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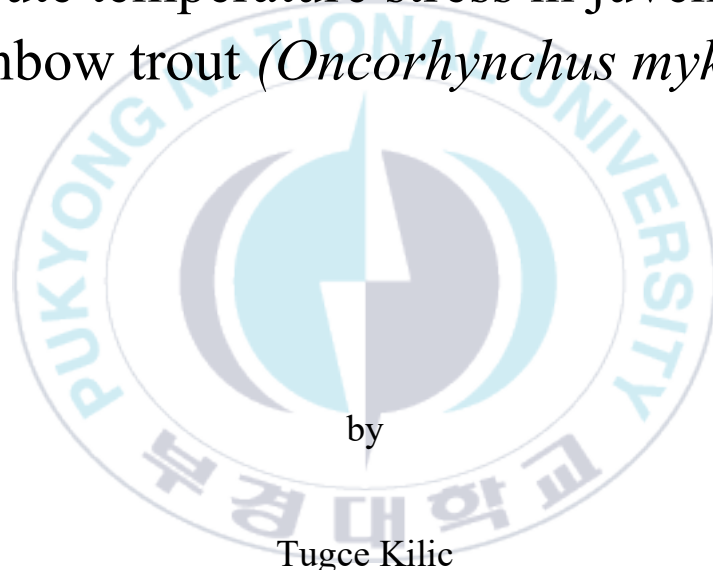
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Thesis for the degree of Master of Fisheries Science

Determination of an optimum dietary
gamma-aminobutyric acid level and
plasma metabolite changes in response to
acute temperature stress in juvenile
rainbow trout (*Oncorhynchus mykiss*)



by

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February 2024

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Supervisor: Prof. Seunghyung Lee



by
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A thesis submitted in partial fulfillment of the requirements
for the degree of

Master of Fisher Science

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

February 2024

Tugce Kilic 의 수산학석사 학위논문을
인준함

2024년 2월 16일



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Determination of an optimum dietary gamma-aminobutyric acid
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A dissertation

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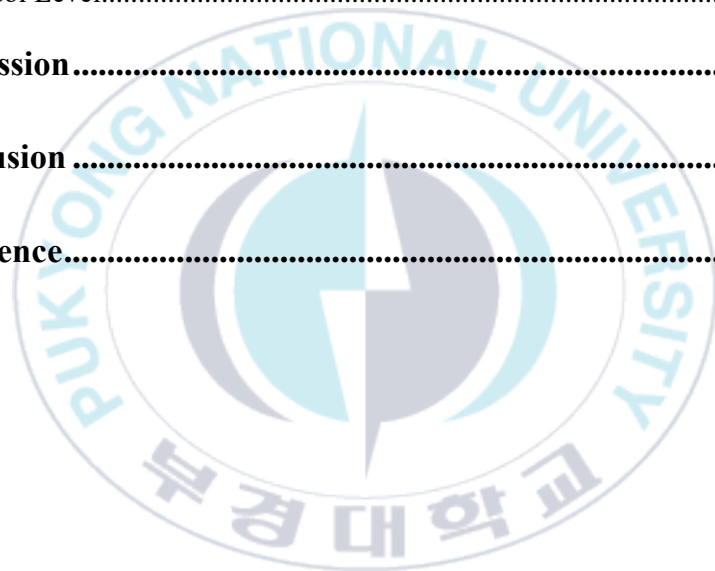
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February 16, 2024

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Determination of an optimum dietary gamma-aminobutyric acid level and plasma metabolite changes in response to acute temperature stress in juvenile rainbow trout
(*Oncorhynchus mykiss*)

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Abstract

Climate change, which is linked to global warming, results in swift environmental alterations, such as rising water temperatures, leading to fatalities, diseases, and epidemics in aquaculture practices. Due to climate change, a study was conducted on rainbow trout (*Oncorhynchus mykiss*), a significant fish species in aquaculture, to determine the optimal level of GABA supplementation in the diet for growth performance and to investigate physiological responses under temperature stress. Gamma-aminobutyric acid (GABA) used in diet, is an important inhibitory neurotransmitter in the nervous system. It has the ability to reduce stress in humans and animals.

The purpose of this research; the current study was conducted to evaluate effects of graded dietary GABA levels on growth performance and stress responses under acute temperature exposure in juvenile rainbow trout.

A total of 315 with an average weight between 18.71 ± 0.08 g (mean \pm SEM) to were randomly divided into 15 groups of , stocked in 15 different glass tanks. For 8 weeks, the 15 tanks represented that were assigned the 5 different GABA (0,50,100,150,200) experimental diets. For the acute stress test, another set of six fish from each tank were exposed to two different temperatures. These 6 fish were distributed in different tanks with water temperatures of 15°C, 20°C, respectively. The fish were exposed to these temperatures for 2 hours followed by a 2 hour recovery period in different tanks filled with water at 15°C.

Broken line analysis for weight gain showed that the optimum dietary GABA level could be approximate 140 mg/kg diet in rainbow trout. The results showed that, final body weight (FBW), weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER)

increased significantly ($p < 0.05$) with GABA level, indicating a dose-dependent relationship. No significant differences were observed Survival (SR), condition factor (CF), hepatosomatic index (HSI), visceral somatic index (VSI) of fish fed all experimental diets ($P > 0.05$).

The addition of GABA had no significant effect on crude protein, lipid, and ash content of fish fed all experimental diets ($P > 0.05$). However, there were significant differences in whole body moisture content; fish fed CON had significantly higher moisture content than fish fed GABA 150 ($P < 0.05$).

Plasma metabolites after of rainbow trout feed the experiment diets 8 weeks, there were no significant differences in glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), total protein (TP), triglyceride (TG), total cholesterol (TCHO), Glucose (GLU) between fish fed all experimental diets ($P > 0.05$).

A total of 25 amino acids were analyzed and the results showed that GABA supplementation did not lead to any significant change in the different inclusion levels ($P > 0.05$). A total of 24 whole brain free amino acids were analyzed, and the results showed that GABA supplementation did not lead to any significant change in the different intake levels ($P > 0.05$).

Acute temperature stress, there is no interaction effect of temperature and GABA supplementation levels on the plasma metabolites analysis ($P > 0.05$). Cortisol, GABA 200 at 20°C showed a significantly higher levels than that of the control diet ($P < 0.05$). All the remaining diets at the temperatures examined showed increased cortisol levels when compared to the control. Alterations in temperature and the levels of GABA supplementation have been noted to exert a pronounced influence on cortisol and plasma concentrations in fish. Collectively, the present study the addition of dietary GABA supplementation appears to enhance the growth performance of rainbow trout. It is possible that GABA plays a role in influencing the fishes stress response and hormonal regulation.

When consider together the study results indicating that GABA supplementation can enhance the growth performance of rainbow trout and that temperature fluctuations may have a significant impact on the levels of GABA supplementation and cortisol levels in fish, these findings provide a meaningful context.

I.Introduction

The world population has recently exceeded 8.0 billion and will reach 9.7 billion in 2050 according to the United Nations (Sekher & Govil, 2022). According to FAO studies, with the increasing population, total fisheries and aquaculture production reached a record 214 million tonnes in 2020, comprising 178 million tonnes of aquatic animals and 36 million tonnes of algae, largely due to the growth of aquaculture, particularly in Asia. The per capita consumption of seafood was 9.9 kg in the 1960s, while it was recorded as 20.5 kg in 2019. Population growth, increasing income, urbanization and increase in fish production are among the reasons that determine this consumption increase, global fisheries and aquaculture production will play an important role in food supply in the future by ensuring the sustainability of growth.

Rainbow trout is one of the most studied fish species with a wide variety of research. The production of rainbow trout produced in the world and in South Korea is shown in figure 1. It is a fish species belonging to the Salmonidae family, which is common in the cold water temperatures of the northwest coast of North America, (MacCrimmon, 1971). Rainbow trout exhibiting partial migrations, some individuals in a population go through sea migrations (anadromy) before returning to fresh water to breed, while others complete their entire life cycle in fresh water. (Jonsson & Jonsson 1993; Pavlov & Savvaitova 2008; Quinn & Myers 2004). Basic biological knowledge is extensive, the rainbow trout, which is widely bred, is common in recreational sport fishing and food (Wolf & Rumsey, 1985; Gall, 1992).

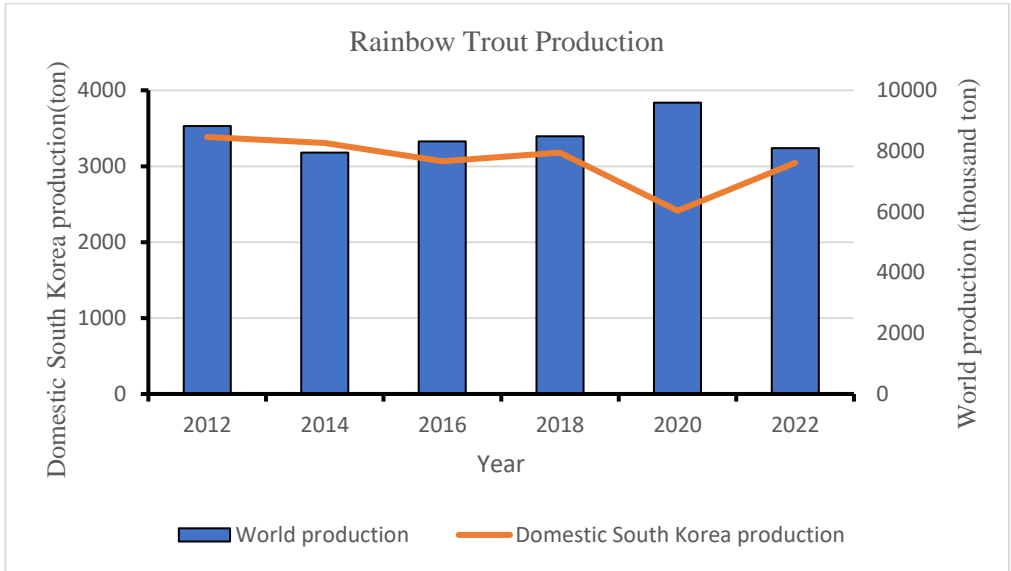
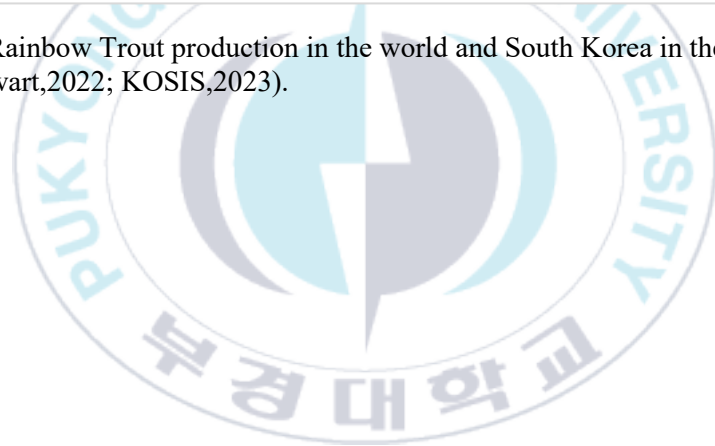


Figure 1. Rainbow Trout production in the world and South Korea in the last 10 years. (Halwart,2022; KOSIS,2023).



Gamma-aminobutyric acid (GABA), widely acknowledged as a safe feed additive, is a non-protein amino acid composed of four carbons, it serves as a key inhibitory neurotransmitter in the central nervous system of animals (Kinnersley & Turano, 2000). Half a century ago, Steward et al. in 1949, GABA was first discovered in plants, then Roberts and Frankel discovered it in the mammalian brain in 1950. Roberts and Heidelberg in 1960 revealed that it plays a role in the neurotransmission of animals.

Neurotransmitters are chemical substances that allow nerve cells (neurons) to communicate with each other. GABA, calms neural activity by stopping or slowing down nerve signals and is a neurotransmitter synthesized in the body is derived from another amino acid called glutamic acid. Glutamic acid is an amino acid found naturally in the body. Glutamic acid is converted to GABA by an enzyme called glutamate decarboxylase. Synthesis of GABA occurs in certain cells in the body, especially nerve cells (Sarasa et al., 2020). GABA, can be found in some nerve cells in the intestines. GABA receptors allow GABA to interact with cells in the intestines. This interaction may regulate neural activity in the intestines. GABA, may help with its calming effect (Hyland & Cryan, 2010). GABA, is naturally found in various foods such as tea, tomatoes, soybeans (Diana et al., 2014; Rashmi et al., 2018). However, in biological tissues GABA is found in low concentrations, and since it is difficult to extract from natural organisms, researchers have developed an alternative method to obtain GABA, higher concentrations of GABA can be produced by lactic acid bacteria (LAB) fermentation (Dhakal et al., 2012).

GABA, has the potential to be an aquatic feed additive that regulates appetite, affects growth performance, and supports the antioxidant status of feed utilization (Li et al., 2015). In a study conducted the inclusion of GABA supplementation in the chicken diet was found to provide protective effects against heat stress, increase the number of goblet cells, improve productivity, enhance egg quality, and elevate serum haptoglobin levels (Zhang et al., 2012). In different research demonstrated a decrease in heat stress-related depression among broilers (Chen et al., 2013). In cows, GABA enhanced feed intake, lactation performance and animal health (Wang et al., 2013). In studies conducted in humans, increased immunity has been observed under stress conditions (Abdou et al., 2006). Dietary GABA at the rate of 100 mg/kg of diet can enhance the growth performance of commercial broilers reared under heat stress conditions (El-Naggar et al., 2019). GABA had been reported to Chinese mitten crabs that were given an optimal dosage of GABA (84-89 mg/kg) had significantly upregulated orexigenic neural signal-related genes which resulted in an increase in feed intake and molting frequency (Zhang et al., 2022). The results obtained in one study showed that GABA supplementation improved growth performance and disease resistance of *L. vannamei*, (Bae et al., 2022). Another study found that supplementing chicken feed with different levels of GABA improved performance characteristics, antioxidant status, and immune response in broiler exposed to cyclic heat stress (Chand et al., 2016). In study showed that the duration of doses of GABA supplementation will affect the growth performance of chickens (Ncho et al., 2021). In a previous study, pigs it was observed that a 0.05% dietary supplement of GABA increased the growth performance (Zhang et al., 2018) and in another study conducted with pigs, it was

observed that a dietary supplement containing 30 mg kg⁻¹ of GABA regulated endocrine hormones and reduced the stress of weaned pigs (Li et al., 2015). These studies on GABA among pigs, poultry and aquatic species, it has ensured that GABA is functional. Research into the effects of GABA has been based on growth and immune responses.

Climate change refers to long-term changes in temperature, precipitation, wind, and other weather conditions around the world; marine ecosystems can be severely impacted by the effects of climate change by increasing the temperature of seawater (Hofmann & Todgham., 2010). It is observed that deserts, heat waves and forest fires spread due to climate change (Mbow et al., 2017). With increasing warming in the Arctic, frozen soils sea ice loss (Pörtner et al., 2019) and high temperatures cause storms, droughts and weather events (Seneviratne et al., 2021). At the same time, ocean warming, ocean acidification, and sea level rise occur (Allen et al., 2018).

Stress is a state caused by a stress factor, or stressor, that deviates from a normal resting or homeostatic state (Barton & Iwama, 1991). In the control activity of the stress situation in the brain, dopamine (DA), noradrenaline (NE) and, particularly, serotonin (5-HT) likely plays a role (Gesto et al., 2013; Øverli et al., 2005; Vindas et al., 2018). The brain follows 2 different neuroendocrine pathways (Alfonso et al., 2021). The first, comprises direct neural stimulation of the chromaffin cells in the anterior kidney (Wendelaar Bonga, 1997), resulting in the release of catecholamines into circulation. A second neuroendocrine route, the hypothalamus–pituitary–interrenal (HPI) axis it starts with the activation of corticotropin-releasing factor (CRF) neurons that discharge CRF into the pars distalis of the anterior pituitary and there, CRF and other peptides

stimulate the release of adrenocorticotrophic hormone (ACTH) into circulation, which in turn stimulates the production and release of cortisol from the interrenal cells in the anterior kidney (Sadoul & Geffroy, 2019).

Various fish species, environments, regions and aquaculture systems are susceptible to extreme temperatures caused by climate change (Islam et al., 2021). The metabolic rate of all ectothermic organisms depends on body size as well as temperature (Clarke & Johnston, 1999). In this context, all of the stress factors perceived as physical and chemical can cause a response in the fish and when fish are exposed to a stressor, the physiological stress response begins when the central nervous system recognizes it as a real threat (Barton, 2002). Changes in temperature affect fish physiology, feed intake, survival, growth, reproduction, behavior and energy (Chang et al., 2018; Islam et al., 2021; Makrinos and Bowden, 2016; Maulvault et al., 2017; Wong & Morris, 2018). The stress effect depends on the lower and upper limit of temperature and exposure time (Crawshaw, 1977; Li et al., 2015; van den Burg et al., 2005). First, as the primary response to both acute and chronic stress, fish activate their brain centers and release large amounts of catecholamines and corticosteroids. Secondary stress response, the release of hormones in the blood and tissue. The tertiary is stress response, growth inhibition, reproduction, reduction of immunity (Barton, 2002; Donaldson et al., 2008; Tort, 2011; Yada & Tort, 2016). Fish under high temperature stress secrete cortisol, increasing the amount of glucose that can be used in the blood, the increased energy metabolism in response to stress negatively affects growth or immunity (Alfonso et al., 2021). As a result of these effects, the release of heat shock proteins (HSP) is affected by endocrine stress systems (Sopinka et al., 2016; Yamashita et al.,

2010). Temperature affects many aspects of the nervous system, from brain organization to neurogenesis and functionality (Amiel et al., 2017; O'Donnell, 2018; Pallotta et al., 2017). Understanding how fish respond to thermal stress at the physiological level and their mechanisms will be meaningful for aquaculture (Faight et al., 2020) .

The results from this experiment performed in our laboratory formed the basis for determining an optimal GABA level in the current trial and we investigated the effects of GABA on fish exposed to changes in temperature. Is important because of the positive effect of GABA among aquatic species and in feed formulation. Since GABA has the above-mentioned properties and important function, it has the potential to be an aquatic feed additive that regulates appetite, supports growth performance, feed efficiency and antioxidant status.

II. Materials and methods

1. Experimental design and diets

The composition of feed ingredients used for the preparation of five experimental diets is provided in Table 1. Anchovy fish meal, squid liver powder, poultry by-product meal, soybean meal, tankage meal, and soy protein concentrate were used as protein sources. Wheat flour and fish oil were used as carbohydrate and lipid sources, respectively. Additional vitamins and minerals, including vitamin C, choline, and vitamin and mineral premixtures were added to make sure the essential vitamin and mineral requirements were met. The GABA premixture was made with the addition of 4 g of GABA (> 99% purity, A2129, Sigma-Aldrich, St. Louis, Missouri, USA) in 196 g of α -cellulose. Five experimental diets were prepared in a basal diet (GABA0), 50 mg (GABA50), 100 mg (GABA100), 150 mg (GABA150), and 200 mg GABA per kg basal diet (GABA200). The analyzed GABA concentration in each diet is provided in Table 1. All dry ingredients were thoroughly mixed with a mixer (Model KB-108, Kimhill, Chia Yi, Taiwan). The mix was moistened with water at approximately 45% of the total weight of the mixed ingredients then fish oil was added slowly until there was complete homogenization throughout the feed mix. The resulting moistened feed mix was then pelleted using a screw-type laboratory pelletizing machine with a 2 mm die (Baokyong Commercial Co., Busan, Korea). This produced homogenous strands that were gently broken into smaller pieces by hand, spread out on trays, and allowed to dry overnight in a room equipped with a dehumidifier. When the pellets were well dried (< 10% of moisture content), they were bagged, labeled, and stored in a freezer at -20°C.

2. Diet and fish whole body proximate composition analysis

The feed analysis was conducted at The Feeds and Foods Nutrition Research Center (FFNRC) to determine the crude protein, moisture, lipid, and ash composition (Table 1) through standard methods (AOAC, 2005). Diet samples were dried at 105°C to determine moisture content, while ash content was determined through incineration at 550°C with a muffle furnace (DAIHAN, Wise Therm®, Seoul, Korea). The crude lipid content was analyzed through the Soxhlet extraction method with the Soxtec system 1046 (Tecator AB, Hogans, Sweeden), while the crude protein was analyzed using the Auto Kjeldahl system (Buchi B 324/435/412, Switzerland; Metrohm 8-719/806, Switzerland) by Kjeldahl nitrogen determination (Nitrogen \times 6.25). (Tecator AB, Sweden) following acid digestion (Table 1). The same methods were also used to determine the whole-body proximate composition of experimental the fish. Furthermore, to determine the naturally occurring concentrations of GABA in the diet, a sample of each diet was analyzed for its total levels of dietary GABA using high-performance liquid chromatography (HPLC) at the Feeds and Foods Nutrition Research Center as shown in Table 1.

Table 1. The composition of feed ingredients for five experimental diets supplemented with graded levels of γ -aminobutyric acid (GABA)

Ingredient (g/kg diet)	Treatment				
	GABA 0	GABA 50	GABA 100	GABA 150	GABA 200
Fish meal (anchovy)	250	250	250	250	250
Squid liver powder	57	57	57	57	57
Soybean meal	51	51	51	51	51
Poultry by-product	58	58	58	58	58
Tankage meal	40	40	40	40	40
Soy protein concentrate	110	110	110	110	110
Wheat flour	240	240	240	240	240
Fish oil	142	142	142	142	142
Mono calcium phosphate	10	10	10	10	10
Mineral premixture ^a	12	12	12	12	12
Vitamin premixture ^b	12	12	12	12	12
Vitamin C	2	2	2	2	2
Choline	6	6	6	6	6
α -cellulose	10	7.5	5	2.5	0
GABA premixture	0	2.5	5	7.5	10
Total	1000	1000	1000	1000	1000
Actual GABA concentration in the diet (ppm)	67.7	108.0	149.1	183.1	217.8
Proximate composition (%; dry matter basis)					
Moisture	8.4	8.3	7.4	8.1	8.2
Crude protein	45.3	44.6	43.9	44.5	44.0
Crude lipid	19.8	20.0	19.5	20.0	19.8
Crude ash	11.1	10.7	11.0	10.5	10.8

^aMineral premix (as mg kg in diets): NaCl, 437; MgSO₄·7H₂O, 1,380; NaH₂P₄·2H₂O, 878; Ca(H₂PO₄) 2H₂O, 1,367; KH₂PO₄, 2,414; ZnSO₄·7H₂O, 299; Fe-Citrate, 3,004; MnSO₄, 0.016; FeSO₄, 0.0378; CuSO₄, 0.00033; Calcium iodate, 0.0006; MgO, 0.00135; NaSeO₃, 0.00025.

^bVitamin premix (as mg/kg in diets): Ascorbic acid, 300; dl-Calcium panthothenate, 150; Choline bitartrate, 3000; Inositol, 150; Menadione, 6; Niacin, 150; Pyridoxine·HCl, 15; Riboflavin, 30; Thiamine mononitrate, 15; dl- α -tocopherol acetate, 201; Retinyl acetate, 6; Biotin, 1.5; Folic acid, 5.4; B₁₂, 0.06.

3. Experimental fish and feeding trial

The eight-week feeding began with the procurement of 360 Rainbow Trout, from An-il fish farm (Republic of Korea) which were transported to the Feed Nutrition Laboratory of the Pukyong National University in plastic bags filled with 1/3rd water and 2/3rd oxygen gas. The fish were carefully distributed into the experimental glass tanks measuring 50cm × 26cm × 60cm and allowed to acclimate to the experimental condition for 14 days during which they were fed the control diet. At the end of the acclimation period, a total of 315 with an average weight between 18.71 ± 0.08 g to 19.13 ± 0.24 g were randomly divided into 15 groups of, stocked in 15 different glass tanks connected to a closed indoor recirculating system with continuously filtered freshwater and aeration to maintain the dissolved oxygen level above saturation. The culture tanks were cleaned daily by siphoning off the accumulated feces and feed remains. Partial water change was done twice a week to ensure proper water quality. Water quality parameters: Dissolved Oxygen (DO), and pH were done by the Multi-Parameter Meter, (YSI Model 58, Yellow Springs, Ohio, USA) and were monitored daily in all tanks, while Nitrogen compounds ($\text{NH}_3/\text{NH}_4^+$, NO_2^- , and NO_3^-) analyses were performed bi-weekly using a Tetra Water quality analyzer (Tetra GMBH, Germany). The corresponding water quality conditions were DO (6.70 ± 1.2 mg/l), pH (7.2 ± 0.5), $\text{NH}_3/\text{NH}_4^+$ (0.25 ± 0.1 mg/l), NO_2^- (0.25 ± 0.2 mg/l), NO_3^- (10 ± 0.24 mg/l). Water Temperature was kept constant at 15°C using the Sun Cool Water Temperature Regulator, Model DH-2010 (Daeil, Korea). Fish were slowly hand-fed to apparent satiation. The experimental feeds were readily consumed by all the groups and no differences in feeding behavior were noticed throughout the experimental period.

4. Sample collection

At the end of the eight-week feeding trial, the fish were fasted for 24 hours, then moved to a small shallow bottom rectangular basin from their respective tanks. All other indices were calculated by using the following equations.

IBW = Initial body weight (g).

FBW = Final body weight (g).

Weight gain (WG, %) = (final weight-initial weight) × 100 / initial weight.

Specific growth rate(SGR, %day) = (ln final weight – ln initial weight) × 100 / days.

Feed efficiency (FE, %) = (wet weight gain/dry feed intake) × 100.

Protein efficiency ratio (PER,%) = (wet weight gain/protein intake).

Survival (SR,%) = (total fish – dead fish) × 100/survival fish

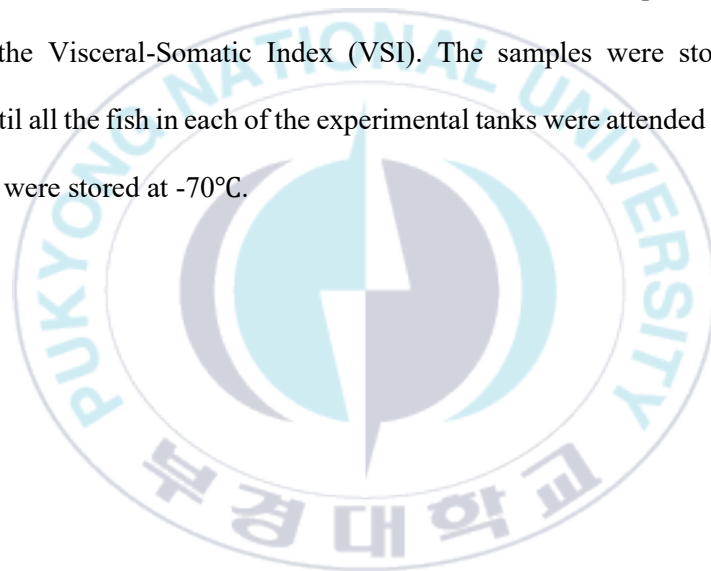
Condition factor = (CF,%)=(wet weight/total length) × 100.

Hepatosomatic index (HSI, %) = liver wt. × 100 / body wt.

Visceral somatic index (VSI, %) = Viscera wt. × 100/ body wt.

The fish in each of the tanks were anesthetized using Phenoxyethanol at 500 ppm/L for 5 to 10mins and six fish were randomly selected from each tank. Each of these selected fish was weighed and the length was measured. Blood samples were taken and pooled using a non-heparinized needle according to the different tanks. The collected blood was allowed to coagulate at room temperature for 30 min. blood serum samples were then centrifuged at 5000× g for 10 min and immediately stored in 200ul EP tube at -70°C for further analysis of non-specific immune response, antioxidant capacity, and blood-chemistry-related parameters, including Glutamic-Oxaloacetic

Transaminase (GOT), Glutamic Pyruvic Transaminase (GPT), Total Protein (TP), Total Cholesterol (TCHO), Glucose, Cortisol. After blood collection, three out of the selected fish were kept in a zip lock bag labeled according to their respective tank and kept at -70°C until they were used for whole-body proximate composition, while the remaining three fish were dissected and the final textured plasma and brain were removed to be used for analysis. Organ samples (except the gill filaments) were weighed, and the measurements taken were used to calculate the Hepato-Somatic Index (HSI) and the Visceral-Somatic Index (VSI). The samples were stored in liquid Nitrogen until all the fish in each of the experimental tanks were attended to after which the samples were stored at -70°C.



5. Plasma metabolites and cortisol analysis

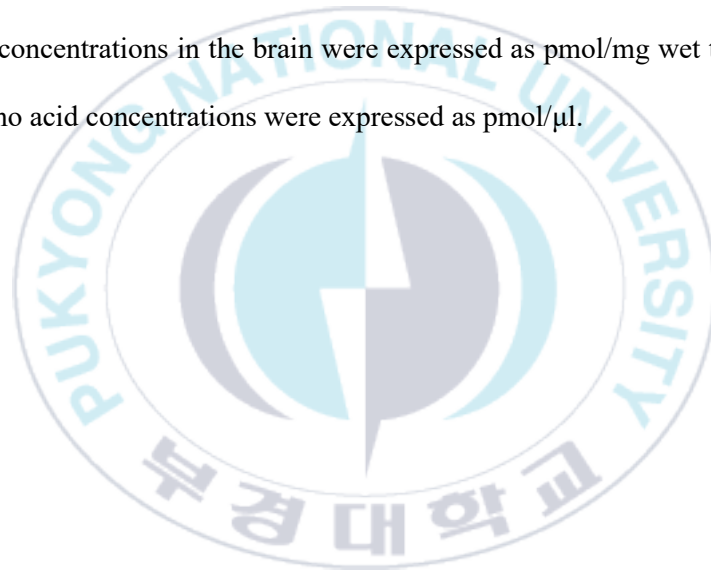
Blood chemistry parameters were analyzed by an automatic chemical analyzer (Fuji DRI-CHEM 3500i, Fuji Photo Film, Ltd., Tokyo, Japan), which was used to determine glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), total protein (TP), triglyceride (TG), total cholesterol (TCHO), Glucose (GLU) levels in serum.

The cortisol level, plasma was removed and stored at -20°C until assayed for cortisol. Plasma samples were thawed at 25°C for 30 min before the assays. The ELISA kit, (BioVision, Milpitas, CA, USA) was then removed from the 4°C refrigerator and brought to room temperature; the reaction plate was removed, and blank wells were separately set. Forty microliters of the testing sample were added to the sample test well, then $50\ \mu\text{L}$ HRP-conjugate reagent and $10\ \mu\text{L}$ antibody were added to each well.

6.Free amino acid profile in plasma and brain analysis

Free amino acid concentrations were analyzed by HPLC according to the method of (Boogers et al., 2008). Plasma was filtered through a 0.2- μ m filter (Millipore, Bedford, MA, USA). The brain were homogenized in an ice-cold 0.2 M perchloric acid solution containing 0.01 mM ethylenediaminetetraacetic acid disodium salt (EDTA·2Na) and left for deproteinization on ice. After 30 minutes, the homogenates were centrifuged at 20,000 g for 15 minutes at 0°C and the supernatants were then adjusted to pH 7 with 1 M sodium hydroxide. Plasma was prepared by centrifuging at 12,000 g for 10 min at 4 °C, and filtrated by ultrafiltration (Millipore, Bedford, USA). Each of the plasma (10 μ L) and brain sample (20 μ L) was subsequently dried completely under reduced pressure. The dried residues were dissolved in 10 μ L of 1 M sodium acetate-methanol-triethylamine (2:2:1) and re-dried under reduced pressure, and then converted to their phenylthiocarbamoyl derivatives by dissolving in 20 μ l of methanol-distilled watertriethylamine-phenylisothiocyanate (7:1:1:1) and allowed to react for 20 min at room temperature. The samples were dried once more and then reconstituted in 200 μ L of Pico-Tag Diluent (Waters, Milford, CT, USA). The diluted samples were filtered through a 0.20-micron filter (Millipore). The same methods were performed on standard solutions which were prepared by diluting a commercially available L-amino acid solution (type ANII, type B, L-asparagine, L - glutamine and L-tryptophan; Wako, Osaka, Japan) with distilled water. The solution containing its derivatives was introduced into a Waters HPLC system. It was equilibrated with buffer A (70 mM sodium acetate adjusted to pH 6.45 with 10% acetic acid-acetonitrile, ratio 975:25) and then eluted using a linear gradient of buffer B

(water-acetonitrile-methanol, 40:45:15) at different concentrations (0%, 3%, 6%, 9%, 40%, and 100%) at a flow rate of 1 ml/min at 46 °C. Absorbances at a wavelength of 254 nm were measured to determine concentrations of free amino acids (leucine, isoleucine, valine, threonine, methionine, phenylalanine, tryptophan, GABA, aspartate, taurine, proline, serine, tyrosine, lysine, 3-methyl-histidine, arginine, alanine, glycine, asparagine, glutamine, citrulline, histidine, β alanine, α -aminoadipic acid, cystathionine, ornithine, hydroxyproline, and glutamate) and phosphoserine. The amino acid concentrations in the brain were expressed as pmol/mg wet tissue and the plasma amino acid concentrations were expressed as pmol/ μ l.



7.Acute Temperature Stress

For the acute stress test, another set of 6 fish from each tank were exposed to different temperatures. These 6 fish were distributed in pairs in different tanks with water temperatures of 15°C 20°C respectively. The fish were exposed to these temperatures for 2 hours followed by a 2 hour recovery period in different tanks filled with water at 15°C. After the recovery period, the fish were euthanized, and the organs were collected as previously described above.



8. Statistical analysis

Results from the 8 week feed restriction trial were analyzed by one-way analysis of variance (ANOVA) accounting for the 5 feed restriction treatments, and comparisons between treatment means were performed through Tukey's studentized range (HSD) test. Results from the temperature exposure trial were analyzed using two way ANOVA to test an interaction between feed restrictions or temperature. The following model was used (Lee et al., 2015).

$$y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + e_{ijk}$$

Where y_{ijk} was the dependent variable measured for the i th feed restriction, the j th temperature, and k th observation where the number of observations is dependent on the response; μ is overall constant; α_i is a fixed effect of the i th feed restriction for $i = 1, 2, 3, 4$ and 5 (GABA0, GABA50, GABA100, GABA150, GABA200 ppm); β_j is a fixed effect of the j th temperature for $j = 1, 2$ (15°C, 20 °C); $\alpha\beta_{ij}$ is an interaction for combinations of the i th feed restriction and j th temperature; and e_{ijk} is error term.

Statistical analyses were performed using the SAS software package (version 9.3, SAS Institute, Cary, NC, USA). The data are presented as the mean and standard error of the mean (mean+SEM).

Determining the optimal feeding rate (OFR; expressed as a percentage of daily body weight) is essential for the success of aquaculture operations. This is significant due to the critical roles played by feeding rate, water temperature, and fish size in the process. The growth response to feeding rate is typically continuous; it increases with

higher feeding rates, peaks at a certain threshold, and then stabilizes beyond that peak feeding rate. Different regression models commonly utilized for estimating OFR and/or nutrient requirement levels include one-slope straight broken-line (one-slope BL), two-slope straight broken-line (two-slope BL), quadratic broken-line (quadratic BL), and quadratic (quadratic) models (Zeitoun et al., 1976; Robbins et al., 2006; Shearer 2000; Pesti et al., 2009; Lee et al., 2014).

Functional equations and visual representations of these models, along with the corresponding codes for fitting each model to a dataset with WG as the dependent variable and feeding rate as the independent variable, can be referenced in the existing literature . The effective model selection criteria, including the adjusted coefficient of determination (R^2 adj) and the corrected Akaike information criterion (AICc), were calculated for comparing model performance in estimating OFR. Higher R^2 adj values and smaller AICc values indicate a better-performing model (Lee et al., 2014). The best predictive model was used for multiple comparisons when significant effects were observed. This meant that some non significant terms were maintained in the model. In the current study, quadratic provides the best model because it has the highest R^2 adj values and the lowest AICc values. Although quadratic has the best model, its $p > 0.05$, it is not significant and therefore it was not included in the discussion. One slope broken line fits better. R 3.0.1 (R Development Core Team 2013) was used for the analyses.

9. Ethics statement

The experiment was carried out following the Institutional Animal Care and Use Committee Regulations, specifically PKNU IACUC 2022 29, as issued by Pukyong. Every possible measure was taken to reduce the suffering of the fish.



III. Results

1. Determination of an optimum γ -aminobutyric acid

1.1. Changes in body weight and feed utilization efficiency and morphology

Table 2, summarizes the Growth performances of rainbow trout (*Oncorhynchus mykiss*) which were measured by weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein productivity ratio for 8 weeks GABA experimental diet (PER) and survival rate (SR). The interpretation of the results for the final body weight is provided below.

The mean FBW, increased significantly ($p < 0.05$) with increasing dietary GABA levels, demonstrating a dose-dependent relationship. Diets containing GABA 100, 150, and 200 had mean FBW values of (56.0 ± 0.7 , 55.8 ± 1.3 , and 57.0 ± 0.4 , respectively) that were significantly higher than the control diet (52.9 ± 0.6). The diet with the lowest GABA concentration (GABA 50) showed slightly higher FBW values (53.9 ± 0.6), but the difference was not statistically significant ($p > 0.05$) compared to the control.

The mean WG, increased significantly with increasing dietary GABA levels, demonstrating a dose-dependent relationship. Diets containing GABA 100, 150, and 200 had mean WG values of (195 ± 5 , 198 ± 1 , and 203 ± 2 , respectively) that were significantly higher than the control diet and GABA 50 (179 ± 1 , 184 ± 1 respectively). The diet with the lowest GABA concentration GABA 50 showed slightly higher WG values (184 ± 1), but the difference was not statistically significant compared to the

control. The reason for the improvement of WG is that the effect of GABA on the growth hormone has been proven (Powers et al, 2008).

The mean SGR, increased significantly with increasing dietary GABA levels, demonstrating a dose-dependent relationship. Diets containing GABA 100, 150, and 200 had mean SGR values of (1.93 ± 0.03 , 1.95 ± 0.63 , and 1.98 ± 0.01 , respectively) that were significantly higher than the Control diet and GABA 50 (1.83 ± 0.00 , 1.86 ± 0.63 respectively). The diet with the lowest GABA concentration GABA 50 showed slightly higher SGR values (1.86 ± 0.63), but the difference was not statistically significant compared to the control.

The mean FE, increased significantly with increasing dietary GABA levels, demonstrating a dose-dependent relationship. In case of feed efficiency (FE), almost all diets performed well, among them GABA 200 (103 ± 2) diets were comparatively better and no significant differences, with other diets but only CON(94.9 ± 0.7) diet showed the lowest FE performance.

The mean PER, increased significantly with increasing dietary GABA levels, demonstrating a dose-dependent relationship. Diets containing GABA 100, 150, and 200 had mean PER values of (2.31 ± 0.03 , 2.28 ± 0.05 , 2.34 ± 0.03 respectively) that were significantly higher than the Control diet and GABA 50 (2.09 ± 0.02 , 2.16 ± 0.03 respectively). The diet with the lowest GABA concentration GABA 50 showed slightly higher PER values (2.16 ± 0.03), but the difference was not statistically significant compared to the control.

There were no significant differences observed in the initial body weight, survival (%), condition factor, hepatosomatic index and viscerosomatic index of fish fed all the experimental diets. Condition factor (CF), hepatosomatic (HSI) and viscerosomatic (VSI) indices measurement and analysis are very important factors to assessment food value in an organism. These values are the values showing the health status of the fish (Ighwela et al., 2014).



Table 2. Growth performance, of rainbow trout feed the experiment diets in 8 weeks.¹

Diets	GABA0	GABA50	GABA100	GABA150	GABA200	P value	Linear	Quadratic
IBW ²	18.93±0.13 ^{ns}	19.00±0.20 ^{ns}	19.02±0.27 ^{ns}	18.76±0.30 ^{ns}	18.80±0.23 ^{ns}	0.5640	0.2594	0.5247
FBW ³	52.9±0.6 ^c	53.9±0.6 ^{bc}	56.0±0.7 ^a	55.8±1.3 ^{ab}	57.0±0.4 ^a	0.0004	<.0001	0.2438
WG ⁴	179±1 ^b	184±1 ^b	195±5 ^{ab}	198±1 ^{ab}	203±2 ^a	0.0096	0.0007	0.7006
SGR ⁵	1.83±0.00 ^b	1.86±0.63 ^b	1.93±0.03 ^{ab}	1.95±0.63 ^{ab}	1.98±0.01 ^a	0.0090	0.0006	0.6756
FE ⁶	94.9±0.7 ^b	96.5±0.6 ^{ab}	101±2 ^{ab}	101±1 ^{ab}	103±2 ^a	0.0237	0.0023	0.4854
PER ⁷	2.09±0.02 ^c	2.16±0.03 ^{bc}	2.31±0.03 ^{ab}	2.28±0.05 ^{ab}	2.34±0.03 ^a	0.0036	0.0004	0.2280
SR ⁸	100±0 ^{ns}	100±0 ^{ns}	100±0 ^{ns}	100±0 ^{ns}	97.8±2.2 ^{ns}	0.4516	0.1877	0.2596
CF ⁹	1.05 ±0.01 ^{ns}	1.05 ±0.01 ^{ns}	1.08 ±0.01 ^{ns}	1.05 ±0.01 ^{ns}	1.07 ±0.03 ^{ns}	0.6656	0.6960	0.6031
HSI ¹⁰	1.03 ±0.03 ^{ns}	1.05 ±0.01 ^{ns}	1.03 ±0.02 ^{ns}	1.05 ±0.01 ^{ns}	0.99 ±0.02 ^{ns}	0.4043	0.1940	0.6364
VSI ¹¹	9.16 ±0.62 ^{ns}	9.76 ±0.57 ^{ns}	9.62 ±0.26 ^{ns}	9.69 ±0.19 ^{ns}	10.3 ±0.2 ^{ns}	0.4453	0.1145	0.8450

¹Values are means (± SEM) from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different (P < 0.05).

²IBW = Initial body weight (g).

³FBW = Final body weight (g).

⁴Weight gain (WG, %) = (final weight-initial weight) × 100 / initial weight.

⁵Specific growth rate (SGR,%/day)= (ln final weight – ln initial weight) × 100 / days.

⁶Feed efficiency (FE, %) = (wet weight gain/ dry feed intake) × 100.

⁷Protein efficiency ratio (PER,%) = (wet weight gain/protein intake).

⁸Survival (SR,%) = (total fish – dead fish) × 100/survival fish

⁹Condition factor(CF) = (wet weight/total length) × 100.

¹⁰Hepatosomatic index (HSI, %) = liver wt. × 100 / body wt.

¹¹Visceralsomatic index (VSI, %) = Viscera wt. × 100/ body wt.

1.2. Whole-body nutrient composition

The whole-body proximate composition of juvenile Rainbow trout GABA fed on the experimental diets for 8 week (% wet weight basis) is shown in table 3. GABA inclusion did not significantly affect whole-body crude protein, lipid and ash content of fish fed all the experimental diets ($P > 0.05$). However, there were significant differences in whole-body moisture content, as fish fed CON had significantly higher moisture content than those of fish fed the GABA 150 diet ($P < 0.05$). There were also no significant differences in the whole-body moisture content among fish fed GABA 50, GABA 100, and GABA 200 diets ($P > 0.05$).

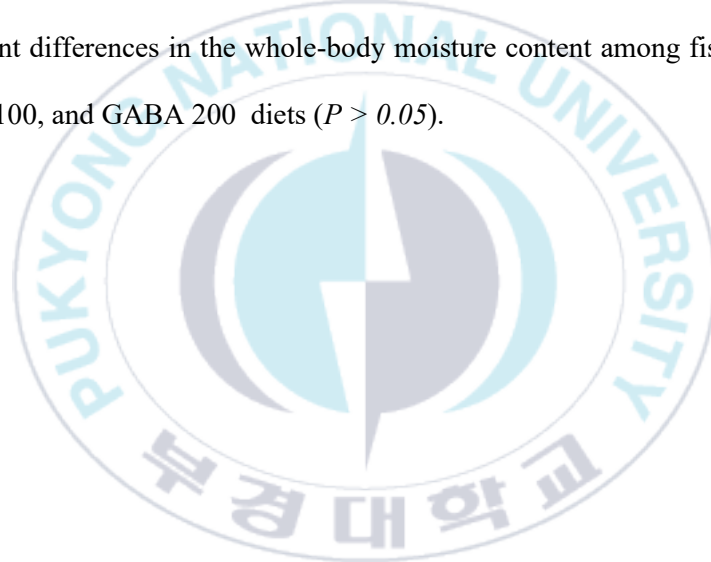


Table 3. Proximate composition of whole body for rainbow trout fed the experiment diets 8 weeks (% of as-is basis)¹

Diets	GABA0	GABA50	GABA100	GABA150	GABA200	P value	Linear	Quadratic
Moisture	71.6±0.2 ^a	70.6±0.4 ^{ab}	69.1±0.9 ^{ab}	69.0±0.4 ^b	69.5±0.6 ^{ab}	0.0338	0.0081	0.0607
Crude protein	15.6±0.2 ^{ns}	16.7±0.2	18.2±1.3	17.3±0.2	16.7±0.1	0.2262	0.5253	0.0611
Crude lipid	10.0±0.1 ^{ns}	9.89±0.49	9.80±0.55	11.0±0.2	11.2±0.4	0.0891	0.0200	0.2586
Crude ash	2.35±0.03 ^{ns}	2.60±0.15	2.57±0.08	2.40±0.03	2.35±0.11	0.0927	0.1665	0.0222

¹Values are means (± SEM) from triplicate groups (n=3) of fish where the values in each row with different superscripts are significantly different ($P < 0.05$)

1.3. Plasma metabolites after the growth trial

Plasma metabolites after growth trial in rainbow trout fed with GABA experimental diets for 8 weeks are summarized in Table 4. There were no significant differences in Glutamic oxaloacetic transaminase(GOT), Glutamic pyruvate transaminase(GPT), Total protein(TP), Triglyceride(TG), Total cholesterol (TCHO), Glucose(GLU) among fish fed all the experimental diets ($P > 0.05$).



Table 4. Plasma metabolites after of rainbow trout feed the experiment diets 8 weeks¹

	Diets					P-value	Linear	Quadratic
	GABA ₀	GABA ₅₀	GABA ₁₀₀	GABA ₁₅₀	GABA ₂₀₀			
GOT ²	203 ± 71 ^{ns}	267 ± 48	387 ± 121	331 ± 13	285 ± 68	0.4999	0.3482	0.1760
GPT ³	14.7 ± 0.3 ^{ns}	27.3 ± 2.4	27.3 ± 5.5	25.3 ± 1.3	23.3 ± 0.7	0.0439	0.1087	0.0124
TP ⁴	2.97 ± 0.15 ^{ns}	3.27 ± 0.13	3.33 ± 0.41	3.6 ± 0.12	3.33 ± 0.33	0.5633	0.2171	0.3529
TG ⁵	283 ± 26 ^{ns}	306 ± 38	337 ± 47	446 ± 67	385 ± 47	0.1822	0.0433	0.6143
TCHO ⁶	226 ± 9 ^{ns}	181 ± 3	199 ± 28	203 ± 12	203 ± 18	0.4749	0.6499	0.2468
GLU ⁷	72.3 ± 2.7 ^{ns}	78 ± 13.9	64 ± 11.4	70.0 ± 4.6	67.3 ± 10.5	0.8684	0.5659	0.9278

¹Values are means (± SEM) from triplicate groups (n=3) of fish where the values in each row with not significantly different ($P > 0.05$)

²GOT:Glutamic oxaloacetic transaminase (U/L)

³GPT:Glutamic pyruvate transaminase (U/L)

⁴TP:Total protein (g/dL)

⁵TG:Triglyceride (mg/dL)

⁶TCHO:Total cholesterol(mg/dL)

⁷GLU:Glucose(mg/dL)

1.4. Free amino acid profile in plasma and brain

Table 5 shows the free Amino Acid profile in plasma of the experimental groups at the end of the experiment. A total of 25 Amino Acids were analyzed and the results indicated that GABA supplementation at the various inclusion levels did not lead to any significant change ($p > 0.05$).



Table 5. Plasma free amino acid profile after of rainbow trout feed the experiment diets 8 weeks.¹

Diets	GABA0	GABA50	GABA100	GABA150	GABA200	P value	Linear	Quadratic
Phosposerine	2.41 ±0.28 ^{ns}	2.26 ±0.09 ^{ns}	2.04 ±0.10 ^{ns}	2.12 ±0.14 ^{ns}	2.17 ±0.11 ^{ns}	0.5725	0.2482	0.2822
Taurine	85.4±1.3 ^{ns}	114±10 ^{ns}	82.2±2.6 ^{ns}	101±6 ^{ns}	91.6±6.2 ^{ns}	0.0524	0.9795	0.2840
Hydroxyproline	15.0 ±2.3 ^{ns}	12.9 ±1.9 ^{ns}	15.8 ±1.1 ^{ns}	17.8 ±0.7 ^{ns}	15.0 ±1.6 ^{ns}	0.3789	0.3613	0.7087
Threonine	17.9 ±1.1 ^{ns}	16.5 ±0.5 ^{ns}	18.8 ±0.5 ^{ns}	18.0 ±1.5 ^{ns}	16.7 ±1.4 ^{ns}	0.5671	0.8356	0.5127
Serine	18.0 ±0.3 ^{ns}	14.8 ±1.1 ^{ns}	17.5 ±0.9 ^{ns}	19.1 ±1.4 ^{ns}	17.1 ±1.4 ^{ns}	0.1585	0.4778	0.7662
Asparagine	21.9±1.4 ^{ns}	16.8±1.9 ^{ns}	23.5±1.4 ^{ns}	22.0±2.9 ^{ns}	22.0±2.3 ^{ns}	0.2473	0.4165	0.8057
Glutamic Acid	48.1±2.2 ^{ns}	42.3±4.3 ^{ns}	48.8±2.5 ^{ns}	50.3±2.7 ^{ns}	47.1±3.6 ^{ns}	0.4934	0.5753	0.9969
Proline	3.39±0.35 ^{ns}	3.23±0.44 ^{ns}	2.76±0.11 ^{ns}	3.29±0.08 ^{ns}	3.03±0.44 ^{ns}	0.6668	0.5262	0.5314
Glycine	39.0±2.4 ^{ns}	34.6±4.4 ^{ns}	36.2±4.5 ^{ns}	43.7±2.6 ^{ns}	34.9±2.8 ^{ns}	0.3725	0.9385	0.8216
Alanine	31.4±0.7 ^{ns}	32.1±6.2 ^{ns}	33.2±4.7 ^{ns}	38.1±2.2 ^{ns}	31.9±3.1 ^{ns}	0.7432	0.5869	0.5127
a-amino-n-butyric acid	7.01±0.40 ^{ns}	6.04±0.47 ^{ns}	4.80±2.40 ^{ns}	5.23±2.63 ^{ns}	5.90±0.66 ^{ns}	0.8937	0.5720	0.4390
Valine	29.9±2.8 ^{ns}	26.9±0.2 ^{ns}	31.6±2.5 ^{ns}	29.5±1.2 ^{ns}	27.3±1.6 ^{ns}	0.4376	0.6478	0.4838
Cystine	1.51±0.09 ^{ns}	1.33±0.13 ^{ns}	1.38±0.04 ^{ns}	1.44±0.09 ^{ns}	1.17±0.11 ^{ns}	0.2072	0.0886	0.6275
Methionine	10.4±0.3 ^{ns}	10.0±0.5 ^{ns}	10.4±0.0 ^{ns}	10.3±0.4 ^{ns}	9.4±0.7 ^{ns}	0.4520	0.2348	0.3657
Isoleucine	14.3±1.4 ^{ns}	13.5±0.4 ^{ns}	14.5±2.1 ^{ns}	14.4±0.7 ^{ns}	13.1±0.6 ^{ns}	0.8975	0.7008	0.6771
Leucine	22.3±2.1 ^{ns}	21.3±0.6 ^{ns}	23.2±2.6 ^{ns}	22.3±0.8 ^{ns}	21.6±0.9 ^{ns}	0.9290	0.9294	0.7059
Tyrosine	9.04±0.26 ^{ns}	9.51±0.47 ^{ns}	9.10±0.32 ^{ns}	9.82±0.33 ^{ns}	9.42±0.51 ^{ns}	0.6236	0.4066	0.6931
Phenylalanine	11.0±0.3 ^{ns}	12.5±0.7 ^{ns}	12.5±0.4 ^{ns}	12.6±0.4 ^{ns}	12.1±0.6 ^{ns}	0.2053	0.1481	0.0682
r-AminoButyric acid	18.6±0.9 ^{ns}	16.6±1.9 ^{ns}	18.7±0.4 ^{ns}	16.9±1.9 ^{ns}	18.7±3.9 ^{ns}	0.9085	0.9498	0.6484
Histidine	22.9±1.3 ^{ns}	20.4±0.0 ^{ns}	23.4±1.7 ^{ns}	23.8±1.4 ^{ns}	22.4±2.3 ^{ns}	0.5638	0.6198	0.9243
Tryptophan	1.37±0.68 ^{ns}	0.72±0.72 ^{ns}	1.60±0.33 ^{ns}	1.95±0.14 ^{ns}	2.24±0.41 ^{ns}	0.3209	0.0909	0.4869
Ornithine	3.59±0.63 ^{ns}	1.23±1.23 ^{ns}	4.35±0.67 ^{ns}	3.29±0.43 ^{ns}	3.04±0.67 ^{ns}	0.1392	0.7031	0.9910
Lysine	3.86±0.54 ^{ns}	3.44±0.18 ^{ns}	4.22±0.32 ^{ns}	3.49±0.22 ^{ns}	3.61±0.47 ^{ns}	0.5938	0.7089	0.7661
Ammonia	11.0±0.7 ^{ns}	9.3±0.2 ^{ns}	10.1±0.8 ^{ns}	8.9±0.9 ^{ns}	9.6±0.4 ^{ns}	0.2540	0.1506	0.2982
Arginine	54.7±1.7 ^{ns}	57.2±6.5 ^{ns}	54.9±2.9 ^{ns}	52.9±0.6 ^{ns}	57.4±6.9 ^{ns}	0.9460	0.9297	0.8034
Total Aminoacid	501±7 ^{ns}	497±34 ^{ns}	503±30 ^{ns}	530±14 ^{ns}	496±37 ^{ns}	0.8907	0.8027	0.7103

¹Values are means (± SEM) from triplicate groups (n=3) of fish where the values in each row not significantly different ($P > 0.05$).

Table 6 shows free amino acid profile in whole brain of rainbow trout fed the experiment diets 8 weeks. A total of 24 free Amino Acids were analyzed and the results indicated that GABA supplementation at the various inclusion levels did not lead to any significant change ($P > 0.05$) in the free amino acid profile of Rainbow trout at the end of the experiment.



Table 6.Brain free amino acid profile of rainbow trout fed the experiment diets 8 weeks¹

Diets	GABA0	GABA50	GABA100	GABA150	GABA200	P value	Linear	Quadratic
Phosposerine	248±10 ^{ns}	303±4	272±20	327±55	249±23	0.2802	0.7823	0.1210
Taurine	4599±215 ^{ns}	5867±148	4730±475	6532±1319	4553±636	0.2384	0.8004	0.2032
Phospho ethanol amine	215±10 ^{ns}	251±7	202±15	260±49	173±12	0.1405	0.3477	0.1526
Aspartic Acid	435±22 ^{ns}	515±3	528±138	574±91	420±57	0.6208	0.9084	0.1713
Hydroxyproline	63±14.2 ^{ns}	85.2±11.7	57.9±9.3	90.8±15.2	65.5±16	0.3825	0.8085	0.5088
Threonine	210±16 ^{ns}	258±5	213±19	290±62	186±25	0.2774	0.8931	0.1606
Serine	242±12 ^{ns}	280±23	226±21	335±54	235±24	0.1416	0.6801	0.3391
Glutamic Acid	3808±198 ^{ns}	4385±42	3872±386	5157±826	3666±500	0.2337	0.7514	0.2166
Proline	27.4±1.2 ^{ns}	32±3	26.7±3.8	33.6±4.9	27.4±2.6	0.5027	0.8800	0.4731
Glycine	330±19 ^{ns}	422±30	313±32	494±101	355±39	0.1710	0.4773	0.4035
Alanine	164±7 ^{ns}	209±2	168±22	215±32	170±18	0.2480	0.7756	0.2432
Citulline	0±0 ^{ns}	0±0	19.3	0±0	0±0	0.4516	1.000	0.2596
Valine	46.8±3.2 ^{ns}	58.1±6.8	44.6±4.9	53.6±10	49.6±5.3	0.6097	0.9585	0.7481
Cystine	69.4±5.6 ^{ns}	83.4±2.5	67.3±8.6	77.2±13.6	61.5±5.4	0.3970	0.4136	0.2957
Methionine	15.6±1.4 ^{ns}	18.2±2.7	13.0±0.9	21.9±3.8	13.9±1.9	0.1335	0.9588	0.4443
Isoleucine	34.6±2.7 ^{ns}	42.0±3.8	37.2±3.4	42.5±6.6	36.0±2.6	0.5712	0.8037	0.2753
Leucine	51.8±3.0 ^{ns}	62.6±6.5	54.8±6.4	59.0±8.1	53.9±2.1	0.6855	0.9759	0.3792
Tyrosine	35.4±3.9 ^{ns}	44.9±1.8	35.2±4.6	42.7±7.7	35.2±4.1	0.4595	0.8606	0.3726
Phenylalanine	50.2±3.8 ^{ns}	62.1±2.7	50.6±6.9	60.2±11.6	49.9±6	0.5766	0.9115	0.3907
b-Amino iso-Butyric acid	0±0 ^{ns}	0±0	235	885	0±0	0.5109	0.5095	0.3990
r-aminobutyric acid	1259±111 ^{ns}	1611±105	1313±145	1486±200	1203±93	0.2606	0.5936	0.1480
Histidine	1575±87 ^{ns}	1660±145	1634±277	1624±212	1578±22	0.9955	0.9564	0.7125
Tryptophan	116±2 ^{ns}	145±7	139±19	175±27	117±15	0.1485	0.5603	0.0601
Lysine	432±18 ^{ns}	557±11	432±50	469±62	438±26	0.1860	0.5546	0.3187
Total amino acid	14000±589	16918± 184	14485±1561	18685±3169	13711±1501	0.2636	0.8341	0.1896

¹Values are means (± SEM) from triplicate groups (n=3) of fish where the values in each row with not significantly different ($P > 0.05$).

1.5. Optimum GABA level

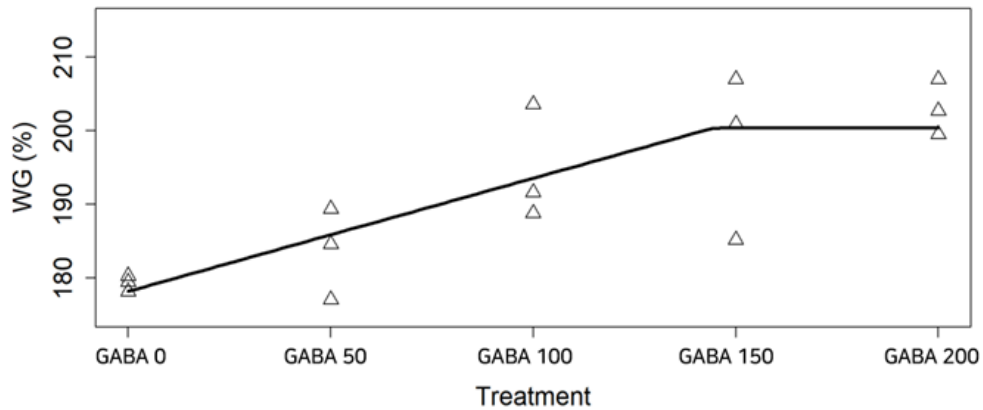


Figure 2: One slope BL figure model describes, the dose-response relationship between nutrient levels (or feeding rate) and growth. It can take the form of a linear or quadratic ascending curve, featuring an ascending, plateau, or descending phase. The point where the curve changes direction represents the optimum nutrient requirement or the Optimum Feeding Rate (OFR). Optimal level of dietary GABA in mg/kg according to WG by one slope broken line analysis in Rainbow Trout fed the experimental diets. Broken line analysis for weight gain showed that the optimum dietary GABA level could be approximate 140 mg/kg diet.

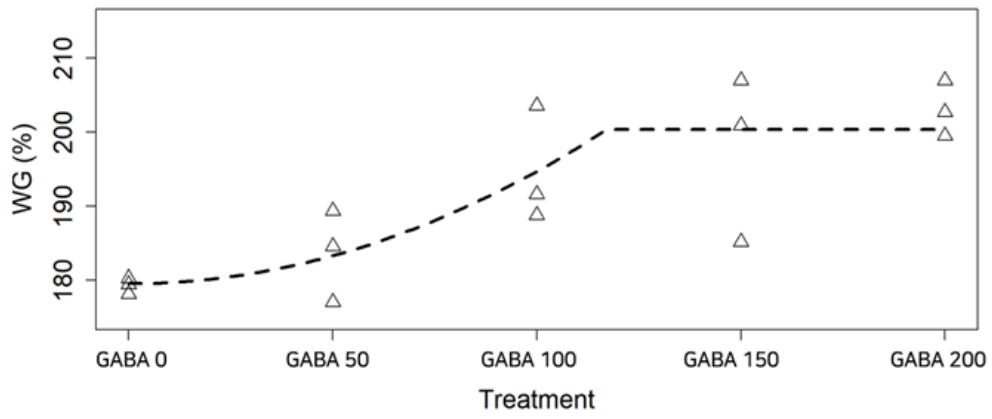
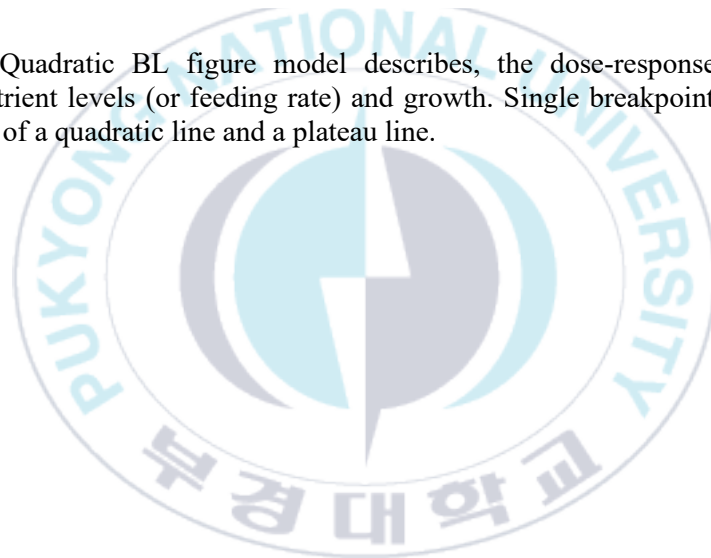


Figure 3: Quadratic BL figure model describes, the dose-response relationship between nutrient levels (or feeding rate) and growth. Single breakpoint which is the intersection of a quadratic line and a plateau line.



2.Acute temperature stress

2.1 Plasma metabolites

Interaction effect, plasma metabolites and stress response of rainbow trout exposed to acute heat stress at 20°C are shown in table 7. Interaction effect; there is no interaction effect of temperature and GABA supplantation levels on the plasma metabolites analyzed ($P>0.05$).

The main effect of temperature; there were no significant different in the main effect of temperature that was recorded for all the metabolites ($P>0.05$).

Main effect of GABA; while all GABA supplementation did not have a significantly different main effect on all other metabolites ($P>0.05$).

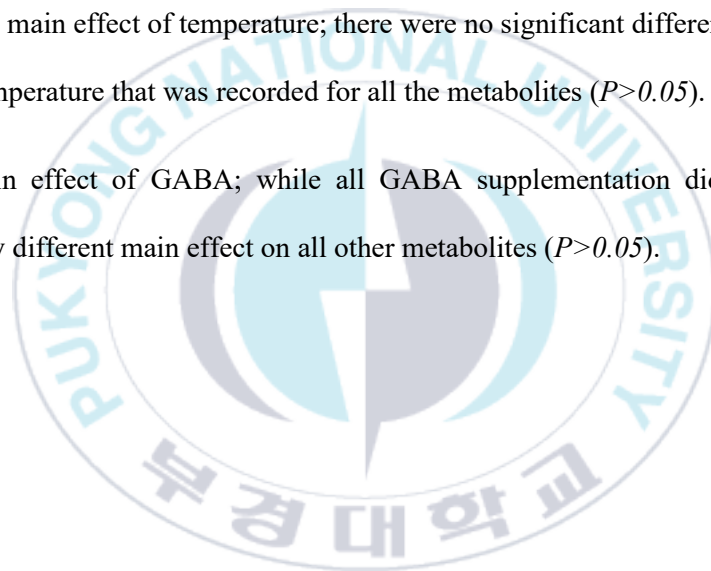


Table 6. Plasma metabolites of rainbow trout exposed to acute temperature stress at 20°C¹

Diets	GOT ²	GPT ³	TP ⁴	TG ⁵	TCHO ⁶	GLU ⁷
Interactive effect between temperature stress and GABA						
T15GABA0	322 ± 54 ^{ns}	26.7±3.8 ^{ns}	2.70±0.09 ^{ns}	335 ± 18 ^{ns}	198 ± 20 ^{ns}	80.5±10.2 ^{ns}
T15GABA50	458 ± 83	36.0 ± 6.8	3.13 ± 0.27	305 ± 36	201 ± 14	116 ± 7
T15GABA100	356 ± 35	27.7 ± 2.3	3.07 ± 0.08	410 ± 45	205 ± 9	104 ± 13
T15GABA150	408 ± 28	26.7 ± 2.2	3.40 ± 0.09	344 ± 20	201 ± 10	107 ± 10
T15GABA200	356 ± 53	25.0 ± 2.7	3.00 ± 0.16	336 ± 35	207 ± 8	127 ± 16
T20GABA0	307 ± 30	66.0±40.8	3.10 ± 0.12	272 ± 17	209 ± 13	86.3 ± 5.4
T20GABA50	339 ± 32	60.0±30.8	3.17 ± 0.10	266 ± 28	202 ± 14	65.2 ± 5.1
T20GABA100	397 ± 81	31.6 ± 2.4	2.88 ± 0.19	337 ± 15	205 ± 14	67.3 ± 12.6
T20GABA150	350 ± 36	48.7±23.1	3.33 ± 0.19	454 ± 80	288 ± 36	131 ± 32
T20GABA200	467 ± 68	31.2 ± 3.0	3.20 ± 0.13	306 ± 18	232 ± 17	121 ± 19
Main effect of temperature						
T15	380 ± 24 ^{ns}	28.4±1.9 ^{ns}	3.06±0.11 ^{ns}	346 ± 17 ^{ns}	202 ± 2 ^{ns}	107 ± 8 ^{ns}
T20	372 ± 28	47.5 ± 7.1	3.14 ± 0.07	327 ± 34	227 ± 16	94.2 ± 13.6
Main effect of GABA						
GABA0	314 ± 8 ^{ns}	46.3±19.7 ^{ns}	2.90 ±15 ^{ns}	304 ± 31 ^{ns}	204 ± 5 ^{ns}	83.4 ± 2.9 ^{ns}
GABA50	398 ± 59	48.0 ± 12.0	3.15±0.02	286 ± 20	201 ± 0	90.5 ± 25.3
GABA100	377 ± 20	29.6 ± 2.0	2.97±0.09	373 ± 36	205 ± 0	85.6 ± 18.3
GABA150	379 ± 29	37.7 ± 11.0	3.37±0.03	399 ± 55	244 ± 44	119 ± 12
GABA200	412 ± 55	28.1 ± 3.1	3.10±0.10	321 ± 15	219 ± 13	124 ± 3
Two-way ANOVA (<i>p</i> -value)						
Temperature	0.8142	0.1135	0.4346	0.4146	0.0575	0.1858
GABA	0.4476	0.7521	0.0530	0.0548	0.0857	0.0161
Temp * GABA	0.2771	0.8785	0.3495	0.0993	0.0809	0.0949

¹Values are means (± SEM) from triplicate groups (n=3) of fish where the values in each row with not significantly different ($P > 0.05$).

²GOT:Glutamic oxaloacetic transaminase (U/L)

³GPT:Glutamic pyruvate transaminase (U/L)

⁴TP:Total protein (g/dL)

⁵TG:Triglyceride (mg/dL)

⁶TCHO:Total cholesterol(mg/dL)

⁷GLU:Glucose(mg/dL)

2.2 Cortisol Level

Interaction effect, GABA 200 at 20 °C showed a significantly higher levels than that of the control diet. This level was however not significantly different from the cortisol response recorded for GABA150 also at 20°C. All the remaining diets at the temperatures examined showed increased cortisol levels when compared to the control ($P < 0.05$). However, the increase was not significantly different from the control.

The main effect of temperature, cortisol in which the main effect of temperature at 20 °C was significantly higher than that of 15 °C ($P < 0.05$). The significant main effect of temperature on cortisol indicates that altering the temperature levels (T15 vs. T20) has led to a statistically significant change in cortisol levels ($P < 0.05$).

Main effect of GABA; cortisol were the only metabolites that showed significantly different main effects of diet (p-values $< .0001$) ($P < 0.05$). The significant main effect of GABA supplementation on cortisol indicates that different levels of GABA supplementation (GABA0, GABA50, GABA100, GABA150, GABA200) have resulted in statistically significant differences in cortisol levels.

Cortisol main effect; cortisol has a significant main effect for both temperature and GABA supplementation. This means that changes in temperature or variations in GABA supplementation levels have a notable impact on cortisol levels in fish ($P < 0.05$).

Table 7. Stress response (cortisol) of rainbow trout after acute temperature change¹

Diets	Cortisol²
T15CON	3.87±0.29 ^d
T15GABA50	5.37±0.29 ^{cd}
T15GABA100	6.32±0.55 ^{bcd}
T15GABA150	7.60±0.45 ^{bc}
T15GABA200	6.24±0.22 ^{bcd}
T20CON	7.27±0.35 ^{bcd}
T20GABA50	7.29±0.42 ^{bcd}
T20GABA100	8.07±1.23 ^{bc}
T20GABA150	9.63±1.51 ^{ab}
T20GABA200	12.4±1.23 ^a
Main effect of TEMP	
T15	5.88±0.06 ^b
T20	8.93±0.24 ^a
Main effect of GABA	
GABA0	5.57±0.03 ^b
GABA50	6.33±0.07 ^b
GABA100	7.19±0.34 ^{ab}
GABA150	8.61±0.53 ^a
GABA200	9.32±0.50 ^a
Two-way ANOVA (<i>p</i>-value)	
TEMP	<.0001
GABA	<.0001
TEMP*GABA	0.0387

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

²Cortisol (ng/ml)

IV. Discussion

In this study, we examined the effects of different dosages of dietary GABA on temperature in rainbow trout in terms of growth, blood plasma indices and stress. The findings of this study showed that the addition of GABA has a positive effect on weight gain, specific growth rate, feed efficiency and protein efficiency rate in rainbow trout, and that GABA can be effective in increasing growth. Broken line analysis for weight gain showed that the optimum dietary GABA level could be approximately 140 mg/kg diet in rainbow trout. Since GABA is an inhibitory neurotransmitter, it has been observed that stress indices decrease in animals (Liu et al., 2015) and GABA improves growth performance by affecting metabolism in animals because it has a normal stimulus control (Turenus et al., 2009). Found that supplementation with GABA significantly improved growth and modulated both aspartate aminotransferase (AST) and superoxide dismutase activity (SOD) in juvenile Nile tilapia (*Oreochromis niloticus*) with a 158 mg/kg determined to be an optimal level in the diet (Temu et al.,2019). On Jian carp (*Cyprinus carpio*) GABA have positive effects on growth performance and support current study (Chen et al.,2021). However, the effect of GABA on growth showed that the level of inclusion was dose-dependent (Wu et al.,2016). It has been observed that 150 mg/kg diet can improve the weight gain of juvenile Pacific white shrimp under ammonia (NH₃) stress (Xie et al., 2017). In another study,it was found that grass carp feed increased the growth rate, superoxide dismutase and total antioxidant level by increasing the GABA level from 50 to 150 mg/kg (Wu et al., 2016). Increase in growth performance was observed in broilers with an optimum GABA level of 100 mg/kg (Zhong et al., 2020). GABA level of 55.3 mg/kg in Pharaoh

cuttlefish the findings of this study demonstrated that dietary GABA had a positive effect on the growth performance (Li et al., 2020). In the current study and other studies, GABA was observed to improve growth performance in animals.

Blood parameters serve reliably for the condition and health of fish (Kader et al., 2012). Changes in the levels of these enzymes in hemolymph can signal hepatopancreatic damage, and they have been observed to be valuable in diagnosing diseases in (Pacheco & Santos, 2002, Zhou et al., 2013). In this study, rainbow trout fed at different diet GABA levels plasma metabolites after growth trial in rainbow trout fed with GABA experimental diets for 8 weeks. There were no significant differences in GOT, GPT, TP, TG, TCHO, GLU. The same results were obtained when olive flounder were fed with different levels of GABA (Farris et al., 2022). In the experiment, where the optimum GABA level in whiteleg shrimp was 100 mg/kg, no significant difference was observed in plasma metabolites (Bae et al., 2022). These experimental studies are ,biochemical parameters that are sufficient to make the effects of GABA seem insignificant. It shows that there could be a stable metabolism that is playing out therefore the dietary interventions did not have any significant effect.

Cortisol is the main glucocorticoid in fishes and the hormone most directly linked to stress is a hormone produced and in fishes (Goikoetxea et al., 2017, Sadoul & Geffroy, 2019). Cortisol is produced by inter-renal cells sparsely distributed within the head kidney and released into the blood (Gamperl et al., 1994). Plasma cortisol is the most widely used indicator of stress in fish. The physiological stress response in fish is driven by the activation of 2 hormonal axes. Brain-sympathetic-chromaffin cell (BSC) axis and the hypothalamic-pituitary-interrenal (HPI) axis. The BSC axis creates

stress with a rapid increase in plasma catechoamines epinephrine and norepinephrine by chromaffin cells, which leads to the oxidation of glucose in the plasma and the need for energy due to stress (Wendelaar Bonga, 1997). The HPI axis is primarily responsible for increased plasma glucocorticoid levels to provide energy under stressful conditions (Balasch&Tort 2019). Increasing water temperatures due to global warming may affect the stress response, metabolism, and energy supply of fish (Pörtner & Knust 2007; Madeira et al., 2016). Since fish are cold-blooded animals, water temperature has an effect on fish physiology (Lv et al., 2021). In the study conducted on juvenile olive flounder, using 200 mg/kg and 0 mg/kg of dietary GABA diet, it was observed that GABA, cortisol and glucose increased as the temperature increased in a study lasting $20 \pm 1^\circ\text{C}$ (normal temperature) and $27 \pm 1^\circ\text{C}$ (high temperature) for 28 days. However, temperature and GABA the interaction was not significant (Lee et al., 2023). In this study, GABA and water temperature had significant effects on cortisol. It was observed that the mean main effect of GABA level had a dose-dependent relationship with the delay of stress responses, with high water temperature having a strong effect. These results support the data from the study of GABA-cortisol and plasma levels in rainbow trout under water temperature stress.

V. Conclusion

In this trial the optimal level of dietary GABA was determined to be approximately 140 mg/kg according to one slope broken line analysis based on WG. Dietary GABA supplementation may improve the growth performance of rainbow trout. The results from this research showed that changes in temperature or variations in GABA supplementation levels have a notable impact on cortisol levels in fish. In practical terms, this suggests that temperature has an influence on the fishes stress response or cortisol regulation. For example, cortisol levels may increase or decrease with changes in temperature. This could also mean that GABA supplementation affects the fishes cortisol levels, and that GABA affects the fishes stress response or hormonal regulation. GABA, increases the growth performance of rainbow trout and other fish in aquaculture feed additive, makes resistant to stress and affects the stress response and hormonal regulation. Considering GABA's cost-effectiveness and its comparatively limited involvement and activity in relation to other components, it is probable that GABA will continue to garner attention and, as a result, warrant further research. Future research is oriented toward the development of novel approaches to investigate GABA's role in fish metabolism. Current study, serves as a strong starting point for future studies on different species and life stages.

VI. Reference

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Acknowledgement

During the preparation of the research, I would like to say a heartfelt thank you to **Prof. Seunghyung Lee**. I am grateful for everything he taught me. I will always remember him with gratitude.

I would also like to thank the International Co-operative Alliance (ICFO) for my scholarship. I express my sincere appreciation and gratitude to my laboratory colleagues.

I would like to thank Serkan Serdaroglu, who always helped me in South Korea.

Finally, I would like to thank my family and friends. accomplishment would not have been possible without them.

Tugce KILIC

