 diet were significantly higher than those of the FM60 diet, whereas no difference in its levels of AMPKβ and PPARγ was observed.

Taken together, the overall pattern of changes in the juveniles fed the low fish meal contained diets when exposed to the chronic stress was, to some extent, incomparable to those from the acute exposure experiment. Thus, it is suggested that physiological responses of homeostasis from temperature stress could be variable between the chronic and acute exposure conditions, and the low fish meal fed olive flounder juveniles could be less tolerable to acute temperature stress.

사료 내 어분 대체가 만성 및 급성 고수온 스트레스에 노출된 넙치(*Paralichthys olivaceus*) 치어의 성장, 에너지 대사 및 스트레스 반응에 미치는 영향

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요약

최근 기후변화로 인한 지구 온난화로 수온 변동이 심해지는 등 급격한 환경 변화가 일어나고 있으며, 이로 인해 양식 어류의 대량 폐사 및 질병 발생이 발생하고 있다. 기후변화에 따른 어획량 감소로 어분 생산량은 시간이 지남에 따라 감소할 것으로 예측된다. 어분은 배합사료의 중요한 원료임에도 불구하고 지속가능한 양식을 위해서는 배합사료 내 어분 함량을 줄일 수밖에 없는 상황이다. 따라서, 본 연구는 한국, 일본, 중국 등 아시아 국가에서 상업적으로 중요한 양식 어종인 넙치(*Paralichthys olivaceus*)의 치어기를 대상으로 사료 내 어분 대체와 만성 및 급성 수온 스트레스에 따른 생리적 반응이 어떤 관계가 있는지 파악하기 위해 수행되었다.

평균 13.6 ± 0.02 g (평균 ± SEM)의 치어기 넙치 480마리를 24개의 직사각형 수조(수조당 20마리씩)에 무작위로 배치했다. 각 12개의 수조는 실험 기간 동안 19.5℃ 의 일정한 수온에서 사육된 그룹(비스트레스 그룹: NS) 또는 19.5℃에서 30℃까지 매주 1.5℃씩 상승된 수온에서 사육된 그룹(만성 수온 스트레스 그룹: CTS)으로 배치했다. 각 NS 및 CTS 그룹은 사료 내 어분 함량이 0, 20, 40, 60%인 네 가지 어분 대체 사료로 구성되었다(2×4 요인 배치, 실험구 당 $N = 3$). 8주간의 사육 실험 후 NS 그룹의 어류는 급성 열 스트레스에 노출되었다(30℃에서 2시간의 수온 스트레스 및 19.5℃에서 2시간 회복: ATS). 스트레스를 받은 치어기 넙치와 스트레스를 받지 않은 치어기 넙치의 성장 성능과 스트레스 및 면역 관련 측정을 조사했다.

만성 노출 실험 결과, CTS 그룹은 NS 그룹에 비해 최종 어체중(FBW), 증체율(WG), 일간 성장률(SGR), 사료 효율(FE), 비만도 및 내장 지수가 유의하게 낮았다(P <0.05). 또한 NS 그룹과 CTS 그룹 모두에서 어분 대체의 메인 이펙트가 있었으며, 어분 대체 수준이 증가함에 따라 FBW, WG, SGR 및 FE가 일반적으로 감소했다. 수온 스트레스가 전어체 일반 성분에 미치는 유의한 메인 이펙트가 있었으며, CTS 그룹의 조단백질 함량이 NS 그룹보다 유의하게 높았다. 혈장 포도당(GLU), 중성 지방(TG), 총 단백질(TP) 및 글루타민산 옥살로초산 트렌스

아미나제(GOT) 수치에 대한 수온에 따른 메인 이펙트가 유의하게 관찰되어 CTS 그룹의 수치가 NS 그룹보다 유의하게 높았음을 보여주었다. 혈장 총 콜레스테롤(TCHO)과 TG에는 어분 대체에 따라 유의한 차이가 있었으며, 어분 대체 수준이 증가함에 따라 TCHO 수치가 유의하게 증가한 반면, TG 수치의 변화 패턴은 반비례하는 것으로 나타났다. 온도 스트레스와 어분 대체 수준은 슈퍼옥시드 디스뮤타아제(SOD), 면역 글로불린 M (IgM), 라이소자임(LZM)에 미치는 영향은 없었지만, 글루타치온 과산화효소(GPX) 활성에는 스트레스와 대체 수준 간 유의한 상호작용이 나타나 FM0 그룹의 수준이 FM20 및 FM60 그룹보다 유의하게 높았지만 온도 스트레스의 영향은 발견되지 않았다. 혈장 코르티솔 수치에 대한 온도 스트레스의 유의한 메인 이펙트가 관찰되어 CTS 그룹이 NS 그룹에 비해 유의하게 높은 코르티솔 수치를 보였다. 간 조직에서 AMP-활성 단백질 효소 베타(AMPK β), 페록시좀 증식체 활성화 수용체 감마(PPAR γ), 열 충격 단백질 70 (HSP70), 포도당 6-인산(G6pase)의 상대적 유전자 발현 수준에는 수온 스트레스나 어분 대체 모두 영향을 미치지 않았다.

급성 노출 실험의 결과는 혈장 효소 및 대사 산물의 전반적인 변화 패턴이 만성 노출 실험의 결과와 어느 정도 비교할 수 없음을 보였다. FM0 실험구의 혈장 TCHO, TG, TP, GOT 수치는 FM60 실험구보다 유의하게 높았지만, FM0 실험구의 혈장 GLU와 글루타민산 피루빈산 트랜스아미나제(GPT) 수치는 FM60 실험구보다 유의하게 낮았다. 실험 사료를 먹은 넙치의 혈장 GPX, SOD, IgM 및 LZM 수준에는 차이가 없었다. 혈장 코르티솔과 HSP70 수치는 실험 사료에 의해 크게 영향을 받지 않았다. FM0 실험구의 HSP70 및 G6pase의 상대적 발현 수준은 FM60 실험구보다 유의하게 높았으나 AMPK β 및 PPAR γ 수준에는 차이가 관찰되지 않았다.

종합하면, 만성 스트레스에 노출되었을 때 어분 함량이 낮은 사료를 공급한 치어기 넙치의 전반적인 영양생화학적 반응 패턴과 급성 노출 실험의 반응 패턴은 일부 다르게 나타났다. 다시 말해, 수온 스트레스로 인한 항상성 유지의 생리적 반응은 만성 노출 조건과 급성 노출 조건에 따라 다를 수 있으며, 치어기 넙치에 저어분 사료를 공급할 시 급성 온도 스트레스에 비교적 취약할 수 있다고 판단된다.

. Introduction

The world population recently passed 8.0 billion in 2022 and will reach 9.7 billion in 2050 (United Nations, 2022). As the population grows, per capita consumption of seafood has increased steadily, from 9.9 kg in the 1960s to a peak of 20.5 kg in 2019 (FAO). Aquaculture production will increase to meet rising seafood consumption, by 2030, 62% of seafood production will come from farmed fish (World bank, 2013).

The rapidly growing aquaculture industry using fish meal as a major protein source in compounded feed. However, fish meal production is declining, and this is due to overfishing and climate change. As an example, Peru, the main source of fish meal and fish oil, has experienced production lag behind levels seen in previous years due to bad weather, area specific fishing restrictions and high catches of juveniles (FAO, 2022). In addition, 25% of the global catch is used for fish meal and fish oil, 90% of which is edible (Centanni, T. M., et al., 2014). Therefore, it is essential to develop fish meal substitutes to meet the demand of the aquaculture industry, reduce production costs, and foster a sustainable aquaculture industry.

Climate change is one of the challenges the aquaculture business needs to take into account. Water temperature is impacted by climate change, and since fish are poikilotherms, water temperature is one of the most crucial environmental elements for them (Lee, D., et al., 2023). Aquatic organisms, which are closely related to changes in aquatic environment, are exposed not only to continuous environmental changes but also to temporary and rapid environmental changes. Fish subjected to temperature

compression release cortisol in the blood, resulting in a primary compression response. Cortisol enhances the activity of the motor system (increases blood pressure and heart rate (Kwon, O., & Kim, T., 2023)) and increases secretion in the blood, generating the energy needed immediately. The creation of heat shock protein (HSP), which normalizes and repairs oxidative damage brought on by heat stress, is also exacerbated by catecholamine release (Alfonso, S., et al, 2021).

Olive flounder (*Paralichthys olivaceus*), one of the most farmed fish species in Korea, accounts for more than 50% of Korean fish production (Korean Statistics Information Service; KOSIS, 2023). Despite the fact that olive flounder is eurythermal (Cho, S. H., et al., 2012), it suffers mass mortality result of rapid water temperature rise due to climate change, especially in summer. The issue of replacing fish meal and the rising water temperature brought on by climate change must be resolved for flounder farming to be sustainable. Therefore, this study was conducted to better understand how flounder fed diets with different fish meal contents responded to energy metabolism, stress and immune response when exposed to chronic or acute water temperature stress.

. Materials and methods

1. Experimental diets preparation

Formulation and chemical composition of the experimental diets are listed in Table 1. All of the dietary ingredients were obtained commercially. On the basis of almost isonitrogenous (55% crude protein), isolipidic (12% total lipid), and isocaloric (3948 kJ kg^{-1} gross energy) diets, four experimental diets were formulated. A 60% fish meal-based diet served as the control diet (FM60). Fish meal protein was gradually replaced at 33, 66 and 100% and designated as FM40 FM20 and FM0 respectively. Experimental diets were formulated by combining animal (tankage meal and poultry by-product meal) and plant (soybean meal and soy protein concentrate; SPC) protein sources to complete amino acid profile of the experimental diets (Table 2) and as attractants and palatability enhancer. The lipid sources included fish oil and lecithin, and the carbohydrate or nitrogen-free extract sources included wheat flour. All of the dry ingredients were completely mixed for 15 minutes in an electric mixer (HYVM-1214, Hanyoung Food Machinery, Republic of Korea) to prepare the diets. Fish oil added to the dry ingredients and mixed for another 15 minutes. Water (45% of the dry ingredients) added to the premixed ingredients and mixed for another 15 minutes. The dough was passed through a pelletizing machine (SFD-GT, Shinsung, Republic of Korea) with a flat die of approximately 2 mm in diameter to prepare pellets, which were then air dried for 48-h until the moisture content of less than 10% (Table 1). The experimental diets were stored at -20 in a fridge until use.

Table 1. Formulation and proximate composition of the experimental diets for juvenile olive flounder (g kg^{-1})

	FM60	FM40	FM20	FM0
Fish meal (Anchovy)[1]	600	400	200	0
Starch[1]	50	50	50	50
Wheat flour[1]	176	145	117	89
Squid liver powder[1]	50	50	50	50
Soybean meal[1]	50	50	50	50
Poultry by product[1]	0	45	90	135
Soy protein concentrate[1]	0	45	90	135
Aquatide[1]	0	55	110	165
Tankage meal[1]	0	55	110	165
Fish oil[1]	30	45	55	65
Lecithin[1]	3	6	9	12
Betain[1]	3	6	9	12
Taurine[1]	3	6	9	12
Monocalcium phosphate[2]	3	6	9	12
Methionine[1]	1	2	4	6
Lysine[1]	1	2	4	6
Mineral mix[3]	12	12	12	12
Vitamin mix[4]	12	12	12	12
Vitamin C[1]	2	2	2	2
Choline[1]	4	6	8	10
Proximate analysis (% of dry matter basis)				
Moisture	9.24	9.89	9.10	9.47
Crude Protein	55.9	55.6	56.1	55.8
Crude Lipid	10.8	12.3	12.5	13.4
Crude Ash	11.9	11.4	10.9	10.7
Energy (kJ/KG)	3988	3978	3935	3892

[1] The feed Co. Goyang, Korea

[2] Duksan pure chemicals CO., LTD, Korea

[3] Mineral mix (as g/kg premix): Ferrous fumarate, 12.50; Manganese sulfate, 11.25; Dried ferrous sulfate, 20.0; Dried cupric sulfate, 1.25; Cobaltous sulfate, 0.75; Zinc sulfate KVP, 13.75; Cancium iodate, 0.75; Magnesium sulfate, 80.20; Aluminum Hydroxide, 0.75.

[4] Vitamin mix (as mg/kg premix): A, 1,000,000 IU; D, 200,000 IU; E, 10,000; B1, 2,000; B6, 1,500; B12, 10; C, 10,000; Calcium pantotenic acid, 5,000; Nicotinic acid 4,500; B-Biotin 10; Choline chloride, 30,000; Inositol, 5,000.

Table 2. Amino acids in the experimental diets (% in each diet sample)

	FM 60	FM 40	FM 20	FM 0
Essential				
Arginine	2.94	2.98	2.72	2.85
Histidine	1.96	1.75	1.59	1.76
Isoleucine	1.92	1.81	1.73	1.64
Leucine	3.27	3.30	3.17	3.28
Lysine	3.40	3.08	2.86	2.55
Methionine	1.14	1.03	1.04	0.96
Phenylalanine	1.82	1.84	1.73	1.78
Threonine	1.71	1.61	1.46	1.40
Valine	2.41	2.25	2.16	2.00
Non-Essential				
Aspartic Acid	3.92	3.76	3.55	3.52
Serine	1.67	1.70	1.64	1.59
Glutamic Acid	6.62	6.87	6.73	6.69
Proline	1.63	1.83	1.99	2.59
Glycine	2.68	2.82	2.88	3.00
Alanine	2.63	2.60	2.56	2.57
Tyrosine	0.86	1.01	0.93	0.83
Cysteine	1.35	1.26	1.18	1.15
	41.9	41.5	39.9	41.0

2. Experimental fish and temperature exposure

2.1 Chronic temperature stress exposure experiment (Experiment I)

The feeding trial was carried out at the Feeds and Foods Nutrition Research Center, Pukyong National University, Busan, Rep. of Korea. Juvenile olive flounders were obtained from a local fish farm located at Boryeong. The fish were acclimated in the lab environment for two weeks. The fish were fed an oxytetracycline-treated control food (FM60) during this period. Figure 1 shows that experimental design, at the beginning of the experiment, four hundred eighty juveniles with an initial body weight averaging 13.6 ± 0.0 g (mean ± SEM) were randomly distributed into each of the 24 rectangular tanks (20 fish per tank two separate systems were used for the experiment on the effects of chronic high temperatures. Each set of 12 tanks was divided into two groups: one raised at a constant temperature of 19.5°C (named as the non-stressed group: NS), and the other raised at a temperature gradually increased from 19.5°C to 30°C (1.5°C increment/week) (named as the chronic temperature-stressed group: CTS). The water temperature of the two groups was controlled by electronic thermostat (DOV-887, Daeil, Busan, Korea), and monitored by HOBO data logger (HOBO water temperature Pro v2 data logger-U22-001, Onset, Bourne, MA, USA) every 10 minutes throughout the trial (Figure 2). A flow rate of 2.7 L min^{-1} was maintained throughout the experimental period. Two times each day, at 9:00 and 17:00, all fish were manually fed their separate experimental diets in accordance with the growth and mortality of the olive flounder and in accordance with 2% of body weight. After feeding, 25% of the water was replaced by siphoning feces (50% every day). Because of the trial for heat

stress, the system was divided into two groups, although the two systems were identical. During the experimental period, the monitored water quality parameters (mean ± SEM) are shown in Table 3. Dissolved oxygen (DO) recorded by YSI DO meter (ProODO/T, Yellow Springs, OH, USA) CTS group has lower DO levels than NS group. As the water temperature increased, the oxygen saturation decreased, and the dissolved oxygen content of CTS group was recorded lower than that of NS Group.

2.2. Acute temperature stress exposure experiment (Experiment II)

After chronic temperature stress experiment for 8 weeks, an acute temperature stress experiment (ATS) was conducted with the NS group. 6 fish from treatment (2 fish per tank) were collected and transferred into a 216 L tank containing 4 sub-rearing units (perforated plastic containers; 30 × 30 × 30 cm^3). The temperatures for acute temperature stress were 30°C. The fish abruptly exposed to acute temperature stress for 2 hours at 30°C and the water temperature was controlled by and artificial thermostat. After exposure, fish were moved to 19.5°C and had a recovery period for 2 hours.

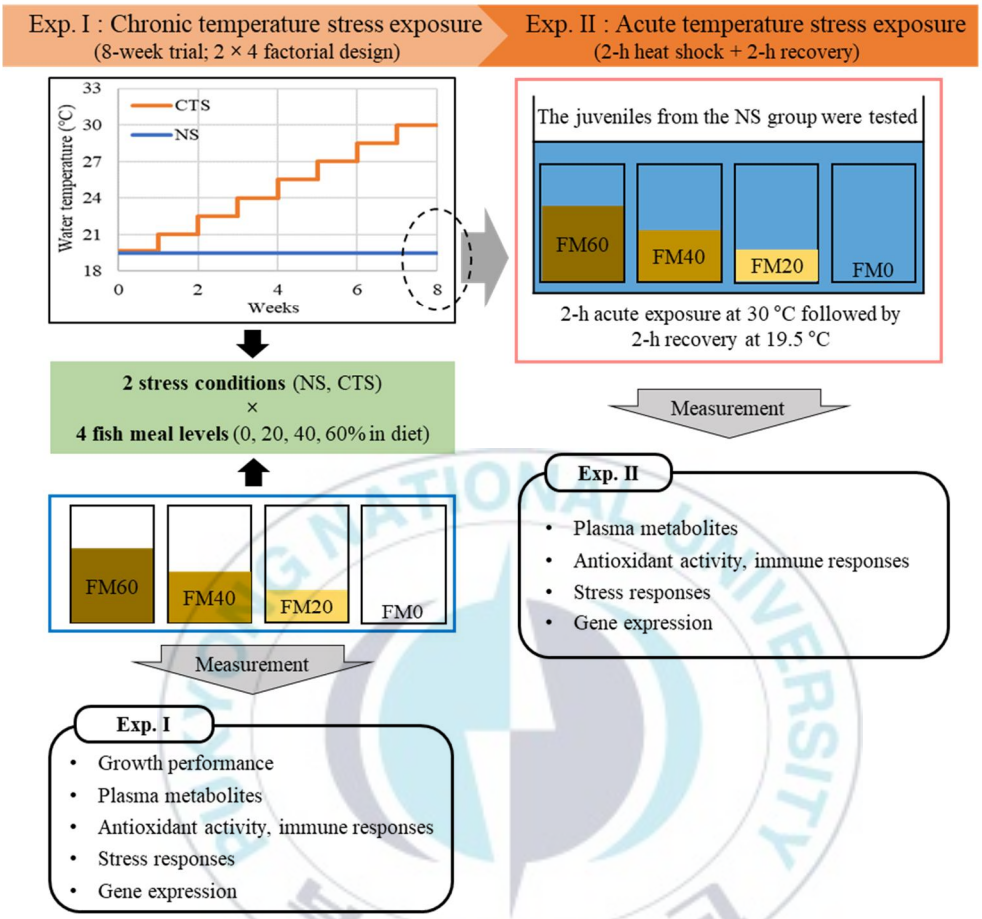


Figure 1. Schematic diagram of chronic (Exp. I) and acute (Exp. II) temperature stress exposure set-ups

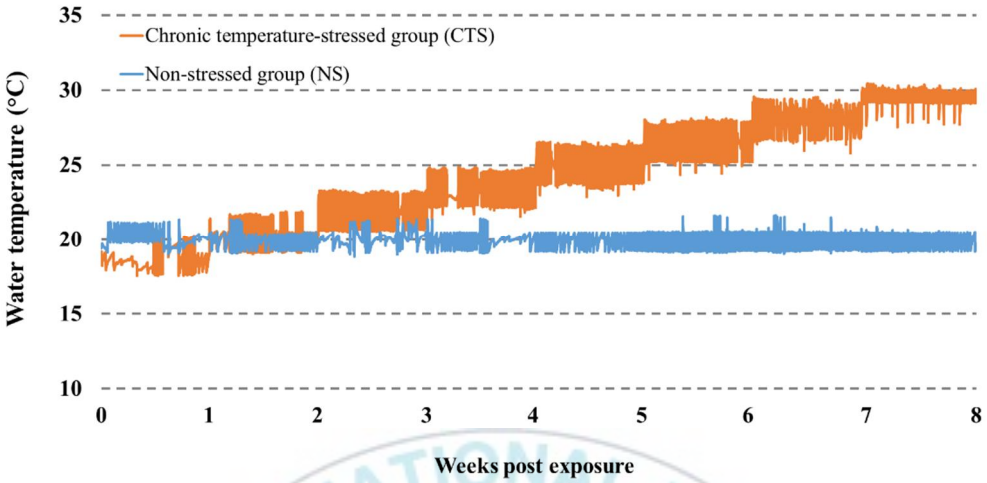


Figure 2. Water temperature records during the chronic temperature stress exposure experiment (Exp. I)

Table 3. Water quality parameters (mean ± SEM)

	Chronic Temperature Stressed Group	Non-Stressed Group
Ammonia[1] (mg/L)	1.21 ± 0.15	1.02 ± 0.15
Nitrite[1] (mg/L)	3.20 ± 0.19	3.45 ± 0.18
Nitrate[1] (mg/L)	64.5 ± 5.1	81.0 ± 5.6
DO[2] (mg/L)	6.47 ± 0.13	7.22 ± 0.04

[1] Tested by saltwater master test kit (API fish care)
[2] Dissolved oxygen tested by YSI DO meter (ProODO/T)

3. Sample collection and analysis

3.1. Growth performance

All fish in each tank were collected and weighed before at the terminal of the eight-week experimental period. Weight gain (WG), specific growth rate (SGR), feed efficiency (FE), survival rate (SR), hepatosomatic index (HSI), viscerosomatic index (VSI), and condition factor (CF) were calculated according to the following equations:

WG (%) = [final weight (g) – initial weight (g)] /initial weight (g) × 100.

SGR (%/day) = [ln final weight (g) – ln initial weight (g)] / days × 100.

FE (%) = (final weight (g) – initial weight (g)) /feed consumed (g) × 100.

SR (%) = (final number of fish/initial number of fish) × 100.

HSI (%) = liver weight (g)/body weight (g) × 100.

VSI (%) = viscera weight (g)/body weight (g) × 100.

CF = wet weight (g)/total length $(cm)^3$ × 100.

3.2. Diet and fish whole body proximate composition analysis

As for the general ingredients, four types of feed used in this experiment and three experimental olive flounder were randomly selected for each type of feed after the experiment, and the whole fish was analyzed. According to the Association of Official Analytical Chemists (AOAC, 2005) method, general component analysis was carried out. After three days of freeze-drying (Advantage 2.0, VirTis, New York, USA), samples were grounded. By drying to constant weight at 105°C for 24 hours and burning at 550°C in a muffle furnace for 3 hours, respectively, moisture and ash were calculated. Nitrogen (N × 6.25) content was measured by acid digestion with Kjeldahl method (2300 Auto analyzer, Foss Tecator. AB, Hoganas, Sweden). Soxtec system 1046 (Tecator AB, Hoganas, Sweden) was used with ether extraction to measure fat content.

3.3. Plasma metabolites analysis

Three fish were randomly taken from each tank for the plasma metabolites analysis. Blood samples were collected from the caudal vein of anesthetized fish using a 1-mL syringe that contained an anticoagulant (dipotassium ethylenediaminetetraacetic acid: EDTA). 1-mL of blood from each tank was collected into 3-ml siliconized vacuum tubes containing 5.4mg K2 EDTA (Becton Dickinson and Company, USA) and divide 1-mL into microtubes and centrifuged at 11,000 × *g* for 5 min and plasma was stored at – 84 until use. Glucose (GLU), total cholesterol (TCHO), triglyceride (TG), total protein (TP), glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) was analyzed using Fuji Film DRI-CHEM SLIDE in a dry biochemical analyzer (Fuji DRI-CHEM nx500i, Fuji Photo Film, Tokyo, Japan).

3.4. Antioxidant reaction, immune responses and stress responses

Glutathione peroxidase (GPX), superoxide dismutase (SOD), lysozyme (LZM), plasma immunoglobulin M (IgM), cortisol and heat shock protein 70 (HSP70) concentration were measured by enzyme-linked immunosorbent assay (ELISA) quantification kits (CUSABIO, Huston, TX, USA). Each standard reagent and plasma sample were analyzed as the manual methods. After entire procedure, the optical density was determined at 450 nm using microplate reader (AMR-100, Allsheng, Hangzhou, China) within 5 min. The measurements of CTS and NS samples were performed in triplicate. The measurements of ATS samples were performed in duplicate.

3.5. Gene expression of liver

Total RNA was extracted from the liver by using Hybrid-R^{TM} (GeneAll, Seoul, Korea). Homogenize ~100 mg of liver tissue samples in 1 mL RiboEx^{TM} using homogenizer. Incubate the homogenate for 5 min at room temperature. Centrifuge at 12,000 × *g* for 10 min at 4 and transfer the supernatant to a fresh tube. Add 200 µl chloroform per 1 mL RiboEx^{TM}. Shake vigorously for 15 sec and incubate for 2 min at room temperature. Centrifuge at 12,000 × *g* for 15 min at 4 and transfer the aqueous phase to a fresh tube. Add 1 volume of buffer RB1 to the sample and mix thoroughly by inverting. Transfer up to 700 µl of the mixture to a mini column type F. Centrifuge at 10,000 × *g* for 30 sec at room temperature. Add 500 µl buffer SW1 to the mini column. Centrifuge at 10,000 × *g* for 30 sec at room temperature. Add 500 µl buffer RNW to the mini column. Centrifuge at 10,000 × *g* for 30 sec at room temperature. Centrifuge at 10,000 × *g* for an additional 1 min at room temperature to remove residual wash buffer. Transfer the mini column to a new 1.5 mL microcentrifuge tube. Add 50~100 µl nuclease-free water to the center of the membrane in the mini column. Centrifuge at 10,000 × *g* for 1 min at room temperature. The concentration and purity of RNAs were checked with a NanoDrop ASP-2680 spectrophotometer (ACTgene, Piscataway, USA). The purified RNA is free of DNA and proteins, and A_{260}/A_{280} was 2.21 ± 0.01 (mean ± SEM).

Following the manufacturer's instructions, complementary DNA (cDNA) was synthesized using the PrimeScriptTM1st strand cDNA synthesis kit (Takara Bio in Kusatsu, Shiga, Japan). The synthesized cDNA was then stored at -20°C until Real-time PCR analysis.

The obtained cDNAs were used as templates for quantitative real-time PCR (qPCR) to assess the mRNA expression levels of AMPKβ, PPARγ, HSP70, and G6pase. B-actin was used as the internal controls and its stability and efficiency have been verified in olive flounder (Zheng, W. J., & Sun, L., 2011; Hu, J., et al., 2014). The reactions were performed on the StepOne Real-Time PCR system (Applied Biosystems, Waltham, Massachusetts, USA) according to the instruction of TB Green© *Premix Ex Taq*™II (Tli RNaseH Plus) (Takara Bio, Kusatsu, Shiga, Japan). The primer sequences for target genes in this study are listed in Table 4. The qPCR program was 95 for 30 sec, followed by 40 cycles of 95 for 5 sec, 60 for 30 sec. After the reaction, a melting curve analysis was carried out to verify the products' specificity. For each selected gene, qPCR reactions were performed on three replicate samples. The mRNA levels of the tested genes were normalized to the corresponding β-actin value and analyzed using the $2^{-\Delta\Delta Ct}$ method. After the PCR run, data analysis was performed with StepOne Software version 2.0 (Applied Biosystems, USA).

Table 4. List of primers used for liver gene expression

Genes	Primer sequences (5'>3')	Product size (bp)	Accession number
β-actin	F: GGAATCCACGAGACCACCTACA R: CTGCTTGCTGATCCACATCTGC	264	XM_020109620.1
AMPKβ[1]	F: CCGGGCCATATCATCAGGAC R: TTGTAGCGATGTGTCGCACT	223	XM_020103523.1
PPARγ[2]	F: GCCATCCTCTCTGGGAAGACCG R: CAGCGCCATGTCACTGTCGTCC	558	XM_020096840.1
HSP70[3]	F: TTCAATGATTCTCAGAGGCAAGC R: TTATCTAAGCCGTAGGCAATCGC	113	XM_020089177.1
G6pase[4]	F: GGGAGCCGCTGGTGTCTAC R: GGCCTTCAGGTACCACTCTTTG	112	XM_020109321.1

[1]Adenosine monophosphate-activated protein kinase beta (AMPKβ)
[2]Peroxidase proliferator-activated receptor gamma (PPARγ)
[3]Heat shock protein 70 (HSP70)
[4]Glucose 6-phosphatase (G6pase)

3.6. Statistical analysis

The data were analyzed by two-way analysis of variance (ANOVA) to determine the effect of the independent variables, i.e., temperature (NS, CTS) and experiment diets (FM60, FM40, FM20, FM0) and the possible interactions between these variables. Data of the ATS group were processed using one-way ANOVA. Tukey's honestly significant difference (HSD) test was used to compare the means. $P < 0.05$ was regarded as statistically significant. All statistical analyses were tested using SAS analytical software, version 9.4 (SAS Institute, Cary, NC, USA). The data are presented as the mean and standard error of the mean (mean ± SEM).

3.7. Ethics Statement

The experiment was followed under the guidelines of Institutional Animal Care and Use Committee Regulations, PKNUIACUC-2022-29, issued by the Pukyong National University, Busan, Rep. of Korea. Every effort was taken to minimize fish suffering.

. Results

1. Chronic temperature stress

NS Group and CTS Group analysis results after 8 weeks of feeding experiment.

1.1. Growth performance

The growth performance parameters of juvenile olive flounder are shown in Table 5. The highest FBW, WG, SGR and FE were obtained in NSFM60. FBW, WG, SGR, FE, CF and VSI significantly reduced when juvenile olive flounder exposed to chronic temperature stress ($P < 0.05$). FBW, WG, SGR and FE were decrease. SR was 91.7%-100% without significant differences ($P > 0.05$).

Table 5. Growth performance of juvenile olive flounder (initial body weight: 13.6 ± 0.02 g ; mean ± SEM) fed the experimental diets (FM60, FM40, FM20, FM0) for 8 weeks

Diets	FBW(g)[1]	WG(%)[2]	SGR(%)[3]	FE(%)[4]	SR(%)[5]	CF[6]	HSI(%)[7]	VSI(%)[8]
Interactive effect between temperature stress and fish meal								
NSFM60	42.8 ± 1.3^{a}	213 ± 8^{a}	2.04 ± 0.05^{a}	132 ± 6^{a}	98.3 ± 1.7^{ns}	1.14 ± 0.02^{a}	1.09 ± 0.11^{ns}	3.70 ± 0.12^{bc}
NSFM40	40.0 ± 0.7^{ab}	194 ± 4^{ab}	1.92 ± 0.02^{ab}	123± 1^{ab}	93.3 ± 3.3	1.04 ± 0.08^{ab}	1.02 ± 0.08	3.47 ± 0.25^{bc}
NSFM20	38.8 ± 0.8^{abc}	184 ± 5^{abc}	1.86 ± 0.03^{ab}	120 ± 4^{ab}	98.3 ± 1.7	1.12 ± 0.04^{a}	1.03 ± 0.06	4.55 ± 0.19^{ab}
NSFM0	35.7 ± 1.0^{bcd}	162 ± 7^{bc}	1.72 ± 0.05^{b}	107 ± 5^{bcd}	100 ± 0.0	1.15 ± 0.04^{a}	1.69 ± 0.57	5.26 ± 0.62^{a}
CTSFM60	34.9 ± 1.3^{cde}	158 ± 9^{cd}	1.69 ± 0.06^{bc}	98.6 ± 4.5^{cde}	98.3 ± 1.7	0.89 ± 0.02^{b}	1.30 ± 0.16	2.55 ± 0.02^{c}
CTSFM40	31.1 ± 0.4^{edf}	128 ± 2de	1.47 ± 0.02^{cd}	85.9 ± 3.7^{de}	96.7 ± 1.7	0.91 ± 0.05^{b}	1.15 ± 0.11	2.44 ± 0.10^{c}
CTSFM20	30.2 ± 1.2^{ef}	122 ± 7^{e}	1.42 ± 0.06^{d}	82.2 ± 5.7^{e}	91.7 ± 3.3	0.90 ± 0.12^{b}	1.29 ± 0.06	3.14 ± 0.25^{bc}
CTSFM0	29.5 ± 1.2^{f}	117 ± 9^{e}	1.38 ± 0.08^{d}	82.0 ± 5.9^{e}	96.7 ± 1.7	0.88 ± 0.01^{b}	1.19 ± 0.25	2.89 ± 0.33^{c}
Main effect of temperature								
Non-stressed	39.3 ± 1.5^{a}	188 ± 11^{a}	1.89 ± 0.07^{a}	120 ± 5^{a}	97.5 ± 1.4^{ns}	1.11 ± 0.02^{a}	1.21 ± 0.16^{ns}	4.25 ± 0.41^{a}
Temperature stressed	31.5 ± 1.2^{b}	131 ± 9^{b}	1.49 ± 0.07^{b}	87.2 ± 3.9^{b}	95.8 ± 1.4	0.90 ± 0.01^{b}	1.24 ± 0.04	2.75 ± 0.16^{b}
Main effect of fish meal								
FM60	38.9 ± 4.0^{a}	186 ± 28^{a}	1.86 ± 0.17^{a}	115 ± 16^{a}	98.3 ± 0.0^{ns}	1.02 ± 0.13^{ns}	1.20 ± 0.11^{ns}	3.12 ± 0.58^{bc}
FM40	35.5 ± 4.4^{b}	161 ± 33^{b}	1.70 ± 0.23^{b}	104 ± 18^{ab}	95.0 ± 1.7	0.98 ± 0.07	1.09 ± 0.07	2.95 ± 0.51^{c}
FM20	34.5 ± 4.3^{b}	153 ± 31^{bc}	1.64 ± 0.22^{bc}	101 ± 19^{b}	95.0 ± 3.3	1.01 ± 0.11	1.16 ± 0.13	3.35 ± 0.70^{ab}
FM0	32.6 ± 3.1^{b}	140 ± 22^{c}	1.55 ± 0.17^{c}	94.7 ± 12.6^{b}	98.3 ± 1.7	1.01 ± 0.13	1.44 ± 0.25	4.08 ± 1.19^{a}
Two-way ANOVA (*p*-value)								
Stress	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.2837	< 0.0001	0.8754	< 0.0001
FM	0.0001	< 0.0001	< 0.0001	0.0026	0.2195	0.7188	0.4958	0.0031
Stress × FM	0.5588	0.4351	0.5225	0.5106	0.1472	0.3364	0.3687	0.1300

[1]Final body weight (FBW, g)
[2]Weight gain (WG, %) = (final weight – initial weight) /initial weight ×100
[3]Specific growth rate (SGR, %/day) = (ln final weight - ln initial weight) / days of trial × 100
[4]Feed efficiency (FE, %) = (final weight – initial weight) × 100/dry feed intake (g)
[5]Survival rate (SR, %) = (total number of fish – number of dead fish) / total number of fish ×100
[6]Condition factor (CF) = final weight / total length^{3} × 100
[7]Hepatosomatic index (HSI, %) = liver weight / final weight × 100
[8]Viscerosomatic index (VSI, %) = viscera weight / final weight × 100

1.2. Whole-body proximate compositions

Results of the proximate chemical composition of juvenile olive flounder fed the experimental diets under chronic temperature stress and non-stress condition are shown in Table 6. There was not significant variation in the crude lipid ($P > 0.05$). Moisture has a main effect at both temperature and fish meal ($P < 0.05$). When the main effect is temperature, the CTS group shows a significantly lower value than the NS group, when the main effect is fish meal, FM60 shows the highest value, and the moisture content tends to decrease as the fish meal content in the feed decreases. Crude protein and crude ash show main effects in temperature, both of which are high in CTS ($P < 0.05$). There is no main effect of fish meal ($P > 0.05$).

Table 6. Whole-body proximate compositions[1] (%, As-is) of juvenile olive flounder (mean ± SEM)

Diets	Moisture	Crude protein	Crude lipid	Crude ash
Interactive effect between temperature stress and fish meal				
NSFM60	76.1 ± 0.2^{ab}	11.9 ± 0.2^{abc}	1.98 ± 0.13^{ns}	3.88 ± 0.08^{c}
NSFM40	76.7 ± 0.4^{a}	11.5 ± 0.2^{c}	2.29 ± 0.19	3.76 ± 0.04^{c}
NSFM20	76.8 ± 0.3^{a}	11.4 ± 0.2^{c}	2.26 ± 0.24	3.78 ± 0.10^{c}
NSFM0	75.9 ± 0.5^{ab}	11.8 ± 0.2^{bc}	2.31 ± 0.03	4.10 ± 0.16^{bc}
CTSFM60	75.8 ± 0.7^{ab}	12.2 ± 0.4^{abc}	1.96 ± 0.29	4.62 ± 0.08^{ab}
CTSFM40	74.2 ± 0.2^{bc}	12.8 ± 0.2^{ab}	2.50 ± 0.35	4.79 ± 0.09^{a}
CTSFM20	74.4 ± 0.0^{bc}	12.8 ± 0.1^{ab}	2.45 ± 0.38	4.73 ± 0.18^{a}
CTSFM0	73.6 ± 0.4^{c}	13.0 ± 0.1^{a}	2.77 ± 0.63	4.87 ± 0.17^{a}
Main effect of temperature				
Non-stressed	75.8 ± 0.1^{a}	11.9 ± 0.0^{b}	2.32 ± 0.04^{ns}	4.07 ± 0.03^{b}
Temperature stressed	74.5 ± 0.2^{b}	12.7 ± 0.1^{a}	2.42 ± 0.07	4.75 ± 0.02^{a}
Main effect of fish meal				
FM60	75.9 ± 0.3^{a}	12.0 ± 0.1^{ns}	1.97 ± 0.08^{ns}	4.25 ± 0.00^{ns}
FM40	75.5 ± 0.1^{ab}	12.2 ± 0.0	2.39 ± 0.08	4.27 ± 0.03
FM20	75.6 ± 0.2^{ab}	12.1 ± 0.1	2.36 ± 0.07	4.25 ± 0.04
FM0	74.7 ± 0.0^{b}	12.4 ± 0.1	2.54 ± 0.3	4.48 ± 0.00
Two-way ANOVA (*p*-value)				
Stress	<.0001	<.0001	0.3744	<.0001
FM	0.0480	0.3949	0.3736	0.2068
Stress × FM	0.0325	0.1015	0.9084	0.5737

[1]After the 8-week feeding experiment was over, 9 fish per experimental group were analyzed (3 fish per tank).

1.3. Plasma metabolites

Table 7 shows the plasma metabolites analysis of fish after feeding experiment. Results revealed that the experimental diets and chronic heat stress significantly affected some plasma metabolites parameters ($P < 0.05$). GLU, TG, TP, and GOT have main effect of temperature ($P < 0.05$). High values of GLU, TCHO, and GOT were detected in the CTS group, while TP decreased. The main effect on fish meal was observed in TCHO and TG ($P < 0.05$). TCHO is the highest value in FM60 and tends to decrease as the fish meal content in experimental feed decreases, and TG shows the opposite tendency.

Table 7. Plasma metabolites of juvenile olive flounder (initial body weight: 13.6 ± 0.02 g ; mean ± SEM) fed the experimental diets (FM60, FM40, FM20, FM0) for 8 weeks

Diets	GLU[1]	TCHO[2]	TG[3]	TP[4]	GOT[5]	GPT[6]
Interactive effect between temperature stress and fish meal						
NSFM60	33.3 ± 7.9^{c}	123 ± 3^{abc}	157 ± 10^{d}	2.83 ± 0.03^{bc}	24.7 ± 0.7^{ns}	18.7 ± 1.2^{ns}
NSFM40	33.3 ± 2.3^{c}	126 ± 5^{ab}	267 ± 21^{bcd}	2.83 ± 0.09^{bc}	28.3 ± 3.5	17.7 ± 1.7
NSFM20	40.7 ± 4.4^{abc}	115 ± 6^{abc}	373 ± 15^{b}	2.93 ± 0.09^{abc}	28.3 ± 1.8	21.3 ± 3.5
NSFM0	38.7 ± 11.3^{bc}	108 ± 5^{abc}	547 ± 50^{a}	2.73 ± 0.07^{c}	27.7 ± 4.1	20.3 ± 3.2
CTSFM60	67.0 ± 5.1^{abc}	133 ± 2^{a}	192 ± 15^{cd}	3.57 ± 0.12^{a}	31.7 ± 22	20.0 ± 1.2
CTSFM40	74.3 ± 6.2^{a}	116 ± 8^{abc}	198 ± 14^{cd}	3.33 ± 0.19^{abc}	32.3 ± 6.4	24.0 ± 3.5
CTSFM20	71.0 ± 5.1^{ab}	98 ± 7^{c}	216 ± 14^{cd}	3.43 ± 0.20^{ab}	41.0 ± 5.3	24.7 ± 2.9
CTSFM0	53.7 ± 8.7^{abc}	103 ± 3^{bc}	304 ± 53^{bc}	3.10 ± 0.21^{abc}	34.0 ± 5.1	23.3 ± 2.4
Main effect of temperature						
Non-stressed	36.5 ± 1.9^{b}	118 ± 4^{ns}	336 ± 83^{a}	2.83 ± 0.04^{b}	27.3 ± 0.9^{b}	19.5 ± 0.8^{ns}
Temperature stressed	66.5 ± 4.5^{a}	113 ± 8	227 ± 26^{b}	3.36 ± 0.10^{a}	34.8 ± 2.1^{a}	23.0 ± 1.0
Main effect of fish meal						
FM60	50.2 ± 16.8	128 ± 5^{a}	175 ± 18^{c}	3.20 ± 0.37^{ns}	28.2 ± 3.5^{ns}	19.3 ± 0.7^{ns}
FM40	53.8 ± 20.5	121 ± 5^{ab}	232 ± 35^{bc}	3.08 ± 0.25	30.3 ± 2.0	20.8 ± 3.2
FM20	55.8 ± 15.2	107 ± 9^{bc}	295 ± 79^{b}	3.18 ± 0.25	34.7 ± 6.3	23.0 ± 1.7
FM0	46.2 ± 7.5	106 ± 2^{c}	426 ± 122^{a}	2.92 ± 0.18	30.8 ± 3.2	21.8 ± 1.5
Two-way ANOVA (*p*-value)						
Stress	<.0001	0.1709	<.0001	<.0001	0.0190	0.0749
FM	0.5327	0.0012	<.0001	0.1966	0.4701	0.5567
Stress × FM	0.3231	0.1109	0.0013	0.6231	0.7489	0.8101

[1] GLU: Glucose (mg/dL)
[2] TCHO: Total cholesterol (mg/dL)
[3] TG: Triglyceride (mg/dL)
[4] TP: Total protein (g/dL)
[5] GOT: Glutamic oxaloacetic transaminase (U/L)
[6] GPT: Glutamic pyruvic transaminase (U/L)

1.4. Antioxidants and immune responses

Result of the immune response and antioxidants of olive flounder exposed to chronic temperature stress for 8 weeks are illustrated in Table 8. There was not significant difference in SOD, LZM, and IgM ($P > 0.05$). Main effects were observed only in GPX, and there is a main effect on fish meal ($P < 0.05$). FM0 was the highest, followed by FM40, FM60, and FM20, with no trend.

Table 8. Antioxidants and immune responses[1] of juvenile olive flounder exposed to chronic temperature stress for 8 weeks (mean ± SEM)

Diets	GPX[2]	SOD[3]	IgM[4]	LZM[5]
Interactive effect between temperature stress and fish meal				
NSFM60	54.8 ± 2.6^{abc}	302 ± 9^{ns}	2.22 ± 0.50^{ns}	1.66 ± 0.12^{ns}
NSFM40	59.7 ± 6.2^{ab}	264 ± 11	2.94 ± 0.36	1.47 ± 0.19
NSFM20	55.1 ± 0.6^{abc}	227 ± 31	2.93 ± 0.22	1.83 ± 0.28
NSFM0	50.4 ± 4.7^{bc}	294 ± 41	2.89 ± 0.80	1.63 ± 0.25
CTSFM60	45.2 ± 4.3^{bc}	222 ± 53	3.34 ± 0.36	1.41 ± 0.30
CTSFM40	56.9 ± 4.4^{ab}	196 ± 13	2.60 ± 0.86	1.46 ± 0.04
CTSFM20	35.6 ± 3.0^{c}	215 ± 78	4.19 ± 0.72	1.63 ± 0.18
CTSFM0	74.8 ± 5.5^{a}	304 ± 27	3.28 ± 0.59	1.79 ± 0.20
Main effect of temperature				
Non-stressed	55.0 ± 1.9^{ns}	272 ± 17^{ns}	2.74 ± 0.17^{ns}	1.65 ± 0.07^{ns}
Temperature stressed	53.1 ± 8.4	234 ± 24	3.35 ± 0.33	1.57 ± 0.09
Main effect of fish meal				
FM60	50.0 ± 4.8^{bc}	262 ± 40^{ns}	2.78 ± 0.56^{ns}	1.54 ± 0.12^{ns}
FM40	58.3 ± 1.4^{ab}	230 ± 34	2.77 ± 0.17	1.46 ± 0.00
FM20	45.3 ± 9.8^{c}	221 ± 6	3.56 ± 0.63	1.73 ± 0.10
FM0	62.6 ± 12.2^{a}	299 ± 5	3.08 ± 0.19	1.71 ± 0.08
Two-way ANOVA (*p*-value)				
Temperature Stress	0.5404	0.2034	0.1652	0.6269
Fish meal	0.0038	0.2258	0.5152	0.5357
Stress*FM	0.0007	0.6278	0.5258	0.7605

[1]Test by Elisa kit (CUSABIO)
[2]GPX: Glutathione peroxidase (mU/mL)
[3]SOD: Superoxide dismutase (ng/mL)
[4]IgM: Immunoglobulin M (μg/mL)
[5]LZM: Lysozyme (renal amyloidosis) (μg/mL)

1.5. Stress response

Cortisol and heat shock protein 70 (HSP70) were analyzed as stress responses (Table 9), and HSP70 had no main effect on temperature and fish meal ($P > 0.05$). Cortisol showed a main effect on temperature, and cortisol levels increased in the CTS group.

Table 9. Stress responses[1] of juvenile olive flounder exposed to chronic temperature stress for 8 weeks (mean ± SEM)

Diets	Cortisol[2]	HSP70[3]
Interactive effect between temperature stress and fish meal		
NSFM60	11.2 ± 2.2^{ns}	73.6 ± 24.7^{ns}
NSFM40	9.37 ± 0.70	58.1 ± 9.2
NSFM20	9.59 ± 0.36	121 ± 33
NSFM0	8.92 ± 0.17	139 ± 3
CTSFM60	11.7 ± 0.5	82.4 ± 15.1
CTSFM40	12.6 ± 0.7	125 ± 43
CTSFM20	12.2 ± 0.6	108 ± 26
CTSFM0	10.6 ± 1.4	73.2 ± 25.1
Main effect of temperature		
Non-stressed	9.76 ± 0.49^{b}	98.0 ± 19.2^{ns}
Temperature stressed	11.7 ± 0.4^{a}	97.3 ± 11.9
Main effect of fish meal		
FM60	11.4 ± 0.2^{ns}	78.0 ± 4.4^{ns}
FM40	11.0 ± 1.6	91.7 ± 33.6
FM20	10.9 ± 1.3	115 ± 6
FM0	9.74 ± 0.82	106 ± 33
Two-way ANOVA (*p*-value)		
Temperature Stress	0.0169	0.9705
Fish meal	0.4499	0.5054
Stress*FM	0.5994	0.1118

[1]Test by Elisa kit (CUSABIO)
[2]Cortisol (ng/mL)
[3]HSP70: Heat shock protein 70 (pg/mL)

1.6. Relative gene expression level of chronic temperature stress exposure

The results of relative gene expression level of chronic temperature exposure are in table 10. There is main effect of temperature in AMPKβ, HSP70, and G6pase ($P < 0.05$). PPARγ has no main effect for both temperature and fish meal ($P > 0.05$). AMPKβ, PPARγ, and G6pase showed no main effect of fish meal ($P > 0.05$), but HSP70 showed a significant difference with fish meal ($P < 0.05$). The highest HSP70 was observed at FM40, followed by FM20, FM60, and FM0.

Table 10. Relative gene expression[1] of chronic temperature stress exposure (mean ± SEM)

Diets	AMPKβ[2]	PPARγ[3]	HSP70[4]	G6pase[5]
Interactive effect between temperature stress and fish meal				
NSFM60	1.01 ± 0.07^{ns}	1.56 ± 0.98^{ns}	1.04 ± 0.19^{d}	1.26 ± 0.48^{ns}
NSFM40	1.15 ± 0.14	0.84 ± 0.27	4.24*^{cd}	2.29 ± 0.88
NSFM20	1.71 ± 0.42	33.8 ± 18.5	3.48 ± 1.21^{d}	1.03 ± 0.31
NSFM0	1.08 ± 0.42	15.0 ± 12.3	1.41 ± 0.39^{d}	3.00 ± 2.41
CTSFM60	6.68 ± 2.84	2.08 ± 1.77	133 ± 7^{bc}	4.07 ± 1.74
CTSFM40	4.78 ± 3.26	0.84 ± 0.58	702 ± 19^{a}	6.56 ± 1.27
CTSFM20	5.47 ± 3.07	0.32 ± 0.21	659 ± 62^{a}	3.05 ± 0.98
CTSFM0	10.6 ± 0.4	0.12 ± 0.01	288*^{b}	5.74 ± 1.21
Main effect of temperature				
Non-stressed	1.24 ± 0.16^{b}	12.8 ± 7.7^{ns}	2.3 ± 0.8^{b}	1.90 ± 0.46^{b}
Temperature stressed	6.54 ± 1.29^{a}	0.91 ± 0.44	468 ± 140^{a}	4.77 ± 0.80^{a}
Main effect of fish meal				
FM60	3.84 ± 2.84^{ns}	1.82 ± 0.26^{ns}	53.9 ± 66.0^{b}	2.67 ± 1.40^{ns}
FM40	2.96 ± 1.81	0.84 ± 0.00	469 ± 349^{a}	4.43 ± 2.13
FM20	3.59 ± 1.88	17.1 ± 16.8	266 ± 328^{a}	2.04 ± 1.01
FM0	4.88 ± 4.74	9.08 ± 7.46	97.1 ± 143.5^{b}	4.10 ± 1.37
Two-way ANOVA (*p*-value)				
Stress	0.0013	0.0621	<.0001	0.0076
FM	0.5743	0.2165	<.0001	0.2294
Stress × FM	0.4925	0.1681	<.0001	0.8544

[1] Relative gene express level (FM60 was set as a control; beta actin used as housekeeping gene)
[2] AMPKβ: Adenosine monophosphate-activated protein kinase beta
[3] PPARγ: peroxisome proliferator-activated receptor gamma
[4] HSP70: heat shock protein 70
[5] G6pase: glucose 6-phosphatase
* Outliers were excluded

2. Acute temperature stress

In acute temperature stress exposure trial, fish were abruptly exposed to temperature at 30 for 2 hours for heat stress then moved to temperature at 19.5 for 2 hours for recovery.

2.1. Plasma metabolites

Plasma metabolites result of juvenile olive flounder exposed to acute temperature stress at 30 are in Table 11. GLU is significantly higher at ATSFM60 followed by ATSFM40, ATSFM20, and ATSFM0 ($P < 0.05$). TCHO and TG are the highest levels in ATSFM0 and shown the opposite trend to GLU ($P < 0.05$). GOT is the highest level in ATSFM40 and there is no trend ($P < 0.05$). GPT is highest in ATSFM60 followed by ATSFM40.

Table 11. Plasma metabolites of juvenile olive flounder exposed to acute temperature stress at 30 (mean ± SEM)

Diets	GLU[1]	TCHO[2]	TG[3]	TP[4]	GOT[5]	GPT[6]
ATSFM60	47.0 ± 1.0^{a}	113 ± 2^{c}	130 ± 3^{d}	2.43 ± 0.07^{b}	15.0 ± 1.0^{c}	13.3 ± 0.3^{a}
ATSFM40	27.3 ± 0.3^{b}	138 ± 2^{b}	319 ± 2^{c}	2.80 ± 0.00^{a}	18.0 ± 0.0^{a}	12.0 ± 0.0^{b}
ATSFM20	26.5 ± 0.3^{bc}	133 ± 0^{b}	404 ± 3^{b}	2.70 ± 0.00^{a}	15.3 ± 0.3^{bc}	10.3 ± 0.3^{c}
ATSFM0	24.3 ± 0.3^{c}	164 ± 2^{a}	1318 ± 9^{a}	2.77 ± 0.03^{a}	17.7 ± 0.3^{ab}	11.0 ± 0.0^{bc}
p-value	<.0001	<.0001	<.0001	0.0005	0.0090	<.0001

[1] GLU: Glucose (mg/dL)
[2] TCHO: Total cholesterol (mg/dL)
[3] TG: Triglyceride (mg/dL)
[4] TP: Total protein (g/dL)
[5] GOT: Glutamic oxaloacetic transaminase (U/L)
[6] GPT: Glutamic pyruvic transaminase (U/L)

2.2. Antioxidants and immune responses

Table 12 shows the analysis results of the antioxidants and immune responses of the experimental fish. There was no significant difference between GPX, SOD, IgM, and LZM ($P > .05$).

Table 12. Antioxidants and immune responses[1] of juvenile olive flounder exposed acute temperature stress at 30 (mean ± SEM)

Diets	GPX[2]	SOD[3]	IgM[4]	LZM[5]
ATSFM60	182 ± 32[ns]	488 ± 8[ns]	1.14 ± 0.02[ns]	5.10 ± 0.24[ns]
ATSFM40	146 ± 26	467 ± 23	1.13 ± 0.02	5.71 ± 0.60
ATSFM20	133 ± 4	450 ± 44	1.16 ± 0.04	4.92 ± 0.71
ATSFM0	132 ± 1	469 ± 17	1.06 ± 0.02	6.45 ± 1.16
p-value	0.1365	0.4015	0.8432	0.5393

[1]Test by Elisa kit (CUSABIO)
[2]GPX: Glutathione peroxidase (mU/mL)
[3]SOD: Superoxide dismutase (ng/mL)
[4]IgM: Immunoglobulin M (μg/mL)
[5]LZM: Lysozyme (renal amyloidosis) (μg/mL)

2.3. Stress response

Table 13 shows the results of stress-related factor analysis of fish exposed to acute temperature stress at 30 . There was no significant difference in cortisol and HSP70 in all experimental treatment ($P > 0.05$).

Table 13. Stress responses[1] of juvenile olive flounder exposed acute temperature stress at 30°C (mean ± SEM)

Diets	Cortisol[2]	HSP70[3]
ATSFM60	4.16 ± 0.59[ns]	2.61 ± 9.46[ns]
ATSFM40	3.90 ± 0.32	5.60 ± 3.94
ATSFM20	4.37 ± 0.33	13.8 ± 4.0
ATSFM0	4.34 ± 0.36	11.4 ± 2.9
p-value	0.5475	0.7925

[1]Test by Elisa kit (CUSABIO)
[2]Cortisol (ng/mL)
[3]HSP70: Heat shock protein 70 (pg/mL)

2.4. Relative gene expression of acute temperature stress at 30

Relative gene expression result of juvenile olive flounder exposed to acute temperature stress at 30 are in Table 14. There is no significant difference in AMPKβ and PPARγ ($P > 0.05$). The expression level of HSP70 and G6pase of the juveniles exposed to acute stress was significantly increased with increasing fish meal replacement ($P < 0.05$).

Table 14. Relative gene expression[1] of acute temperature stress at 30 (mean ± SEM)

	AMPKβ[2]	PPARγ[3]	HSP70[4]	G6pase[5]
ATSFM60	1.04 ± 0.14^{ns}	1.14 ± 0.26^{ns}	1.08 ± 0.21^{b}	1.02 ± 0.08^{b}
ATSFM40	1.01 ± 0.16	1.59 ± 0.87	2.37 ± 0.96^{b}	0.89 ± 0.28^{b}
ATSFM20	1.10 ± 0.19	0.21 ± 0.07	2.83 ± 1.34^{b}	3.60 ± 1.28^{ab}
ATSFM0	0.62 ± 0.12	0.05 ± 0.01	7.65 ± 1.57^{a}	6.42 ± 1.71^{a}
p-value	0.1532	0.0788	0.0035	0.0047

[1] Relative gene express level (ATSFM60 was set as a control; beta actin used as housekeeping gene)
[2] AMPKβ: AMP-activated protein kinase beta
[3] PPARγ: peroxisome proliferator-activated receptor gamma
[4] HSP70: heat shock protein 70
[5] G6pase: glucose 6-phosphatase

. Discussion

1. Growth performance

In this study, the control group contained 60% fish meal in the diet, which is the typical fish meal content of olive flounder (Lee J., et al, 2012; Kim H. S., et al, 2014). FBW, WG, SGR, and FE showed main effects according to fish meal content and water temperature, respectively ($P < 0.05$). The main effect of fish meal, showing that WG, SGR, FE decreased with decreasing fish meal content. In addition, the main effect of temperature stress exhibiting that, the CTS group had reduced growth performance compared to the NS group. In CF, there was only the main effect by water temperature, and the group without temperature stress was significantly higher ($P < 0.05$). There was no significant difference between experimental groups in HSI ($P > 0.05$). In VSI, the main effect was shown for water temperature and fish meal replacement, respectively ($P < 0.05$). It was significantly lowered when exposed to chronic water temperature stress. FM40 showed the lowest value and showed a tendency to increase as the fish meal content in feed decreased.

2. Plasma metabolites

There was a difference tendency between TCHO and TG according to the fish meal content in the experimental feed. Both TCHO and TG are plasma metabolites related to lipid metabolism. However, the two showed opposite trends. As fish meal content decreased, TCHO decreased and TG increased. It has been reported that dietary soy products can inhibit lipogenic enzyme activity and consequently lower TG levels as well as TCHO levels in other fish species (Dias, J., et al., 2005; Lim, S. J., & Lee, K. J., 2009; Lim, S. J., et al., 2011). However, in the study of Yaghoubi, M., et al. (2016), TCHO decreased and TG increased as fish meal content decreased. This is because methionine and lysine are the main precursors of carnitine involved in fatty acid transport (Harpaz, 2005), so if the diet is deficient in methionine and lysine, it causes potential disorders in lipid metabolism in the liver. As shown in the table 1 and 2, although the contents of methionine and lysine were gradually increased in the feed formulation as fish meal was replaced in this study, the experimental feed amino acid analysis results gradually decreased, so it is judged that the corresponding result appeared. TCHO was the highest in the control group (FM60) with the highest fish meal content, and decreased as fish meal was replaced. Takagi, S., et al. (2001) found a similar trend of higher TCHO in fish meal based diets, while it was decreased in red sea bream (*Pagrus major*) fed high SPC based diets. Kader, M. A., & Koshio, S. (2012) also found that TCHO decreased with increasing soybean meal content in sea bream.

Regarding water temperature stress, GLU, TG, TP, and GOT showed significant differences ($P < 0.05$), and the values of parameters except TG significantly increased with exposure to chronic water temperature stress. GLU and GOT appear to

increase in response to stress (Abdel-Ghany, H. M., et al., 2023). When exposed to acute water temperature stress, unlike the results of the chronic stress exposure experiment, significant differences were shown in fish meal content for all plasma metabolites parameters. This indicates that juvenile responds differently to chronic or acute exposure to high temperature stress (Islam, M. J., et al., 2022). Studies on the effects of different fish meal contents in feed and stress exposure are not sufficient, so further research is needed.

3. Antioxidants, immune responses and stress responses

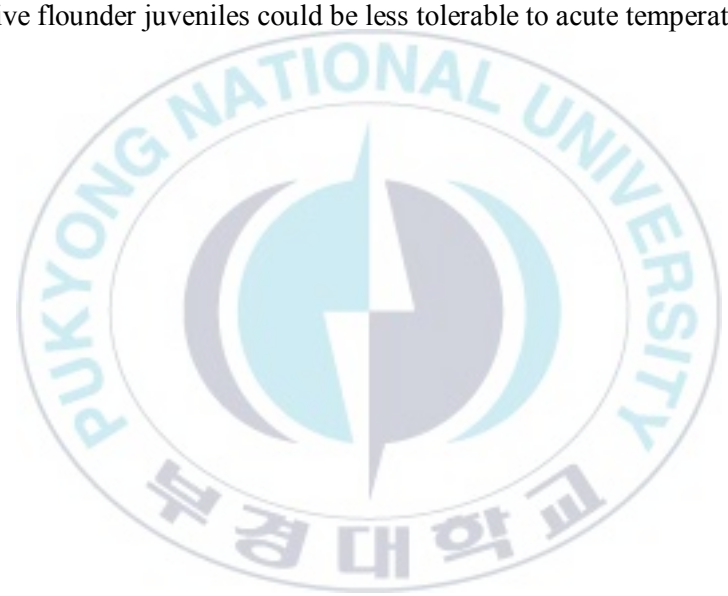
In the results of chronic water temperature stress, only GPX showed significant differences according to fish meal content. However, no trend was observed. Cortisol is a steroid hormone produced and secreted by the adrenal glands. It is an essential hormone that regulates the animal's stress response and glucose. Cortisol and glucose are reliable indicators of stress in fish, reflecting the severity and duration of the stress response, showing similar trends (Jentoft, S., et al., 2005; Lee, S., et al., 2023). Glucose and cortisol showed similar trends in the results of the chronic water temperature stress test in this study as well. As a stress response, cortisol showed the main effect on temperature stress, and when juvenile was exposed to chronic water temperature stress, its value significantly increased, indicating that it was responding to stress. In addition, when cortisol was exposed to acute water temperature stress, there was no significant difference in fish meal content in all parameters. In the case of cortisol, the concentration increases and then decreases according to the time exposed to stress (Nakano, T., et al., 2014). Due to these characteristics and the acute water temperature stress exposure method and sampling method performed in this study, differences by acute stress exposure may not have been found. Further research is needed to find out the trend of cortisol expression in flounder fry according to the high temperature stress response time.

4. Relative gene expression

The results of the chronic water temperature stress exposure experiment were based on the NSFM60, and the gene expression results according to the fish meal content and water temperature. There was no main effect of fish meal on AMPKβ, PPARγ, G6pase ($P > 0.05$). However, HSP70 has main effect of fish meal content ($P < 0.05$). There was main effect of temperature on AMPKβ, HSP70, G6pase ($P < 0.05$). HSP70 is an important stress-related protein and its expression level increases when fish are exposed to stressors such as pathogenic infection, water temperature, and water quality (Ming, J., et al, 2012; Huang, L., et al, 2015; Chaklader, M. R., et al, 2020). In this study, when juveniles were exposed to chronic stress, the level of HSP70 increased. Furthermore, acute temperature stress, the level of HSP70 increased significantly as the fish meal content decreased, suggesting that the replacement of fish meal in the feed may operate as a stressor when exposed to acute high-temperature conditions. However, the tendency for HSP70 to increase with decreasing fish meal content is different than when exposed to chronic stress. Therefore, it is possible that fish are responding differently to chronic and acute stress, and further research is needed. Gluconeogenesis and glycolysis are controlled by G6Pase (Song, Z., et al, 2018). According to Basto, A., et al (2023), as fish meal was replaced in feed, the expression level of G6pase considerably decreased. Although it rose in this study, it may have responded differently because it was subjected to acute water temperature stress, suggesting further research.

. Conclusion

We confirmed that the fish meal replacement result in alteration on the stress responses in both stress conditions. However, the degree of alteration was different between the chronic and acute temperature stressed, showing that the higher susceptibility of the juveniles fed the low fish meal diets was observed when they were exposed to the acute temperature stress. Therefore, it can be concluded that low-fish meal fed olive flounder juveniles could be less tolerable to acute temperature stress.



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감사의 글

사료영양학 실험실에 학·석사 과정으로 들어와서 많은 것들을 배웠습니다. 학문적인 영역 뿐만 아니라 가족보다도 더 오랜 시간을 함께하는 사람들과 지내며 여러 방면으로 많이 배우고, 함께 좋은 추억도 많이 쌓았습니다.

학·석사 과정을 마무리하는데 도움을 주신 많은 분들이 떠오릅니다. 우선, 학·석사 과정 동안 많은 가르침을 주신 이승형 교수님께 진심으로 감사드립니다. 바쁘신 가운데에도 학위논문 심사위원을 맡아 주신 김종명 교수님과 최윤희 교수님께 감사드립니다. 교수님들의 조언 덕분에 학위를 잘 마무리할 수 있었습니다. 강의를 통해 학문적인 지식을 넓히게 도와주신 박정환 교수님, 김찬희 교수님께도 감사를 드립니다. 또한 학위를 시작하는데 있어 용기를 주셨던 선문대학교 박영진 교수님께도 감사드립니다.

석사 과정 동안 든든한 버팀목이 되어주며 늘 동생들 생각이 우선인 사료방의 첫째 하함 오빠, 석사 입학 동기로써 같은 고민을 나눴던 현철 오빠, 동생이지만 배울 점이 많았던 수현이, 학부생이지만 많은 일에 잘 참여하는 준혁이, 그리고 Abayomi, Tugce, Deni에게 고마운 마음을 전합니다.

그리고 학위 과정 동안 힘낼 수 있게 버팀목이 되어준 가족들, 친구들 덕분에 잘 마무리할 수 있었습니다. 모두 감사합니다.