Thesis for Degree of Master of fisheries science

Effects of dietary protein and lipid levels on stress responses of northern snakehead (*Channa argus*) under acute temperature stress

by

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Pukyong National University

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치어기 가물치(*Channa argus*) 사료 내 단백질과 지질 함량이 급성 수온 스트레스 반응에 미치는 영향

Advisors : Prof. Seunghyung Lee

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A dissertation

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Abstract

Due to the importance of major nutrients such as protein and lipid for optimal growth and physiological performances of fishes, many studies have been conducted to determine its requirement for cultured fishes. However, little is known about interactive effects of dietary protein and lipid levels on temperature stress responses in cultured fishes. Northern snakehead is a commercially important fish species in Asia but has faced with continuously increasing water temperature associated with climate change. Thus, a 3 × 3 factorial experiment was designed to evaluate the relationship between interaction of dietary protein and lipid levels and physiological performance of juvenile northern snakehead responding to acute temperature stress.

Four hundred five juveniles (initial body weight: 19.3 ± 0.03 g; mean ± SEM) were randomly distributed into each of 27 tanks (15 fish per tank; N = 3 tanks per treatment). Nine diets were prepared to contain three levels of crude protein (41, 44, and 47%) in combination with three levels of crude lipid (6, 9, and 12%). Following the 60-days of feeding trial, the juveniles from each of the nine treatments were abruptly exposed to higher water temperature at 35 ℃ for 2 hours and were recovered at ambient water temperature for 2 hours.

Results showed that a significant interactive effect of dietary protein and lipid levels on weight gain (WG), specific growth rate (SGR), feed efficiency (FE), and condition factor (CF), showing the improved values as the protein increased but the lipid level decreased (P < 0.05). There was a main effect of both protein and lipid levels on WG, SGR, FE, and CF. Based on results of the three-way (3 protein levels × 3 lipid level × 2 stress condition) ANOVA test on plasma metabolites, there was neither the interactive effects between the dietary nutrient levels and stress condition nor the interactive effect between the protein and lipid levels on plasma metabolites. However, a significant main effect of temperature stress was detected in plasma glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), total protein (TP), and triglyceride (TG). There was a significant main effect of dietary protein levels on the relative gene expression of heat shock protein 70 (*hsp70*) in head kidney, whereas no change in heat shock protein 60 (*hsp60*) and *hsp70* in other tissues, including gill, liver, and spleen was

observed.

In summary, the tested dietary protein and lipid levels resulted in significant alteration of the growth responses; however, those did not induce dramatic changes in plasma metabolites and the expression of the key stress markers *(hsp60* and *hsp70)* in gill, liver, and spleen with the exception in the head kidney when exposed to the acute temperature stress.

치어기 가물치(*Channa argus*) 사료 내 단백질과 지질 함량이 급성 수온 스트레스 반응에 미치는 영향

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요약

어류의 최적 성장과 생리적 성능을 위해서는 단백질, 지질과 같은 주요 영양소가 중요하므로 양식 어류에 대한 주요 영양소 요구량을 결정하기 위해 많은 연구가 수행되었다. 그러나 양식 어류의 온도 스트레스 반응에 대한 사료 내 단백질과 지질 수준의 상호 작용 효과에 대해서는 알려진 바가 거의 없다. 북부 가물치(*Channa argus*)는 아시아에서 상업적으로 중요한 어종이며, 기후 변화로 인해 지속적으로 상승하는 수온에 직면해 있다. 따라서 치어기 북부 가물치의 지속 가능한 양식을 위해서는 사료 내 다양한 단백질과 지질 수준이 급성 온도 스트레스에 반응하여 이 종의 생리적 성능에 어떻게 영향을 미치는지 이해하는 것이 중요하다.

사료 내 단백질과 지질 수준의 상호 작용과 급성 온도 스트레스에 반응하는 생리적 성능 사이의 관계를 평가하기 위해 3 × 3 (단백질 수준 3가지 × 지질 수준 3가지) 요인 실험이 설계되었다. 평균 19.3 ± 0.03 g (평균 ± SEM)의 치어기 가물치 455마리의 새끼를 27개의 직사각형 수조 (수조당 15마리씩)에 무작위로 배치했다. 세 가지 수준의 조단백질(41, 44, 47%)과 함께 세 가지 수준의 조지질(6, 9, 12%)을 함유하도록 9가지 실험구를 구성하였다. 60일간의 사육 실험 후(비스트레스 그룹: NS), 실험구 당 3마리의 가물치 치어는 급성 온도 스트레스에 노출되었다(35℃에서 2시간 수온 스트레스 및 사육 수온 27℃에서 2시간 회복 그룹: TS).

사육 실험 결과(NS 그룹), 사료 내 단백질과 지질 수준이 증체율(WG), 일간 성장률(SGR), 사료 효율(FE), 및 비만도(CF)에 대한 유의미한 상호 작용 효과를 보여주었으며, 사료 내 단백질 수준이 증가함에 따라 지질 수준은 감소함에 따라 유의하게 높은 값을 보여주었다 (P <0.05). WG, SGR, FE 및 CF에서는

사료 내 단백질과 지질 수준이 메인 이펙트가 있었다. 혈장대사산물 분석에서 3원 분산분석(단백질 수준 3가지 x 지질 수준 3가지 x 스트레스 조건 2가지) 결과에 따르면, 사료 내 영양 수준과 스트레스 조건 사이의 상호작용 효과나 혈장대사산물에 대한 단백질과 지질 수준 사이의 상호작용 효과가 나타나지 않았다. 그러나 온도 스트레스의 중요한 주요 영향은 혈장 글루타민산 옥살로초산 트랜스아미나제(GOT), 글루타민산 피루빈산 트랜스아미나제(GPT), 총 단백질(TP) 및 중성지방(TG)에서 감지되었다. 신장에서 열 충격 단백질 70(*hsp70*)의 상대적 유전자 발현에 대해서는 사료 내 단백질 수준에서 메인 이펙트가 있었던 반면, 아가미, 간 및 비장을 포함한 다른 조직에서는 열 충격 단백질 60(*hsp60*)과 *hsp70*의 변화가 없었습니다.

요약하면, 사료 내 단백질과 지질 수준은 성장 반응에 상당한 변화를 가져왔다. 그러나 이는 급성 온도 스트레스에 노출되었을 때 신장을 제외하고 아가미, 간 및 비장의 혈장 대사 산물과 주요 스트레스 인자(*hsp60 및 hsp70*)의 발현에 극적인 변화를 유발하지 않았다고 판단된다.

I. Introduction

Protein, the most expensive ingredient in fish feed (Fagbenro et al., 1992) and is an essential nutrient for growth, reproduction, and the synthesis or maintenance of new proteins (NRC, 2011), so ensuring sufficient dietary supply is necessary for optimal growth. Inadequate of protein in the feed reduces growth, but excess can cause waste, increased nitrogenous waste can reduce water quality, and increase production cost (Webb & Gatlin, 2003; Wu and Gatlin, 2014; Kim & Lee, 2005). To prevent this and enhance protein utilization for growth, dietary protein can be partly substituted with lipids or carbohydrates (Cho and Kaushik, 1990; Kaushik and Medale, 1994). Carnivorous fish species prefer lipids as energy sources between lipids and carbohydrates, dietary lipids supply essential fatty acids and serve as a carrier of fat-soluble vitamins (Watanabe, 1982; Mai et al., 1995; Lee et al., 2002). This has been shown to lower dietary protein use and increase dietary protein utilization, known as the “protein sparing effect” (Lee et al., 2002). However, high dietary lipids can lead to excessive accumulation of lipids in fish and reduced growth performance (Kim et al., 2004). Therefore, it is important to optimize the level of dietary protein and lipid levels for good growth and development of cost-effective feed.

Increasing dietary levels of all essential nutrients increases physiological responses to the point where dietary requirements are met, and adequate nutritional status allows fish to maintain homeostasis (Hardy, 1991). Beyond a certain level of nutrient availability, there is no concomitant increase in physiological responses and can be toxic, while at levels below that requirement, signs of deficiency appear.

Stress is a state in which the homeostasis of an organism is threatened by endogenous or exogenous stimulation (stressor), and the stress response is known as a strategy for the organism to overcome these stimulations (Chrousos and Gold, 1992). Stressors can reduce the capacity of exposed fish to maintain homeostasis (Schreck, 1982). Previous studies have reported that the behavioral and physiological responses of fish to stressors vary depending on the fish species and type of stressor (Barton and Iwama, 1991; Iwama et al., 2006). As an environmental stressor, changes in water temperature cause stress to fish and affect their physiological activity, and excessive water temperature stimulation can cause death (Cossins et al., 1995; Ellis, 2001; Bowden, 2008). When fish respond to water temperature stress, cortisol secretion in the blood primarily increases (Wedemeyer, 1981; Perry and Reid, 1993; Chang and Hur, 1999). This increases the activity of gluconeogenic enzymes and increases the secretion of glucose (Hur et al., 2019). Also, there is a report that the expression of heat shock proteins (Hsps), a biomarker of cellular stress, is increased (Choi et al., 2007).

Under unstressed nutritional status, fish use energy for maintenance, activity, growth, reproduction, development and store the remaining energy. However, depending on stress level, more energy is used to maintain homeostasis. This indicates a correlation between nutritional status and stress response (Sokolova et al., 2012).

The northern snakehead (*Channa argus*) is a common and economically important freshwater species widely cultured in China, with a total annual production of over 500,000 tons (Sun et al., 2023). In addition, it is a carnivorous fish species, a top predator in the aquatic ecosystem, that is characterized by rapid growth, high nutritional value, and strong resistance to environmental stress.

However, because of global warming, the China Sea coast, their main habitat,

has continuously increasing water temperature associated with climate change (Cai et al., 2021). Currently, little is known about the interactive effects of dietary protein and lipid level requirements and temperature stress responses in cultured fish.

Therefore, we conducted this study to determine the relationship between the interaction of protein and lipid levels in the diet of juvenile snakehead (*channa argus*) and their physiological performance in response to acute temperature stress.

II. Materials and methods

1. Ethical Statement

The experiment was conducted following the guidelines of Institutional Animal Care and Use Committee Regulations, PKNUIACUC-2022-50, issued by the Pukyong National University, Busan, Republic of Korea. Every effort was taken to minimize the suffering of fish.

2. Experimental design and diets

Feed formulation and proximate composition of the nine experimental diets are provided in Table 1, and analyzed amino acids, fatty acids compositions are given in Table 2 and 3, respectively. This study involved a 3 × 3 factorial design with three levels of dietary crude protein (CP) (41%, 44%, and 47%) and three levels of dietary crude lipid (CL) (6%, 9%, and 12%). The formulated nine diets were named as P41L6, P41L9, P41L12, P44L6, P44L9, P44L12, P47L6, P47L9, and P47L12 (P-Protein, L-Lipid), respectively. The levels of protein and lipid on the formulation were based on earlier studies (Sagada et al., 2017). Fish meal, squid liver powder, poultry offal meal, blood meal, and soybean meal were used as the main protein sources, and wheat flour was used as the carbohydrate source, while fish oil was used as the lipid source. Other dietary ingredients were added to fulfill the nutritional requirements of C. argus (Liu et al., 1998). All ingredients were weighed and mixed for 15 minutes in an electronic mixer (HYVM-1214, Hanyoung Food Machinery, Republic of Korea), followed by the addition of water for 15 minutes and fish oil for another 15 minutes. This mixture was formed into dough which was placed in a screw-type pelleting machine (SFD-GT, Shinsung, Republic of Korea) and extruded through a 2mm in diameter die. Compounded feed pellets were dried in an oven (KE-010, Dongwon, Korea) at 45 ℃ for 14 hours. After drying, packed separate and stored at -20 ℃ until used during the feeding trial.

Table 1. Formulation and proximate composition of the nine experimental diets for juvenile snakehead

Ingredient (%)	Diets[1]								
	P41L6	P41L9	P41L12	P44L6	P44L9	P44L12	P47L6	P47L9	P47L12
Fish meal	32.2	32.2	32.2	36.2	36.2	36.2	40.2	40.2	40.2
Squid liver powder	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Poultry offal meal	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Blood meal	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Soybean meal	17.0	17.0	17.0	17.5	17.5	17.5	18.0	18.0	18.0
Wheat flour	20.7	20.7	20.7	20.7	20.7	20.7	20.7	20.7	20.7
Fish oil	1.60	4.60	7.60	1.20	4.20	7.20	0.80	3.80	6.80
Others[2]	9.90	9.90	9.90	7.90	7.90	7.90	5.90	5.90	5.90
Cellulose (binder)	10.6	7.60	4.60	8.50	5.50	2.50	6.40	3.40	0.40
Proximate composition (%, dry matter basis)									
Moisture	2.94	4.06	3.54	3.56	3.33	3.01	3.24	3.47	4.17
Crude Protein	42.3	42.7	42.7	46.5	45.7	46.4	50.1	50.5	51.0
Crude Lipid	5.39	9.13	12.73	6.13	9.23	12.38	5.77	8.99	12.82
Crude Ash	16.1	15.7	15.6	15.0	14.8	14.6	13.7	13.2	13.4
Gross energy (kJ/g)[3]	16.5	17.6	18.8	17.3	18.4	19.6	18.1	19.3	20.4
Protein:Energy ratio (mg/kJ)	25.9	24.1	22.6	26.5	24.8	23.3	27.1	25.5	24.0

[1] Diets: P41L6 = 41% crude protein, 6% crude lipid; P41L9 = 41% crude protein, 9% crude lipid; P41L12 = 41% crude protein, 12% crude lipid, etc.
[2] Feed stuffs not mentioned here are the same feed stuffs as the domestic aquaculture feed companies are using currently.
[3] Calculated based on 17.2 kJ/g carbohydrate; 23.6 kJ/g protein and 39.5 kJ/g lipid.

Table 2. Amino acids composition of the nine experimental diets (% of dry matter basis)

Amino acids	Diets								
	P41 L6	P41 L9	P41 L12	P44 L6	P44 L9	P44 L12	P47 L6	P47 L9	P47 L12
Essential amino acids (EAA)									
Arginine	2.95	2.83	3.05	3.42	2.97	3.13	3.19	3.24	3.22
Histidine	1.96	1.89	1.99	2.22	2.01	1.89	2.03	1.98	1.90
Isoleucine	1.52	1.63	1.67	2.01	2.21	1.95	2.00	2.05	2.07
Leucine	2.88	3.02	3.16	3.59	3.59	3.63	3.70	3.76	3.81
Lysine	2.35	2.56	2.64	3.20	2.92	2.92	3.06	3.10	3.11
Phenylalanine	1.88	1.91	2.03	2.32	2.11	2.17	2.25	2.30	2.29
Threonine	1.45	1.55	1.60	1.84	2.00	2.00	1.89	1.92	1.94
Valine	2.04	2.15	2.20	2.55	2.40	2.43	2.57	2.54	2.65
Non-Essential amino acids (NEAA)									
Alanine	2.48	2.54	2.64	2.95	2.69	2.90	3.04	3.00	3.10
Aspartic acid	3.53	3.77	3.83	4.37	4.31	4.53	4.61	4.70	4.77
Glycine	2.54	2.58	2.73	3.09	2.31	3.19	3.26	3.27	3.36
Glutamic acid	6.02	6.36	6.53	7.35	7.39	7.50	7.66	7.70	7.75
Proline	1.75	1.79	1.97	2.12	1.95	2.57	2.61	2.65	2.66
Serine	1.62	1.66	1.74	1.84	1.85	1.97	1.95	1.99	2.02
Tyrosine	1.45	1.55	1.60	1.84	2.00	2.00	1.89	1.92	1.94
Total	36.2	37.3	39.1	44.2	42.4	44.1	45.1	45.6	46.0

Table 3. Fatty acids composition of the nine experimental diets

Fatty acids	Diets								
	P41 L6	P41 L9	P41 L12	P44 L6	P44 L9	P44 L12	P47 L6	P47 L9	P47 L12
C6:0	0.00	0.19	0.00	0.00	0.00	0.04	0.09	0.00	0.06
C8:0	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00
C12:0	0.19	0.17	0.15	0.19	0.18	0.16	0.23	0.22	0.21
C13:0	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00
C14:0	9.25	14.4	16.2	9.86	13.1	16.1	10.5	17.4	18.9
C14:1	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00
C15:0	0.62	0.67	0.69	0.66	0.66	0.74	0.74	0.76	0.76
C16:0	49.9	49.8	49.8	50.5	47.9	44.7	48.5	45.0	42.5
C16:1	3.36	4.78	5.30	3.36	4.45	5.44	3.35	3.82	4.30
C17:0	0.70	0.57	0.49	0.71	0.57	0.54	0.69	0.92	0.73
C17:1	0.10	0.09	0.10	0.09	0.09	0.09	0.11	0.13	0.12
C18:0	11.4	9.16	8.30	11.3	9.43	7.93	10.9	9.11	8.31
C18:1n9t	0.14	0.10	0.00	0.13	0.11	0.09	0.15	0.13	0.10
C18:1n9c	14.8	13.0	10.5	14.1	13.0	11.5	13.1	11.5	12.6
C18:2n6c	5.16	2.80	3.09	4.79	4.41	3.74	5.91	5.71	5.30
C20:0	0.43	0.43	0.44	0.43	0.43	0.46	0.41	0.41	0.37
C18:3n6	0.00	0.00	0.00	0.00	0.00	0.05	0.04	0.00	0.00
C20:1	1.57	2.04	2.18	1.64	1.98	2.20	1.48	0.74	1.30
C18:3n3	0.37	0.18	0.28	0.33	0.42	0.47	0.52	0.00	0.00
C21:0	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00
C20:2	0.07	0.06	0.00	0.10	0.12	0.19	0.14	0.17	0.23
C22:0	0.18	0.16	0.16	0.19	0.16	0.16	0.19	0.19	0.22
C20:3n6	0.00	0.00	0.00	0.00	0.00	0.05	0.04	0.00	0.00
C22:1n9	0.40	0.60	0.74	0.42	0.57	0.62	0.26	0.32	0.52
C20:3n3	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.03	0.04
C23:0	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.04
ARA	0.09	0.00	0.00	0.08	0.11	0.15	0.15	0.20	0.23
C24:0	0.10	0.08	0.00	0.11	0.08	0.07	0.11	0.12	0.11
EPA	0.67	0.34	0.96	0.54	1.35	2.78	1.24	1.84	1.70
C24:1	0.23	0.28	0.30	0.22	0.33	0.36	0.25	0.29	0.39
DHA	0.35	0.10	0.27	0.25	0.59	1.22	0.76	0.95	1.00

3. Experimental fish and feeding trial

3.1. Growth trial (Experiment I)

Juvenile snakehead (*Channa argus*) were obtained from a commercial hatchery (Busan, South Korea). The feeding trial was conducted for 60 days at Pukyong National University (Busan, Republic of Korea). Before starting the feeding trial, the fish were fed with the basal diet containing 48% CP and 12%CL for 4 weeks for acclimatized to experimental conditions. After acclimation, fish were starved for 24h to measure the initial body weight. At start of feeding trial, four hundred and fifty juvenile fish (initial mean weight 19.3 ± 0.03 g/fish, n = 15) were randomly selected and distributed into 27 tanks. Each tank was then randomly assigned as one of the three replicates of the nine dietary treatments. The feeding trial was conducted by using a semi-recirculating system with 27 tanks (18.4 L) receiving fresh water at the rate 1.8 L min^{-1} from center tank. During the experiment, Fish were hand-fed the experimental diets to apparent satiation at twice daily (09:00 and 17:00) also recorded daily dietary intake per tank. Water temperature (27.0 ± 0.4 ℃) and Dissolved oxygen (7.52 ± 0.02 mg/L) values were recorded by YSI DO meter (ProODO/T,Yellow Springs, OH, USA) while pH (7.4 ± 0.1), total ammonia (0.04 ± 0.01 mg/L), nitrite (0.01 ± 0.00 mg/L), and nitrates (1.00 ± 0.38 mg/L) were also tested by fresh water master test kit (API fish care, Chalfont, PA, USA) twice a day. Twenty five percent of water was exchanged every day using pre-aerated tap water. Siphoning was conducted two times per day to remove feces in tanks. Mortality was also monitored daily, and any dead fish was removed, weighed and recorded accordingly.

3.2. Acute temperature stress experiment (Experiment II)

At the end of the feeding experiment for 60 days, an acute temperature stress experiment was conducted by randomly selected 3 fish per tank. 3 fish were collected and transferred to 18.4L tank under the same conditions as the growth trial except for water temperature. The temperature for acute temperature stress experiment were 35℃. The fish abruptly exposed to acute temperature stress for 2 hours at 35℃. Water temperature and dissolved oxygen were maintained at 35.0 ± 0.1℃, 7.32 ± 0.5 mg/L, respectively. After exposure, fish were removed to 27℃ and had a recovery period for 2 hours. The water temperature 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 5 minutes throughout the trial (Figure 1, 2).

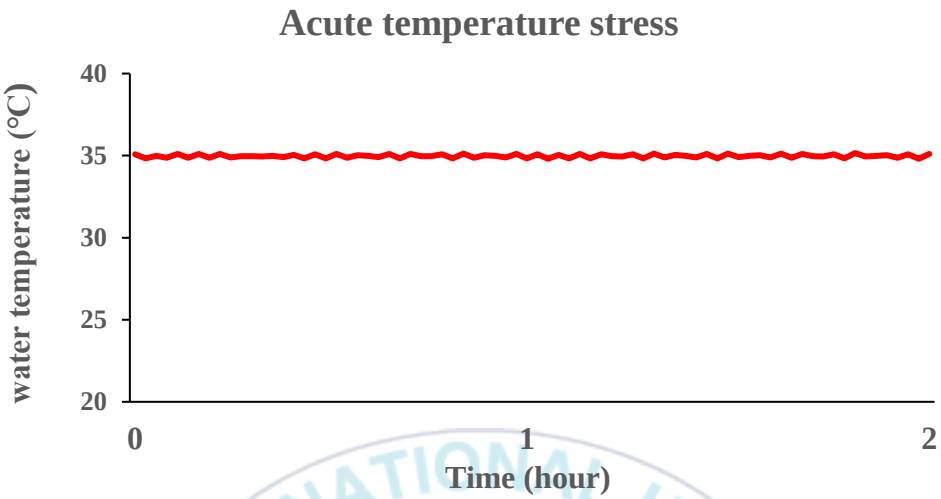

Figure 1. Water temperature records during the acute temperature stress exposure experiment (Exp. II)

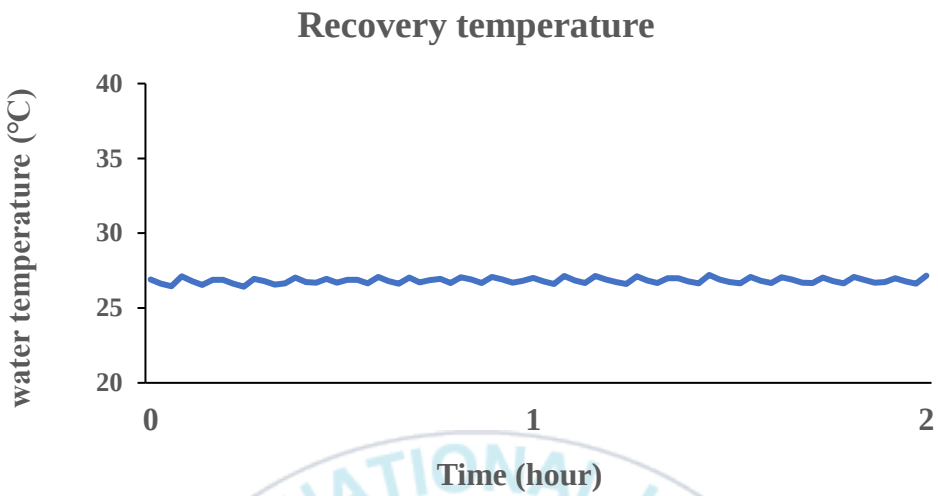

Figure 2. Water temperature records during the recovery after acute temperature stress exposure experiment (Exp. II)

4. Sample collection and analysis

4.1. Growth performance

At the end of 60 days of the feeding trial, fish in each tank were counted and weighed for calculation of final body weight (FBW), weight gain (WG), specific growth rate (SGR), feed efficiency (FE), and survival rate (SR).

Fish were anaesthetized with 2-phenoxy ethanol (300 mL/L), individual weight and length measured. Three to five fish from each tank were dissected to obtain liver and viscera samples for determination of hepatosomatic index (HSI), viscerosomatic index (VSI), and condition factor (CF). In addition, the gut was removed by cutting just anterior to the stomach and at the anus. The gut was then uncoiled, without stretching, and the total gut length and gut mass were measured. Then relative gut length (RGL) and relative gut weight (RGW) were calculated.

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

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

FE (%) = wet weight gain (g) / dry feed intake (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.

RGL = gut length (mm) / standard length (mm).

RGW = gut weight (g) / body weight (g).

4.2. Diet and fish whole body proximate composition analysis

At the beginning of the trial, five fish were collected for initial whole body composition analyses. After the experiment, five snakehead randomly selected for each tank were used for whole body composition analysis. Proximate composition analyses of the experimental diets and fish whole bodies performed by the standard methods of AOAC (Association of Official Analytical Chemists, 2005). Samples of diets and fish were dried at 105℃ for 24 hours to constant weight to determine their moisture content. Ash content was determined by incineration at 550℃ in muffle furnace for 3 hours. Crude protein ($N \times 6.25$) content was determined using the kjeldahl method (2300 Auto analyzer, Foss Tecator. AB, Hoganas, Sweden) after acid digestion, and crude lipid was ascertained by Soxhlet extraction using the Soxtec system 1046 (Tecator AB, Hoganas, Sweden) after freeze-drying the samples for two days. Amino acid analysis was performed by ninhydrin method (Sykam Amino Acid Analyzer S433, Sykam; Eresing, Germany).

4.3. Plasma collection and analysis

Blood samples were obtained from the caudal vein of eight to ten fish in experiment I (named as the non-stressed group: NS), three fish in acute temperature stress experiment (experiment II, named as the acute temperature stressed group: TS), from each tank by using a 1-mL syringe that contained an anticoagulant (dipotassium ethylenediaminetetraacetic acid: EDTA). Blood was pooling by each tank into 3-ml siliconized vacuum tubes containing 5.4mg K2 EDTA (Becton Dickinson and Company, USA) and divided into 1.5 mL microtubes, centrifuged at 11,000 x g for 5 min and plasma was stored at -84℃ for determination of blood biochemical parameters including glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT), total protein (TP), triglyceride (TG), total cholesterol (TCHO), and plasma glucose (GLU) was analyzed using Fuji Film DRI-CHEM SLIDE in a dry biochemical analyzer (Fuji DRI-CHEM nx500i, Fuji Photo Film, Tokyo, Japan).

4.4. Antioxidant reaction, immune responses, and stress responses

In experiment I, Glutathione peroxidase (GPx) and lysozyme (LZM) and in experiment II, 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 all samples were performed in triplicate.

4.5. Quantitative real-time PCR

At the end of experiment II, from three fish by tank, the gill, liver, head kidney and spleen were dissection, into ep tube and quickly frozen in liquid nitrogen. Once frozen, samples were stored at -80℃ until use. Total RNA was extracted from the gill, liver, head kidney, and spleen by using TM Hybrid-R^{TM} (GeneAll, Seoul, Korea) after homogenized the ~100 mg of tissues sample in 1 mL RiboEx^{TM}. The quantity and quality of the extracted RNA were assessed using a Nanodrop ASP-2680 spectrophotometer (ACTgene, Piscataway, USA; the A_{260}/A_{280} ratio was greater than 1.8. The extracted RNA was treated with DNase (DNase I, RNase-free, Thermo Fisher Scientific, Baltics UAB, Vilnius, Lithuania), then 1 μg of total RNA was reverse-transcribed 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. Real-time quantitative PCR was carried out on a StepOne Real-Time PCR system (Applied Biosystems, Waltham, Massachusetts, USA) in a 25 μL total volume reaction using TB Green© *Premix Ex Taq*^{TM}II (Tli RNaseH Plus) (Takara Bio, Kusatsu, Shiga, Japan) and 800 nmol primers according to the protocol provided by the manufacturer. The details of specific primer for target genes in this study were shown in Table 4. PCR recycling conditions for all genes were as follows: initial denaturation step of 95℃ for 30 sec, followed by 40 cycles of 95℃ for 5 sec, 60℃ for 30 sec. Relative expression levels of the target genes transcripts (*hsp60* and *hsp70*), with *β-actin* as a normalized control, were calculated using the $2^{-\triangle\triangle Ct}$ method. After the PCR run, data analysis was performed with StepOne Software version 2.0 (Applied Biosystems, USA). In all cases, each PCR test was performed in triplicate.

Table 4. List of primers used for head kidney, gill, liver, and spleen gene expression

Genes	Primer sequences (5'>3')	Product size (bp)	Accession number
β-actin	F: CACTGTGCCCATCTACGAG R: CCATCTCCTGCTCGAAGTC	196	EF452499
hsp60[1]	F: CAACCAGCACCGCAAACCT R: ACCACCTGAAGCCCAACCT	189	KU883612.1
hps70[2]	F: ATTTTGAATGTGTCTGCGGT R: ACTTGCTGATGATGGGGTTA	232	KU883613.1

[1]Heat shock protein 60 (*hsp*60)
[2]Heat shock protein 70 (*hsp*70)

4.6. Statistical analysis

All experiment data were presented as mean ± SEM. All data subjected to one-way analysis of variance (ANOVA) to determine the significance due to effects of dietary treatments, and two-way ANOVA to determine the significance due to levels of protein, lipid, or their interaction. To investigate the effect of acute heat stress on plasma metabolites, applied a three-way ANOVA (3 protein levels × 3 lipid level × 2 stress condition). Post Hoc was analyzed by Tukey's honestly significant difference (HSD) test with statistical significance determined at $P < 0.05$. All statistical analyses were carried out using SAS analytical software, version 9.4 (SAS Institute, Cary, NC, USA).

III. Results

Experiment I. Growth trial

1.1 Growth performance

Growth performance of northern snakehead juveniles fed the different experimental diets after 60 days of feeding are shown in Table 5. P47L6 produced significantly the highest ($P < 0.05$) WG, SGR, FE, CF, and VSI values than the rest. Based on two-way ANOVA, dietary CP and CL levels significantly affected ($P < 0.05$) WG, SGR, FE, and CF of fish. Significant improvements in WG, SGR, FE, and CF values were observed by increment of dietary CP levels from 41% to 47% ($P < 0.05$) and dietary CL levels decreased from 12% to 6%. There was also a significant effect of interaction ($P < 0.05$) between dietary protein and lipid on WG, SGR, not on FE and CF. Fish survival rate varied from 87% to 100% and no significant differences were observed among dietary treatment. HSI, VSI, RGL, and RGM of experimental fish were not significantly affected by different levels of dietary CP, but were significantly affected by dietary CL.

Table 5. Growth performance of snakehead fed the nine experimental diets for 8 weeks[1]

Diets	FBW[2]	WG[3]	SGR[4]	FE[5]	SR[6]	CF[7]	HSI[8]	VSI[9]	RGL[10]	RGM[11]
Interactive effect between protein and lipid										
P41L6	22.6 ± 1.2^{bc}	16.3 ± 5.3^{bc}	0.27 ± 0.08^{bc}	14.1 ± 4.9^{bc}	100 ± 0^{ns}	0.63 ± 0.01^{ab}	1.00 ± 0.13^{a}	4.38 ± 0.13^{ab}	0.42 ± 0.02^{ns}	0.015 ± 0.001^{a}
P41L9	20.6 ± 0.7^{c}	6.55 ± 3.26^{c}	0.11 ± 0.06^{c}	6.23 ± 3.10^{c}	95.6 ± 2.2	0.61 ± 0.01^{b}	0.86 ± 0.08^{ab}	3.59 ± 0.08^{bc}	0.43 ± 0.01	0.013 ± 0.001^{ab}
P41L12	20.3 ± 0.1^{c}	4.06 ± 2.4^{c}	0.07 ± 0.04^{c}	3.80 ± 2.12^{c}	86.7 ± 0.0	0.60 ± 0.01^{b}	0.72 ± 0.08^{ab}	3.48 ± 0.18^{c}	0.40 ± 0.01	0.010 ± 0.001^{b}
P44L6	26.0 ± 0.8^{b}	34.1 ± 3.3^{b}	0.52 ± 0.04^{b}	28.5 ± 3.0^{b}	95.6 ± 2.2	0.66 ± 0.01^{ab}	0.95 ± 0.02^{ab}	3.94 ± 0.07^{abc}	0.41 ± 0.02	0.013 ± 0.001^{ab}
P44L9	23.5 ± 0.4^{bc}	21.9 ± 1.5^{bc}	0.35 ± 0.02^{bc}	19.5 ± 1.1^{bc}	97.8 ± 2.2	0.64 ± 0.01^{ab}	0.88 ± 0.04^{ab}	3.98 ± 0.15^{abc}	0.40 ± 0.03	0.013 ± 0.000^{ab}
P44L12	20.3 ± 0.6^{c}	5.20 ± 4.41^{c}	0.09 ± 0.07^{c}	4.85 ± 4.04^{c}	88.9 ± 4.4	0.61 ± 0.02^{b}	0.68 ± 0.01^{ab}	3.66 ± 0.36^{abc}	0.39 ± 0.00	0.011 ± 0.001^{ab}
P47L6	33.1 ± 0.5^{a}	70.2 ± 3.0^{a}	0.95 ± 0.03^{a}	49.5 ± 0.5^{a}	97.8 ± 2.2	0.70 ± 0.02^{a}	0.96 ± 0.05^{ab}	4.47 ± 0.04^{a}	0.44 ± 0.02	0.014 ± 0.001^{ab}
P47L9	26.3 ± 2.0^{b}	36.3 ± 9.3^{b}	0.54 ± 0.12^{b}	29.3 ± 6.6^{b}	97.8 ± 2.2	0.65 ± 0.02^{ab}	0.74 ± 0.06^{ab}	3.77 ± 0.09^{abc}	0.39 ± 0.02	0.012 ± 0.001^{ab}
P47L12	23.1 ± 0.3^{bc}	19.0 ± 0.3^{bc}	0.31 ± 0.00^{bc}	16.8 ± 0.4^{bc}	95.6 ± 4.4	0.62 ± 0.01^{b}	0.66 ± 0.02^{b}	3.52 ± 0.23^{bc}	0.42 ± 0.02	0.011 ± 0.001^{ab}
Main effect of protein										
CP41	21.1 ± 0.7^{c}	8.98 ± 3.74^{c}	0.15 ± 0.06^{c}	8.03 ± 3.10^{c}	94.1 ± 3.9^{ns}	0.61 ± 0.01^{b}	0.86 ± 0.08^{ns}	3.82 ± 0.29^{ns}	0.42 ± 0.01^{ns}	0.013 ± 0.001^{ns}
CP44	23.3 ± 1.7^{b}	20.4 ± 8.4^{b}	0.32 ± 0.13^{b}	17.6 ± 6.9^{b}	94.1 ± 2.7	0.64 ± 0.01^{ab}	0.84 ± 0.08	3.86 ± 0.10	0.40 ± 0.01	0.012 ± 0.001
CP47	27.5 ± 2.9^{a}	41.8 ± 15.0^{a}	0.60 ± 0.19^{a}	31.9 ± 5.1^{a}	97.0 ± 0.7	0.66 ± 0.02^{a}	0.79 ± 0.09	3.92 ± 0.28	0.42 ± 0.01	0.012 ± 0.001
Main effect of lipid										
CL6	27.2 ± 3.1^{a}	40.2 ± 15.9^{a}	0.58 ± 0.20^{a}	30.7 ± 10.3^{a}	97.8 ± 1.3^{a}	0.66 ± 0.02^{a}	0.97 ± 0.01^{a}	4.27 ± 0.16^{a}	0.42 ± 0.01^{ns}	0.014 ± 0.001^{a}
CL9	23.4 ± 1.6^{b}	21.6 ± 8.6^{b}	0.34 ± 0.13^{b}	18.4 ± 6.7^{b}	97.0 ± 0.7^{a}	0.63 ± 0.01^{b}	0.83 ± 0.04^{b}	3.78 ± 0.11^{b}	0.41 ± 0.01	0.013 ± 0.000^{a}
CL12	21.2 ± 0.9^{c}	9.42 ± 4.80^{c}	0.16 ± 0.08^{c}	8.48 ± 4.16^{c}	90.4 ± 2.7^{b}	0.61 ± 0.01^{b}	0.69 ± 0.02^{c}	3.55 ± 0.05^{b}	0.40 ± 0.01	0.011 ± 0.000^{b}

Table 5 continued. Growth performance of snakehead fed the nine experimental diets for 8 weeks[1]

Two-way ANOVA (*p*-value)										
CP	<.0001	<.0001	<.0001	<.0001	0.3643	0.0082	0.4027	0.7691	0.2792	0.8653
CL	<.0001	<.0001	<.0001	<.0001	0.0196	0.0007	0.0003	0.0003	0.2697	0.0012
CP*CL	0.0165	0.0102	0.0491	0.0722	0.4363	0.4579	0.8615	0.1488	0.4160	0.4976

[1]Values are presented as mean ± SEM (Tukey test at alpha=0.05)
[2]FBW: Final body weight (g)
[3]WG: Weight gain (%) = (final weight (g) - initial weight (g)) / initial weight (g) × 100
[4]SGR: Specific growth rate (% day-1) = (ln final weight (g) - ln initial weight (g)) / days × 100
[5]FE: Feed efficiency (%) = (wet weight gain (g) / dry feed intake (g)) × 100
[6]SR: Survival rate (%) = (number of fish at the beginning – number of dead fish) / number of fish at the beginning × 100
[7]CF: Condition factor = (wet weight (g) / total length (cm)3) × 100
[8]HSI: Hepatosomatic index (%) = liver weight (g) × 100/ body weight (g)
[9]VSI: Visceral somatic index (%) = Viscera weight (g) × 100/ body weight (g)
[10]RGL: Relative gut length = gut length (mm) / standard length (mm)
[11]RGM: Relative gut mass = gut weight (g) / body weight (g)

1.2 Whole-body composition

Whole-body proximate composition of snakehead is shown in Table 6. There were no significant differences ($P > 0.05$) in moisture, CP, CL, and crude ash contents of all the experimental diets. Amino acids and fatty acids profile of whole body in juvenile snakehead fed the diets containing various protein and lipid levels are presented in Table 7 and 8.

Table 6. Proximate composition of snakehead fed the nine experimental diets for 8 weeks (% of as-is basis)[1]

Diets	Moisture	Crude protein	Crude lipid	Crude ash
Interactive effect between protein and lipid				
P41L6	75.9 ± 1.6^{ns}	16.5 ± 1.0^{ns}	1.58 ± 0.22^{ns}	5.98 ± 0.32^{ns}
P41L9	77.3 ± 1.1	15.8 ± 0.8	1.32 ± 0.02	5.62 ± 0.29
P41L12	75.6 ± 1.3	17.0 ± 0.9	1.29 ± 0.15	6.05 ± 0.26
P44L6	76.7 ± 1.1	16.3 ± 0.7	1.47 ± 0.10	5.47 ± 0.27
P44L9	74.8 ± 1.3	17.4 ± 0.9	1.48 ± 0.14	6.27 ± 0.32
P44L12	74.6 ± 1.0	17.6 ± 0.6	1.53 ± 0.11	6.18 ± 0.29
P47L6	75.6 ± 1.5	16.8 ± 1.1	1.67 ± 0.19	5.85 ± 0.30
P47L9	76.9 ± 0.3	16.0 ± 0.2	1.52 ± 0.13	5.59 ± 0.14
P47L12	77.6 ± 0.8	15.5 ± 0.5	1.48 ± 0.12	5.32 ± 0.25
Main effect of protein				
CP41	76.2 ± 0.5^{ns}	16.4 ± 0.3^{ns}	1.39 ± 0.09^{ns}	5.88 ± 0.13^{ns}
CP44	75.4 ± 0.7	17.1 ± 0.4	1.49 ± 0.02	5.97 ± 0.25
CP47	76.7 ± 0.6	16.1 ± 0.4	1.55 ± 0.06	5.58 ± 0.15
Main effect of lipid				
CL6	76.0 ± 0.3^{ns}	16.5 ± 0.2^{ns}	1.57 ± 0.06^{ns}	5.77 ± 0.15^{ns}
CL9	76.3 ± 0.8	16.4 ± 0.5	1.44 ± 0.06	5.83 ± 0.22
CL12	75.9 ± 0.9	16.7 ± 0.6	1.43 ± 0.07	5.85 ± 0.27
Two-way ANOVA (*p*-value)				
CP	0.3785	0.3000	0.3945	0.2265
CL	0.9259	0.9130	0.3969	0.9343
CP*CL	0.3837	0.4333	0.7780	0.1290

[1]Values are presented as mean ± SEM (Tukey test at alpha=0.05)

Table 7. Amino acids profile of snakehead fed the nine experimental diets for 8 weeks (% of as-is basis)[1]

Diets	Essential amino acid (EAA)								Non-Essential amino acid (NEAA)						
	Arginine	Histidine	Isoleucine	Leucine	Lysine	Phenylalanine	Threonine	Valine	Aspartic acid	Serine	Glutamic acid	Proline	Glycine	Alanine	Tyrosine
Interactive effect between protein and lipid															
Initial	1.17±0.05	0.54±0.03	0.72±0.03	1.20±0.05	1.33±0.05	0.70±0.02	0.57±0.02	0.85±0.03	1.59±0.06	0.37±0.01	2.45±0.09	0.94±0.04	1.55±0.06	1.17±0.04	0.50±0.02
P41L6	1.04±0.05^{ns}	0.54±0.06^{ns}	0.67±0.06^{ns}	1.17±0.13^{ns}	1.13±0.05^{ns}	0.64±0.04^{ns}	0.47±0.03^{ns}	0.75±0.05^{ns}	1.47±0.10^{ns}	0.27±0.01^{ns}	2.18±0.13^{ns}	0.89±0.04^{ns}	1.50±0.09^{ns}	1.10±0.08^{ns}	0.25±0.06^{ns}
P41L9	0.97±0.08	0.50±0.03	0.65±0.05	1.09±0.07	1.13±0.06	0.61±0.04	0.41±0.03	0.66±0.05	1.42±0.08	0.26±0.01	2.01±0.12	0.81±0.04	1.41±0.08	1.08±0.05	0.26±0.07
P41L12	1.00±0.07	0.54±0.03	0.75±0.03	1.28±0.09	1.21±0.05	0.64±0.03	0.37±0.01	0.68±0.04	1.59±0.10	0.28±0.01	2.06±0.12	0.88±0.05	1.47±0.07	1.22±0.04	0.25±0.02
P44L6	0.97±0.04	0.52±0.02	0.66±0.06	1.14±0.11	1.15±0.05	0.61±0.02	0.41±0.03	0.68±0.04	1.47±0.12	0.26±0.01	2.03±0.09	0.87±0.05	1.41±0.05	1.12±0.06	0.22±0.02
P44L9	1.03±0.06	0.57±0.02	0.73±0.06	1.30±0.09	1.22±0.07	0.66±0.03	0.44±0.04	0.73±0.05	1.59±0.13	0.28±0.01	2.17±0.09	0.88±0.05	1.55±0.07	1.25±0.05	0.23±0.03
P44L12	1.03±0.07	0.56±0.02	0.79±0.04	1.30±0.05	1.27±0.04	0.66±0.02	0.40±0.03	0.72±0.05	1.65±0.08	0.28±0.01	2.12±0.08	0.93±0.04	1.51±0.05	1.27±0.03	0.25±0.01
P47L6	1.03±0.05	0.55±0.02	0.71±0.08	1.21±0.14	1.21±0.08	0.65±0.04	0.43±0.02	0.70±0.02	1.57±0.14	0.27±0.01	2.08±0.07	0.90±0.03	1.52±0.10	1.19±0.11	0.23±0.03
P47L9	0.96±0.04	0.53±0.02	0.68±0.01	1.17±0.01	1.12±0.03	0.62±0.01	0.43±0.03	0.68±0.05	1.46±0.01	0.26±0.00	2.04±0.08	0.82±0.02	1.42±0.03	1.13±0.03	0.20±0.01
P47L12	0.93±0.02	0.44±0.01	0.63±0.06	1.00±0.06	1.05±0.00	0.56±0.02	0.39±0.03	0.66±0.01	1.35±0.05	0.24±0.01	1.88±0.06	0.80±0.01	1.31±0.05	0.99±0.03	0.30±0.07
Main effect of protein															
CP41	1.00±0.02^{ns}	0.53±0.01^{ns}	0.69±0.03^{ns}	1.18±0.06^{ns}	1.16±0.03^{ns}	0.63±0.01^{ns}	0.42±0.03^{ns}	0.70±0.03^{ns}	1.49±0.05^{ns}	0.27±0.01^{ns}	2.08±0.05^{ns}	0.86±0.03^{ns}	1.46±0.03^{ns}	1.13±0.04^{ns}	0.25±0.00^{ns}
CP44	1.01±0.02	0.55±0.02	0.73±0.04	1.25±0.05	1.21±0.03	0.64±0.02	0.42±0.01	0.71±0.02	1.57±0.05	0.27±0.01	2.11±0.04	0.89±0.02	1.49±0.04	1.21±0.05	0.23±0.01
CP47	0.97±0.03	0.51±0.03	0.67±0.02	1.13±0.06	1.13±0.05	0.61±0.03	0.42±0.01	0.68±0.01	1.46±0.06	0.26±0.01	2.00±0.06	0.84±0.03	1.42±0.06	1.10±0.06	0.24±0.03
Main effect of lipid															
CL6	1.01±0.02^{ns}	0.54±0.01^{ns}	0.68±0.02^{ns}	1.17±0.02^{ns}	1.16±0.02^{ns}	0.63±0.01^{ns}	0.44±0.02^{ns}	0.71±0.02^{ns}	1.50±0.03^{ns}	0.27±0.00^{ns}	2.10±0.04^{ns}	0.89±0.01^{ns}	1.48±0.03^{ns}	1.14±0.03^{ns}	0.23±0.01^{ns}
CL9	0.99±0.02	0.53±0.02	0.69±0.02	1.19±0.06	1.16±0.03	0.63±0.02	0.43±0.01	0.69±0.02	1.49±0.05	0.27±0.01	2.07±0.05	0.84±0.02	1.46±0.05	1.15±0.05	0.23±0.02
CL12	0.99±0.03	0.51±0.04	0.72±0.05	1.19±0.10	1.18±0.07	0.62±0.03	0.39±0.01	0.69±0.02	1.53±0.09	0.27±0.01	2.02±0.07	0.87±0.04	1.43±0.06	1.16±0.09	0.27±0.02
Two-way ANOVA (*p*-value)															
CP	0.8048	0.5122	0.4725	0.3644	0.2663	0.5392	0.9029	0.8917	0.4488	0.2915	0.6749	0.4521	0.6591	0.1248	0.7523
CL	0.8403	0.9009	0.5842	0.8841	0.7181	0.9832	0.4013	0.8227	0.9039	0.9257	0.9701	0.3288	0.8532	0.7805	0.7008
CP*CL	0.6764	0.2991	0.3055	0.2330	0.2290	0.3864	0.4427	0.6384	0.3091	0.2928	0.6375	0.6053	0.3231	0.0586	0.9186

[1]Values are presented as mean±SEM (Tukey test at alpha=0.05)

Table 8. Fatty acids profile of snakehead fed the nine experimental diets for 8 weeks[1]

Diets	C12:0	C14:0	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1n9t	C18:1n9c	C18:2n6c	C20:0	C18:3n6
Interactive effect between protein and lipid													
Initial	0.13±0.00	5.87±0.04	0.55±0.01	41.5±0.1	2.48±0.01	0.25±0.00	0.09±0.01	8.66±0.07	0.08±0.01	16.4±0.0	15.1±0.1	0.22±0.10	0.12±0.01
P41L6	0.22±0.03^{b}	7.46±0.47^{a}	0.71±0.01^{c}	43.3±0.4^{c}	3.61±0.05^{ab}	0.51±0.02^{ns}	0.15±0.01^{cd}	8.94±0.37^{b}	0.03±0.03^{a}	17.6±0.4^{c}	9.69±0.52^{c}	0.47±0.03^{a}	0.24±0.01^{bc}
P41L9	0.46±0.23^{bc}	7.92±0.34^{e}	0.74±0.01^{c}	43.3±0.3^{a}	3.71±0.06^{c}	0.50±0.02	0.16±0.01^{abc}	9.24±0.36^{e}	0.03±0.03^{b}	17.0±0.2^{ab}	8.66±0.18^{c}	0.52±0.01^{bc}	0.23±0.01^{c}
P41L12	0.15±0.01^{bc}	7.16±0.33^{de}	0.73±0.02^{bc}	42.8±0.7^{b}	3.41±0.05^{bc}	0.51±0.01	0.14±0.01^{a}	9.35±0.55^{e}	0.08±0.00^{b}	16.7±0.4^{a}	9.53±0.20^{a}	0.49±0.04^{cd}	0.28±0.05^{ab}
P44L6	0.46±0.23^{bc}	7.22±0.28^{ab}	0.72±0.02^{a}	44.2±0.7^{b}	3.50±0.12^{a}	0.49±0.01	0.14±0.00^{ab}	9.27±0.21^{e}	0.03±0.03^{b}	16.9±0.4^{b}	8.57±0.12^{bc}	0.47±0.02^{ab}	0.22±0.02^{abc}
P44L9	0.39±0.24^{a}	7.42±0.36^{cd}	0.76±0.01^{ab}	43.6±0.0^{b}	3.62±0.14^{abc}	0.53±0.01	0.15±0.01^{abc}	8.98±0.27^{c}	0.02±0.02^{b}	17.1±0.2^{c}	9.06±0.42^{c}	0.49±0.03^{a}	0.23±0.01^{bc}
P44L12	0.20±0.05^{bc}	7.45±0.22^{bc}	0.72±0.01^{c}	43.3±1.2^{d}	3.60±0.07^{bc}	0.48±0.02	0.14±0.01^{d}	9.15±0.71^{a}	0.09±0.00^{a}	16.6±0.4^{d}	8.90±0.13^{bc}	0.47±0.04^{a}	0.23±0.01^{abc}
P47L6	0.20±0.05^{c}	7.02±0.29^{de}	0.72±0.01^{bc}	44.9±0.8^{a}	3.59±0.12^{ab}	0.49±0.01	0.15±0.01^{ab}	8.86±0.44^{f}	0.06±0.03^{a}	17.2±0.3^{bc}	8.67±0.08^{bc}	0.43±0.03^{d}	0.22±0.03^{abc}
P47L9	0.18±0.00^{bc}	7.61±0.37^{de}	0.74±0.03^{abc}	42.9±0.8^{b}	3.59±0.11^{bc}	0.51±0.01	0.15±0.02^{abc}	9.20±0.63^{d}	0.00±0.00^{a}	17.1±0.6^{c}	9.49±0.40^{b}	0.48±0.03^{c}	0.24±0.01^{a}
P47L12	0.19±0.05^{b}	7.35±0.52^{cd}	0.70±0.01^{ab}	44.4±0.9^{b}	3.58±0.13^{bc}	0.50±0.02	0.14±0.01^{bcd}	9.13±0.48^{d}	0.05±0.03^{a}	17.2±0.2^{c}	8.67±0.07^{bc}	0.46±0.04^{bc}	0.21±0.01^{abc}
Main effect of protein													
CP41	0.28±0.09^{b}	7.51±0.22^{b}	0.73±0.01^{b}	43.13±0.19^{a}	3.57±0.09^{ns}	0.51±0.00^{ns}	0.15±0.01^{ns}	9.18±0.12^{b}	0.05±0.02^{b}	17.1±0.2^{a}	9.29±0.32^{ns}	0.49±0.01^{b}	0.25±0.02^{b}
CP44	0.35±0.08^{a}	7.36±0.07^{a}	0.73±0.01^{a}	43.71±0.28^{b}	3.57±0.04	0.50±0.02	0.14±0.01	9.14±0.08^{a}	0.05±0.02^{b}	16.9±0.1^{c}	8.84±0.14	0.48±0.01^{a}	0.23±0.00^{ab}
CP47	0.19±0.01^{b}	7.32±0.17^{b}	0.72±0.01^{a}	44.07±0.59^{a}	3.59±0.00	0.50±0.00	0.15±0.00	9.06±0.11^{c}	0.04±0.02^{a}	17.2±0.0^{b}	8.94±0.27	0.46±0.01^{c}	0.22±0.01^{a}
Main effect of lipid													
CL6	0.29±0.08^{b}	7.23±0.13^{a}	0.72±0.00^{ns}	44.15±0.44^{b}	3.57±0.03^{a}	0.50±0.01^{ns}	0.15±0.00^{ns}	9.02±0.13^{b}	0.04±0.01^{a}	17.3±0.2^{a}	8.98±0.36^{b}	0.46±0.01^{ns}	0.22±0.01^{ns}
CL9	0.34±0.08^{a}	7.65±0.15^{c}	0.75±0.01	43.27±0.19^{a}	3.64±0.03^{b}	0.52±0.01	0.15±0.00	9.14±0.08^{a}	0.02±0.01^{b}	17.1±0.0^{ab}	9.07±0.24^{b}	0.50±0.01	0.23±0.00
CL12	0.18±0.01^{b}	7.32±0.09^{b}	0.72±0.01	43.51±0.50^{c}	3.53±0.06^{b}	0.50±0.01	0.14±0.00	9.21±0.07^{a}	0.07±0.01^{a}	16.9±0.2^{b}	9.04±0.26^{a}	0.47±0.01	0.24±0.02
Two-way ANOVA (*p*-value)													
CP	<.0001	<.0001	0.0005	<.0001	0.1095	0.6841	0.1820	<.0001	<.0001	<.0001	0.0771	<.0001	0.0176
CL	<.0001	<.0001	0.7102	<.0001	<.0001	0.1279	0.0712	<.0001	0.0062	0.0423	<.0001	0.3103	0.2414
CP*CL	<.0001	<.0001	<.0001	<.0001	0.0497	0.0372	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0037

Table 8 continued. Fatty acids profile of snakehead fed the nine experimental diets for 8 weeks[1]

Diets	C20:1	C18:3n3	C20:2	C22:0	C20:3n6	C22:1n9	C20:3n3	C20:4n6	C24:0	C20:5n3	C24:1	C22:6n3
Interactive effect between protein and lipid												
Initial	0.93±0.00	1.98±0.02	0.37±0.01	0.07±0.00	0.08±0.00	0.00±0.00	0.09±0.01	0.26±0.01	0.00±0.00	0.74±0.03	0.00±0.00	4.06±0.01
P41L6	1.61±0.02^{ab}	0.44±0.04^{ab}	0.30±0.00^{ab}	0.03±0.03^{a}	0.16±0.01^{bc}	0.15±0.02^{ns}	0±0	0.43±0.04^{b}	0.11±0.01^{a}	0.59±0.04^{ab}	0.04±0.04^{ab}	3.18±0.14^{d}
P41L9	1.73±0.08^{ab}	0.41±0.05^{ab}	0.32±0.01^{b}	0.03±0.03^{b}	0.17±0.01^{d}	0.18±0.01	0±0	0.48±0.03^{c}	0.13±0.01^{c}	0.62±0.03^{abc}	0.09±0.05^{d}	3.39±0.09^{e}
P41L12	1.56±0.03^{b}	0.43±0.02^{ab}	0.33±0.01^{ab}	0.05±0.03^{b}	0.18±0.00^{ab}	0.13±0.01	0±0	0.54±0.06^{d}	0.11±0.02^{b}	0.53±0.03^{bcd}	0.11±0.01^{d}	4.71±0.29^{f}
P44L6	1.67±0.01^{a}	0.48±0.12^{ab}	0.32±0.02^{b}	0.02±0.02^{b}	0.12±0.06^{ab}	0.17±0.01	0±0	0.47±0.02^{cd}	0.08±0.04^{ab}	0.61±0.03^{bcd}	0.08±0.04^{d}	3.72±0.4^{e}
P44L9	1.73±0.07^{ab}	0.39±0.05^{b}	0.34±0.02^{a}	0.00±0.00^{b}	0.17±0.00^{ab}	0.15±0.01	0±0	0.46±0.03^{b}	0.11±0.01^{a}	0.59±0.04^{a}	0.05±0.05^{a}	3.64±0.38^{d}
P44L12	1.68±0.03^{b}	0.41±0.04^{b}	0.33±0.01^{ab}	0.07±0.01^{ab}	0.17±0.01^{a}	0.14±0.01	0±0	0.51±0.09^{a}	0.11±0.03^{a}	0.57±0.03^{abc}	0.12±0.02^{a}	4.50±0.43^{a}
P47L6	1.67±0.04^{ab}	0.39±0.03^{ab}	0.29±0.03^{ab}	0.02±0.02^{ab}	0.10±0.05^{c}	0.09±0.05	0±0	0.43±0.03^{d}	0.06±0.03^{b}	0.54±0.04^{d}	0.06±0.03^{bc}	3.84±0.35^{c}
P47L9	1.64±0.07^{b}	0.47±0.07^{a}	0.30±0.02^{ab}	0.00±0.00^{ab}	0.17±0.01^{ab}	0.16±0.01	0±0	0.49±0.09^{b}	0.08±0.04^{ab}	0.58±0.02^{cd}	0.04±0.04^{c}	3.83±0.76^{b}
P47L12	1.68±0.02^{ab}	0.38±0.01^{ab}	0.32±0.01^{ab}	0.03±0.03^{ab}	0.10±0.05^{ab}	0.15±0.01	0±0	0.45±0.03^{b}	0.06±0.04^{ab}	0.56±0.04^{bcd}	0.07±0.04^{abc}	3.56±0.18^{b}
Main effect of protein												
CP41	1.63±0.05^{b}	0.43±0.01^{ns}	0.32±0.01^{b}	0.04±0.01^{ns}	0.17±0.00^{c}	0.15±0.01^{ns}	0±0	0.48±0.03^{b}	0.12±0.01^{c}	0.58±0.02^{a}	0.08±0.02^{b}	3.76±0.48^{c}
CP44	1.70±0.02^{a}	0.43±0.03	0.33±0.01^{a}	0.03±0.02	0.15±0.02^{a}	0.16±0.01	0±0	0.48±0.02^{a}	0.10±0.01^{a}	0.59±0.01^{a}	0.08±0.02^{a}	3.95±0.28^{b}
CP47	1.66±0.01^{b}	0.41±0.03	0.30±0.01^{ab}	0.02±0.01	0.12±0.02^{b}	0.13±0.02	0±0	0.46±0.02^{b}	0.07±0.01^{b}	0.56±0.01^{b}	0.06±0.01^{a}	3.74±0.09^{a}
Main effect of lipid												
CL6	1.65±0.02^{a}	0.44±0.02^{ns}	0.30±0.01^{ns}	0.03±0.00^{ns}	0.13±0.02^{b}	0.14±0.02^{ns}	0±0	0.44±0.01^{c}	0.08±0.01^{a}	0.58±0.02^{b}	0.06±0.01^{ns}	3.58±0.20^{c}
CL9	1.70±0.03^{ab}	0.42±0.02	0.32±0.01	0.01±0.01	0.17±0.00^{c}	0.17±0.01	0±0	0.48±0.01^{b}	0.11±0.01^{b}	0.60±0.01^{a}	0.06±0.02	3.62±0.13^{b}
CL12	1.64±0.04^{b}	0.41±0.01	0.32±0.00	0.05±0.01	0.15±0.02^{a}	0.14±0.01	0±0	0.50±0.03^{a}	0.10±0.02^{a}	0.56±0.01^{ab}	0.10±0.02	4.26±0.35^{a}
Two-way ANOVA (*p*-value)												
CP	0.0079	0.1006	0.0344	0.2952	<.0001	0.0559		<.0001	<.0001	<.0001	0.0002	<.0001
CL	0.0285	0.9535	0.1004	0.2465	<.0001	0.6681		<.0001	0.0097	0.0390	0.4084	<.0001
CP*CL	0.0522	0.0023	0.0139	0.0015	<.0001	0.3182		<.0001	<.0001	0.0013	<.0001	<.0001

[1]Values are presented as mean ± SEM (Tukey test at alpha=0.05)

1.3 Antioxidant enzyme and immunity

Result of the antioxidant enzyme and non-specific immune responses of snakehead are shown in table 9. GPx and LZM activities were not significantly affected by dietary levels of protein or lipid ($P > 0.05$).

Table 9. Antioxidant enzyme and immunity of snakehead fed the experimental diets for 8 weeks[1]

Diets	GPx[2]	LZM[3]
Interactive effect between protein and lipid		
P41L6	85.78 ± 3.64[ns]	3.46 ± 0.51[ns]
P41L9	81.09 ± 3.26	2.71 ± 1.01
P41L12	72.10 ± 3.76	4.51 ± 1.54
P44L6	81.03 ± 4.87	5.56 ± 2.70
P44L9	81.37 ± 1.37	6.74 ± 0.73
P44L12	73.71 ± 4.12	5.55 ± 1.09
P47L6	73.93 ± 3.51	6.67 ± 1.63
P47L9	68.34 ± 5.62	6.62 ± 2.65
P47L12	73.80 ± 0.62	4.55 ± 0.56
Main effect of protein		
CP41	79.66 ± 4.01[ns]	3.56 ± 0.52[ns]
CP44	78.70 ± 2.50	5.95 ± 0.40
CP47	72.02 ± 1.84	5.95 ± 0.70
Main effect of lipid		
CL6	80.25 ± 3.45[ns]	5.23 ± 0.94[ns]
CL9	76.93 ± 4.29	5.36 ± 1.32
CL12	73.20 ± 0.55	4.87 ± 0.34
Two-way ANOVA (*p*-value)		
CP	0.0438	0.1321
CL	0.0955	0.9529
CP*CL	0.2619	0.7308

[1]Values are presented as mean ± SEM (Tukey test at alpha=0.05)
[2]Glutathione peroxidase (U/mL)
[3]Lysozyme (ng/dl)

1.4 Plasma metabolites

Plasma metabolites analysis of juvenile snakehead after growth trial (Exp. Ⅰ) are presented in Table 10. There were no significant differences in GOT and GPT levels of fish fed all the experimental diets ($P > 0.05$). TP and TG values in plasma increased with increasing dietary protein levels and decreased dietary lipid levels. Increasing the dietary CL level from 6% to 12% significantly reduced TCHO, GLU ($P < 0.05$) but main effect of dietary CP levels was not significant ($P > 0.05$).

Table 10. Plasma metabolites of snakehead fed the nine experimental diets for 8 weeks[1]

Diets	GOT[2]	GPT[3]	TP[4]	TG[5]	TCHO[6]	GLU[7]
Interactive effect between protein and lipid						
P41L6	13.0 ± 1.7^{ns}	12.7 ± 1.5^{ns}	1.93 ± 0.09^{b}	37.0 ± 6.1^{bc}	57.3 ± 3.0^{abc}	169 ± 36^{ns}
P41L9	12.7 ± 2.0	11.3 ± 1.9	1.67 ± 0.18^{b}	19.0 ± 2.0^{c}	52.7 ± 3.3^{abc}	145 ± 11
P41L12	13.0 ± 0.6	12.7 ± 0.3	1.87 ± 0.13^{b}	14.7 ± 5.6^{c}	44.3 ± 2.0^{c}	88.7 ± 6.7
P44L6	13.3 ± 1.2	13.0 ± 0.6	2.17 ± 0.12^{ab}	44.0 ± 2.1^{b}	60.0 ± 2.0^{ab}	167 ± 28
P44L9	11.7 ± 0.7	11.3 ± 0.3	2.03 ± 0.13^{ab}	36.3 ± 5.8^{bc}	54.0 ± 2.1^{abc}	156 ± 20
P44L12	14.0 ± 1.0	13.7 ± 0.3	1.80 ± 0.06^{b}	21.7 ± 4.9^{bc}	46.7 ± 2.4^{bc}	111 ± 5
P47L6	13.7 ± 1.2	11.3 ± 0.3	2.60 ± 0.17^{a}	71.7 ± 5.5^{a}	64.3 ± 5.9^{a}	194 ± 39
P47L9	13.3 ± 0.9	12.3 ± 0.9	2.10 ± 0.10^{ab}	36.3 ± 5.9^{bc}	54.0 ± 2.3^{abc}	134 ± 14
P47L12	14.0 ± 0.6	12.7 ± 0.3	1.90 ± 0.12^{b}	26.7 ± 3.5^{bc}	49.3 ± 0.3^{bc}	106 ± 4
Main effect of protein						
CP41	12.9 ± 0.1^{ns}	12.2 ± 0.4^{ns}	1.82 ± 0.08^{b}	23.6 ± 6.8^{c}	51.4 ± 3.8^{ns}	134 ± 24^{ns}
CP44	13.0 ± 0.7	12.7 ± 0.7	2.00 ± 0.11^{ab}	34.0 ± 6.6^{b}	53.6 ± 3.9	145 ± 17
CP47	13.7 ± 0.2	12.1 ± 0.4	2.20 ± 0.21^{a}	44.9 ± 13.7^{a}	55.9 ± 4.4	145 ± 26
Main effect of lipid						
CL6	13.3 ± 0.2^{ns}	12.3 ± 0.5^{ns}	2.23 ± 0.20^{a}	50.9 ± 10.6^{a}	60.6 ± 2.0^{a}	176 ± 9^{a}
CL9	12.6 ± 0.5	11.7 ± 0.3	1.93 ± 0.13^{b}	30.6 ± 5.8^{b}	53.6 ± 0.4^{b}	145 ± 6^{ab}
CL12	13.7 ± 0.3	13.0 ± 0.3	1.86 ± 0.03^{b}	21.0 ± 3.5^{b}	46.8 ± 1.4^{c}	102 ± 7^{b}
Two-way ANOVA (*p*-value)						
CP	0.6954	0.7280	0.0070	0.0002	0.2088	0.7989
CL	0.5187	0.2177	0.0046	<.0001	<.0001	0.0023
CP*CL	0.9264	0.5779	0.1395	0.0580	0.8997	0.8116

[1]Values are presented as mean ± SEM (Tukey test at alpha=0.05)
[2]GOT: Glutamic oxaloacetic transaminase (U/l)
[3]GPT: Glutamic pyruvic transaminase (U/l)
[4]TP: Total protein (g/dl)
[5]TG: Triglyceride (mg/dl)
[6]TCHO: Total cholesterol (mg/dl)
[7]GLU: Glucose (mg/dl)

Experiment II. Acute temperature stress experiment

Results of juvenile snakehead after acute temperature stress (35℃, 2 h) and recovery (27℃, 2 h) treatment.

2.1. Plasma metabolites

Table 11 shows the effects of temperature manipulation, dietary CP and CL on plasma metabolites analyzed TS group compared with the NS group. The levels of GOT, GPT, TP, and TG were detected significant main effect ($P < 0.05$) of stress condition. GOT and GPT were significantly higher at the acute temperature stress compared to the non stress treatment groups in fish. There appeared to be opposite tendency in the activities of TP and TG. However, the interaction of the main factors (stress condition, crude protein and lipid) failed to have any observable effect on any metabolites in the plasma ($P > 0.05$).

Table 11. Plasma metabolites of snakehead fed the nine experimental diets for 8 weeks and after acute temperature stress experiment[1]

Diets	GOT[2]	GPT[3]	TP[4]	TG[5]	TCHO[6]	GLU[7]
NSP41L6	13.0 ± 1.7^{ns}	12.7 ± 1.5^{ns}	1.93 ± 0.09^{ns}	37.0 ± 6.1^{ns}	57.3 ± 3.0^{ns}	169 ± 36^{ns}
NSP41L9	12.7 ± 2.0	11.3 ± 1.9	1.67 ± 0.18	19.0 ± 2.0	52.7 ± 3.3	145 ± 11
NSP41L12	13.0 ± 0.6	12.7 ± 0.3	1.87 ± 0.13	14.7 ± 5.6	44.3 ± 2.0	88.7 ± 6.7
NSP44L6	13.3 ± 1.2	13.0 ± 0.6	2.17 ± 0.12	44.0 ± 2.1	60.0 ± 2.0	167 ± 28
NSP44L9	11.7 ± 0.7	11.3 ± 0.3	2.03 ± 0.13	36.3 ± 5.8	54.0 ± 2.1	156 ± 20
NSP44L12	14.0 ± 1.0	13.7 ± 0.3	1.80 ± 0.06	21.7 ± 4.9	46.7 ± 2.4	111 ± 5
NSP47L6	13.7 ± 1.2	11.3 ± 0.3	2.60 ± 0.17	71.7 ± 5.5	64.3 ± 5.9	194 ± 39
NSP47L9	13.3 ± 0.9	12.3 ± 0.9	2.10 ± 0.10	36.3 ± 5.9	54.0 ± 2.3	134 ± 14
NSP47L12	14.0 ± 0.6	12.7 ± 0.3	1.90 ± 0.12	26.7 ± 3.5	49.3 ± 0.3	106 ± 4.2
TSP41L6	17.3 ± 3.0	16.0 ± 2.5	1.97 ± 0.23	28.7 ± 4.8	51.7 ± 3.5	149 ± 7
TSP41L9	14.0 ± 1.0	14.3 ± 0.3	1.70 ± 0.25	9.67 ± 8.67	50.3 ± 4.3	134 ± 10
TSP41L12	12.7 ± 0.9	13.0 ± 1.5	1.17 ± 0.07	5.00 ± 4.00	34.0 ± 3.5	62.0 ± 21.4
TSP44L6	19.0 ± 3.1	17.3 ± 2.7	1.90 ± 0.15	29.3 ± 6.8	52.3 ± 2.3	156 ± 30
TSP44L9	14.7 ± 0.9	16.3 ± 1.3	1.73 ± 0.09	12.5 ± 2.0	48.7 ± 8.4	107 ± 17
TSP44L12	17.0 ± 0.6	16.7 ± 3.3	1.63 ± 0.22	2.50 ± 1.50	48.3 ± 10.4	67.7 ± 21.1
TSP47L6	17.7 ± 4.7	16.3 ± 4.4	2.23 ± 0.09	55.3 ± 4.1	66.7 ± 5.2	219 ± 28
TSP47L9	16.0 ± 1.2	15.3 ± 1.2	1.97 ± 0.28	18.3 ± 10.1	48.7 ± 4.8	116 ± 38
TSP47L12	16.3 ± 1.5	16.7 ± 4.2	1.93 ± 0.03	31.7 ± 8.1	57.7 ± 2.3	137 ± 22
Main effect of stress						
NS	13.2 ± 0.2^{b}	12.3 ± 0.3^{b}	2.01 ± 0.09^{a}	34.1 ± 5.7^{a}	53.6 ± 2.1^{ns}	141 ± 11^{ns}
TS	16.1 ± 0.7^{a}	15.8 ± 0.5^{a}	1.80 ± 0.10^{b}	21.4 ± 5.6^{b}	50.9 ± 2.9	128 ± 16
Main effect of protein						
CP41	13.8 ± 0.7^{ns}	13.3 ± 0.7^{ns}	1.72 ± 0.12^{b}	19.0 ± 4.9^{b}	48.4 ± 3.3^{b}	125 ± 17^{ns}
CP44	14.9 ± 1.1	14.7 ± 1.0	1.88 ± 0.08^{b}	24.4 ± 6.3^{b}	51.7 ± 2.0^{ab}	127 ± 16
CP47	15.2 ± 0.7	14.1 ± 0.9	2.12 ± 0.11^{a}	40.0 ± 8.1^{a}	56.8 ± 3.1^{a}	151 ± 18
Main effect of lipid						
CL6	15.7 ± 1.1^{ns}	14.4 ± 1.0^{ns}	2.13 ± 0.11^{a}	44.3 ± 6.8^{a}	58.7 ± 2.5^{a}	176 ± 11^{a}
CL9	13.7 ± 0.6	13.5 ± 0.9	1.87 ± 0.08^{b}	22.0 ± 4.7^{b}	51.4 ± 1.0^{b}	132 ± 7^{b}
CL12	14.5 ± 0.7	14.2 ± 0.8	1.72 ± 0.12^{b}	17.0 ± 4.8^{b}	46.7 ± 3.1^{b}	95.4 ± 11.6^{c}

Table 11 continued. Plasma metabolites of snakehead fed the nine experimental diets for 8 weeks and after acute temperature stress experiment[1]

Main effect of stress & protein						
NSCP41	12.9 ± 0.1^{ns}	12.2 ± 0.4^{ns}	1.82 ± 0.08^{ns}	23.6 ± 6.8^{ns}	51.4 ± 3.8^{ns}	134 ± 24^{ns}
NSCP44	13.0 ± 0.7	12.7 ± 0.7	2.00 ± 0.11	34.0 ± 6.6	53.6 ± 3.9	145 ± 17
NSCP47	13.7 ± 0.2	12.1 ± 0.4	2.20 ± 0.21	44.9 ± 13.7	55.9 ± 4.4	145 ± 26
TSCP41	14.7 ± 1.4	14.4 ± 0.9	1.61 ± 0.24	14.4 ± 7.2	45.3 ± 5.3	115 ± 27
TSCP44	16.9 ± 1.3	16.8 ± 0.3	1.76 ± 0.08	14.8 ± 7.8	49.8 ± 1.3	110 ± 26
TSCP47	16.7 ± 0.5	16.1 ± 0.4	2.04 ± 0.09	35.1 ± 10.8	57.7 ± 5.2	157 ± 31
Main effect of stress & lipid						
NSCL6	13.3 ± 0.7^{ns}	12.3 ± 0.5^{ns}	2.23 ± 0.12^{ns}	50.9 ± 5.8^{ns}	60.6 ± 2.2^{ns}	176 ± 18^{ns}
NSCL9	12.6 ± 0.7	11.7 ± 0.6	1.93 ± 0.10	30.6 ± 3.8	53.6 ± 1.3	145 ± 8
NSCL12	13.7 ± 0.4	13.0 ± 0.2	1.86 ± 0.06	21.0 ± 2.9	46.8 ± 1.2	102 ± 4
TSCL6	18.0 ± 1.8	16.6 ± 1.7	2.03 ± 0.10	37.8 ± 5.1	56.9 ± 3.1	175 ± 16
TSCL9	14.9 ± 0.6	15.3 ± 0.6	1.80 ± 0.12	13.6 ± 4.1	49.2 ± 3.1	119 ± 13
TSCL12	15.3 ± 0.8	15.4 ± 1.7	1.58 ± 0.13	13.1 ± 5.4	46.7 ± 4.7	89.0 ± 16.0
Three-way ANOVA (*p*-value)						
ST	0.0018	0.0009	0.0087	<.0001	0.2074	0.2171
CP	0.3739	0.4997	0.0003	<.0001	0.0091	0.1023
CL	0.1899	0.7030	0.0002	<.0001	0.0002	<.0001
ST*CP	0.6046	0.6668	0.8832	0.2297	0.3030	0.2030
ST*CL	0.3351	0.7413	0.7259	0.3776	0.6817	0.6557
CP*CL	0.7974	0.9340	0.8649	0.0539	0.1780	0.2710
ST*CP*CL	0.9824	0.9674	0.0944	0.4279	0.4787	0.8279

[1]Values are presented as mean ± SEM (Tukey test at alpha=0.05)
[2]GOT: Glutamic oxaloacetic transaminase (U/l)
[3]GPT: Glutamic pyruvic transaminase (U/l)
[4]TP: Total protein (g/dl)
[5]TG: Triglyceride (mg/dl)
[6]TCHO: Total cholesterol (mg/dl)
[7]GLU: Glucose (mg/dl)

2.2. Stress responses

Result of stress responses of snakehead exposed to acute temperature stress were analyzed (Table 12). Cortisol and HSP70 had no main effect for both dietary CP and CL levels ($P > 0.05$).

Table 12. Stress responses (HSP70, cortisol) of snakehead after acute temperature stress

Diets	HSP70[2]	Cortisol[3]
Interactive effect between protein and lipid		
P41L6	26.9 ± 0.6[ns]	16.5 ± 6.0[ns]
P41L9	27.1 ± 1.6	12.0 ± 1.8
P41L12	22.1 ± 0.9	19.2 ± 4.0
P44L6	25.7 ± 2.0	23.8 ± 2.3
P44L9	23.9 ± 1.9	16.7 ± 1.7
P44L12	22.7 ± 0.7	17.6 ± 3.2
P47L6	24.7 ± 2.2	18.9 ± 2.0
P47L9	26.2 ± 1.0	17.3 ± 3.2
P47L12	26.4 ± 2.5	20.7 ± 3.1
Main effect of protein		
CP41	25.4 ± 1.6[ns]	15.9 ± 2.1[ns]
CP44	24.1 ± 0.9	19.3 ± 2.2
CP47	25.8 ± 0.5	19.0 ± 1.0
Main effect of lipid		
CL6	25.8 ± 0.6[ns]	19.7 ± 2.1[ns]
CL9	25.7 ± 1.0	15.3 ± 1.7
CL12	23.7 ± 1.3	19.2 ± 0.9
Two-way ANOVA (*p*-value)		
CP	0.4437	0.3931
CL	0.2474	0.2327
CP*CL	0.3028	0.6455

[1]Values are presented as mean ± SEM (Tukey test at alpha=0.05)
[2]Heat shock protein 70 (pg/dl)
[3]Cortisol (ng/ml)

2.3. Relative gene expression level of temperature stress exposure

Results of relative gene expression levels by tissues (haed kidney, gill, liver, and spleen) of snakehead following temperature stress exposure are presented in Tables 13, 14, 15, and 16, respectively. Two-way ANOVA showed that treatments fed 47% dietary CP had significantly higher ($P < 0.05$) *hsp70* gene expression in head kidney of fish than those 41% and 44% CP diets, but main effect of dietary CL had no effect on *hsp60* and *70* expression (Table 13). The interactive effect between dietary CP and CL levels were not significant for head kidney, gill, liver, and spleen ($P > 0.05$).

Table 13. Relative gene expression levels[1] in head kidney after acute temperature stress (mean ± SEM)

Diets	*hsp60*[2]	*hsp70*[3]
Interactive effect between protein and lipid		
P41L6	2.00 ± 1.10^{ns}	2.30 ± 1.51^{b}
P41L9	5.12 ± 3.17	1.05 ± 0.55^{b}
P41L12	2.68 ± 1.22	6.26 ± 5.61^{ab}
P44L6	1.89 ± 0.87	4.94 ± 4.12^{ab}
P44L9	1.67 ± 0.78	4.26 ± 2.43^{b}
P44L12	1.50 ± 0.77	3.65 ± 1.80^{b}
P47L6	17.9 ± 12.0	24.0 ± 6.51^{a}
P47L9	9.84 ± 6.01	9.51 ± 4.53^{ab}
P47L12	7.07 ± 4.24	15.8 ± 3.8^{ab}
Main effect of protein		
CP41	3.27 ± 0.95^{ns}	3.20 ± 1.57^{b}
CP44	1.69 ± 0.11	4.28 ± 0.37^{b}
CP47	11.6 ± 3.2	16.4 ± 4.2^{a}
Main effect of lipid		
CL6	7.26 ± 5.31^{ns}	10.4 ± 6.8^{ns}
CL9	5.54 ± 2.37	4.94 ± 2.47
CL12	3.75 ± 1.70	8.56 ± 3.68
Two-way ANOVA (*p*-value)		
CP	0.0480	0.0013
CL	0.6821	0.2710
CP*CL	0.7141	0.3302

[1] Relative gene express level (P41L6 was set as a control; beta actin used as housekeeping gene)
[2] Heat shock protein 60 (pg/dl)
[3] Heat shock protein 70 (pg/dl)

Table 14. Relative gene expression levels[1] in gill after acute temperature stress (mean ± SEM)

Diets	*hsp60*[2]	*hsp70*[3]
Interactive effect between protein and lipid		
P41L6	1.01 ± 0.10[ns]	1.70 ± 1.10[ns]
P41L9	12.4 ± 5.4	22.5 ± 19.7
P41L12	56.0 ± 53.4	28.8 ± 14.3
P44L6	8.59 ± 7.29	4.03 ± 3.83
P44L9	23.6 ± 20.7	5.48 ± 2.75
P44L12	3.05 ± 1.83	13.7 ± 9.93
P47L6	1.47 ± 0.20	3.87 ± 2.46
P47L9	1.32 ± 0.49	1.32 ± 1.04
P47L12	8.45 ± 6.78	11.6 ± 4.89
Main effect of protein		
CP41	23.2 ± 16.8[ns]	17.6 ± 8.2[ns]
CP44	11.7 ± 6.13	7.73 ± 3.00
CP47	3.75 ± 2.35	5.61 ± 3.10
Main effect of lipid		
CL6	3.69 ± 2.45[ns]	3.20 ± 0.75[ns]
CL9	12.4 ± 6.4	9.76 ± 6.47
CL12	22.6 ± 16.9	18.0 ± 5.4
Two-way ANOVA (*p*-value)		
CP	0.2983	0.1720
CL	0.2974	0.0831
CP*CL	0.2262	0.5757

[1] Relative gene express level (P41L6 was set as a control; beta actin used as housekeeping gene)
[2] Heat shock protein 60 (pg/dl)
[3] Heat shock protein 70 (pg/dl)

Table 15. Relative gene expression levels[1] in liver after acute temperature stress (mean ± SEM)

Diets	*hsp60*[2]	*hsp70*[3]
Interactive effect between protein and lipid		
P41L6	1.01 ± 0.12[ns]	1.00 ± 0.05[ns]
P41L9	0.89 ± 0.06	0.48 ± 0.14
P41L12	0.52 ± 0.19	0.003 ± 0.001
P44L6	0.80 ± 0.14	0.78 ± 0.66
P44L9	0.63 ± 0.05	0.18 ± 0.05
P44L12	0.60 ± 0.20	0.03 ± 0.02
P47L6	0.63 ± 0.05	0.33 ± 0.19
P47L9	0.57 ± 0.07	0.06 ± 0.06
P47L12	0.93 ± 0.20	1.03 ± 0.81
Main effect of protein		
CP41	0.81 ± 0.15[ns]	0.49 ± 0.29[ns]
CP44	0.68 ± 0.06	0.33 ± 0.23
CP47	0.71 ± 0.11	0.47 ± 0.29
Main effect of lipid		
CL6	0.81 ± 0.11[ns]	0.70 ± 0.20[ns]
CL9	0.70 ± 0.10	0.24 ± 0.12
CL12	0.69 ± 0.13	0.35 ± 0.34
Two-way ANOVA (*p*-value)		
CP	0.4753	0.8275
CL	0.4555	0.2792
CP*CL	0.0596	0.1589

[1] Relative gene express level (P41L6 was set as a control; beta actin used as housekeeping gene)
[2] Heat shock protein 60 (pg/dl)
[3] Heat shock protein 70 (pg/dl)

Table 16. Relative gene expression levels[1] in spleen after acute temperature stress (mean ± SEM)

Diets	*hsp60*[2]	*hsp70*[3]
Interactive effect between protein and lipid		
P41L6	1.04 ± 0.22[ns]	1.01 ± 0.10[ns]
P41L9	0.88 ± 0.22	0.46 ± 0.22
P41L12	0.74 ± 0.08	0.23 ± 0.22
P44L6	1.87 ± 0.90	1.91 ± 1.90
P44L9	0.90 ± 0.12	3.08 ± 2.22
P44L12	0.66 ± 0.05	0.38 ± 0.22
P47L6	1.89 ± 1.04	0.88 ± 0.25
P47L9	0.56 ± 0.08	0.13 ± 0.06
P47L12	1.20 ± 0.68	1.53 ± 0.93
Main effect of protein		
CP41	0.89 ± 0.09[ns]	0.57 ± 0.23[ns]
CP44	1.14 ± 0.37	1.79 ± 0.78
CP47	1.21 ± 0.38	0.85 ± 0.41
Main effect of lipid		
CL6	1.60 ± 0.28[ns]	1.27 ± 0.33[ns]
CL9	0.78 ± 0.11	1.22 ± 0.93
CL12	0.86 ± 0.17	0.71 ± 0.41
Two-way ANOVA (*p*-value)		
CP	0.7314	0.3165
CL	0.1387	0.7466
CP*CL	0.7454	0.3376

[1] Relative gene express level (P41L6 was set as a control; beta actin used as housekeeping gene)
[2] Heat shock protein 60 (pg/dl)
[3] Heat shock protein 70 (pg/dl)

IV. Discussion

In the present study, the growth performance of juvenile snakehead *(channa argus)* was significantly affected by dietary protein and lipid levels. WG, SGR, and FE increased with the increase of dietary protein level from 41% to 47% and decrease of dietary lipid level from 12% to 6%, and the highest growth was obtained when fish fed diets containing 47% CP with 6% CL. According to a previous study, the optimal protein and lipid content of the same species, northern snakehead (*channa argus*), was found to be 48% and 12% or 15% (Sagada et al., 2017). Also, the protein requirements recommended to other snakehead species fingerlings such as striped (*channa striata*) and hybrid snakehead (*C. maculates* ♀ × *C. argus* ♂) were 45 ~ 47.2%, 47.9~5.5%, respectively, which showed protein requirements similar to the results of this study (Aliyu-Paiko et al., 2010a, 2010b; Hua et al., 2019; Zhang et al., 2017).

Adequate levels of nonprotein nutrients in the feed, such as lipids and carbohydrates, can minimize the use protein for energy (NRC, 2011) and lead to protein-sparing effect (De Silva et al., 1991; Lee et al., 2002). In this study, the growth of *C. argus* was found to decreased with increasing dietary lipid, meaning that no protein sparing effect by dietary lipids. In agreement with our results, several other studies have shown a similar trend toward a lack of protein-sparing effect. For example, European sea bass (Peres and Oliva-Teles, 1999), Amberjack (Takakuwa et al., 2006), Sea bream (Ozorio et al., 2006), Cuneate drum (Wang et al., 2006), Malaysian mahseer (Ng et al. 2008), Cobia (Craig et al., 2006), and Giant croaker (Chai et al., 2013). In contrast, Sagade et al. (2017) observed a protein-sparing effect on the same species. In addition to the lack of protein-sparing effect, in this study, *C. argus* seemed to be unable to effectively utilize high dietary lipid levels. Aliyu-Paiko et al. (2010) observed that the growth performance of juvenile striped snakehead (*Channa striata*) fed with three lipid levels (6.5, 9, and 11.5%) decreased linearly with increasing lipid levels, showing a similar trend to present study.

After experiment I, no significant ($P > 0.05$) differences in whole-body proximate

composition among dietary treatments with respect to moisture, crude protein, crude lipid, and crude ash. Gallagher (1999) also reported that Sunshine Bass, *Morone chrysops* × *M. saxatilis* fed diets of various protein levels no significant differences in whole-body proximate composition.

The aim of this study is to determine the correlation between the dietary nutrient content and the physiological response due to acute temperature stress in juvenile northern snakehead. Changes in water temperature are one of the environmental stressors and are known to directly affect the physiological response and growth of fish (Elliott 1982; Barton and Iwama 1991; Dutta 1994; Bhikajee and Gobin 1998; Hur et al. 2008; Lee et al. 2014; Shin et al. 2018). In particular, Previous studies have reported that rapid changes in water temperature change metabolism and hematology of fish (Lankford et al. 2003; Jaxion-Harm & Ladich 2014; Amin & Khan 2016). Therefore, after a growth trial based on dietary protein and lipid levels (Exp I, NS group), acute temperature stress experiment was conducted (Exp II, TS group). Hematological parameters such as Glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), total protein (TP), triglyceride (TG), total cholesterol (TCHO), and glucose (GLU) are known as biological indicators that reflect the physiological status of fish (Adhikari et al., 2004). The analyzes hematological parameters in plasma may also be used as a condition and stress indicator.

GOT and GPT playing important roles in protein metabolism and are present in the liver cells of most vertebrates. When the liver is damaged, GOT and GPT exporting into the blood stream and are used indicators of liver function (Ming et al., 2012). Short-term and long-term destruction of liver cells caused by various stressors such as rapid changes in water temperature, hypoxia, pH, ammonia, and heavy metal pollution causes the levels of these enzymes in the blood increase (Kang et al., 2007; Pan et al., 2003). In the present study (NS group), the absence of differences in GOT and GPT in plasma among different dietary protein and lipid levels indicated that the nutrient levels in the feed did not have a negative effect on

liver status or physical function. However, the GOT and GPT of the TS group were significantly higher than the NS group. This suggests that acute temperature stress may have contributed to liver damage in this study. Similar results were also reported by Kumar et al. (2011) in *L. rohita* exposed to heat shock and by Das et al. (2006) in *L. rohita* exposed to different temperatures.

Plasma total protein content reflects the nutritional and metabolic status of fish (Sidduqui, 1977; Byrne et al., 1989), and the effects of stress caused by water temperature, water quality, and other factors have been reported (McLeay and Brown, 1979). In this study, the TS group subjected to acute temperature stress showed a significant decrease in TP and TG compared to the NS group. A review paper (Islam et al., 2022) reported that GOT, GPT tended to increase and TP, TG decreased in the plasma metabolites stress response of fish exposed to water temperature stress. And the results of plasma metabolites analysis after Exp II in this study indicated the same trend.

Cortisol is an important stress hormone secreted from the hypothalamus-pituitary-interrenal (HPI) axis when fish is subjected to external stimulation, and regulates osmotic pressure, metabolism, and immune responses (Vizzini et al., 2007). Elevated plasma cortisol levels have been reported due to stressors in many bony fish, such as starry flounder (*Platichthys stellatus*) and Korean rockfish (*Sebastes Schlegell*) (Kim et al. 2009; Do et al. 2016). The trend of increase in plasma cortisol by stress promotes gluconeogenesis and increases the secretion of glucose into the blood, Increased glucose helps maintain homeostasis by meeting the increased energy demands caused by stress (Vijayan & Tan, 1997). On the other hand, there is a lack of research on the effect of fish nutritional status on plasma cortisol concentration when fish are stressed. In Abdel-Tawwab. (2012), a study on the correlation between dietary protein levels and stocking density of Nile tilapia (*Oreochromis niloticus*) found that there was a main effect on plasma cortisol concentration, but there was no interaction. they also reported that the lower difference in cortisol level in the high protein-fed group (40% and 45%) after and before heat shock in *L. rohita* clearly indicates a lower state of stress compared with 20 and 30% CP-fed

group (Kumar et al., 2011). In current study, as a result of analyzed plasma cortisol in the acute temperature stressed group (TS), there was no main effect or interaction between the two factors of dietary protein and lipid. This suggests that differences in nutrient content between experimental diets had no effect on stress response.

Relative gene expression by tissue was conducted on the group exposed to acute temperature stress (TS group) after a growth trial experiment based on protein and lipid levels in the feed. Heat shock proteins (Hsp) are well known as stress-related proteins expressed by water temperature stimulation, environmental changes, and pollution (Beckmann et al., 1990). In particular, it has been reported as a protein that reacts sensitively to rapid changes in water temperature (Feder & Hofmann, 1999) and plays a major role in maintaining homeostasis in the body from stress factors (Sanders, 1993; Suzue & Young, 1996; Iwama et al., 1998; Ackerman & Iwama, 2001). As a result of this study, there was no significant difference in the relative gene expression levels of *hsp60* and *hsp70* between the experimental groups in the gill, liver, and spleen of the TS group. However, as an exception, when analyzing the relative gene expression levels of *hsp70* between the experimental groups in the head kidney, the experimental feed containing 47% protein and 6% lipid showed the most significantly higher levels compared to the rest diets. HSPs gene expression responses can vary depending on tissue, HSPs family, and stressor, and the sensitivity of HSPs gene expression can vary depending on species, developmental stage, and season (Kayhan and Duman, 2010). Currently, there is a lack of research on tissue-specific expression differences in heat shock protein genes depending on nutritional status when fish are exposed to water temperature stress, and additional research is deemed necessary.

V. Conclusion

In juvenile northern snakehead (*channa argus*), the nutritional status, that is, the dietary protein and lipid levels interaction with the growth performance. However, when exposed to acute water temperature stress, the nutritional status of snakehead appears to have little interaction with the stress response to acute water temperature stress.

Considering the characteristics of the species, they live in extreme environments, such as large changes in water temperature, and have excellent environmental resistance, which can be explained by their high resistance to stressful environments and low sensitivity.

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