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Effect of type and dosage of carotenoid in feed on plasma cortisol and glucose of Kurumoi rainbowfish (*Melanotaenia parva* Allen) due to transportation stress

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Abstract
This study was conducted to investigate the effect of carotenoid source and dosages on plasma cortisol and glucose of Lake Kurumoi rainbowfish due to transport stress. The juvenile fish (weight 1.84 ± 0.03g, total length: 5.2 ± 0.09 cm [mean ± SE]) were fed different carotenoid synthetics such as astaxanthin (AS), canthaxanthin (CS), lutein (LS) at different dosages (130 and 260 mg kg^{-1}) for two months included basal diet (without carotenoids) which was considered as control. After feed treatment was performed, simulation of transport stress was conducted for 15 hours. Plasma cortisol and glucose concentration of blood were measured during transport stress at 0 hour before transport (H0-); and at 0 (H0+), 24 (H24), 48 hours (H48) and 72 hours (H72) after transport. Fishes were fasted during measurement periods. The present study indicates that there are different variation of plasma cortisol and glucose on different carotenoids source and dosages in Lake Kurumoi rainbowfish. Fish fed with carotenoid source at different dosages provided different pattern from each other. The increase of plasma cortisol on basal fish diet was the fastest one, that was directly at 0 hour after transport (increased by 186%) compared to fish fed carotenoid that was slower and slightly increased at 24 hours after stress. It was contrary to the plasma glucose. Fish fed carotenoid diets tended to suppress stress than basal diet (without carotenoid).

Keywords: carotenoids, cortisol, glucose, *Melanotaenia parva*, transport stress

Introduction
Melanotaenia parva or commonly known as Kurumoi rainbowfish is an ornamental fish candidate with economic value and potential aesthetic. This fish has been developed in aquaculture system in aquarium [1]. Today, Kurumoi rainbowfish are demanded by consumers both local farmer and international hobbyist with quite competitive price. The development of this fish continues to be directed into production, quality improvement, and other aquaculture technology. To meet both local and international market, Kurumoi rainbowfish should have the ability to adapt well towards handling and transportation. It is conducted to maintain high survival rate until received by consumer. Handling and transportation could trigger stress in fish that eventually leads to fish death and decreases profit for fish farmer [2].

In monitoring stress response, blood characteristics can be used to evaluate physiological response in fish [3] through changes in the level of cortisol hormone and blood glucose [4, 5]. Cortisol is a hormone secreted by glucocorticoid through interrenal tissue in teleost fish [6] and released by the activation of hypothalamus-pituitary interrenal axis (HPI axis) [7]. Cortisol not only activates the process of glycogenolysis and gluconeogenesis in fish, but also causes chromaffin cells to increase the release from catecholamine that further will increase glycogenolysis [8]. This process rises substrate (glucose) level to produce enough energy as needed [2]. Therefore, some studies also used glucose level as stress indicator [7, 9].

In accordance with this finding, if stress response occurs through the mechanism of cortisol hormone and glucose change, fish will experience hyperactivity which is a gradual response against stress, and if the stress is excessive, fish will be dead. The occurrence of stress response indicates adaptation to unexpected changes and to return to homeostatic condition [6].

Research concerning the effect of source and dosage of carotenoid on fish stress response is difficult to search, and in Kurumoi rainbowfish, this study has not yet done. Administration of

several sources and dosages of carotenoid is expected to decrease physiological response in Kurumoi rainbowfish due to transportation stress.

Material and Methods

Initial fish used were the seed of ornamental Kurumoi rainbowfish produced from natural spawning with uniform initial size of fish (weight of 1.08±0.13 g; 4.60±0.20 cm). About 30 fish were placed in square aquarium with water volume of 30 liters and reared during 56 days. Aquarium were arranged in an indoor recirculation system with photoperiod (10 hours of light: 14 hours of dark). Fish were fed experimental diet to satiation with frequency of 2 times a day at 8 am and 3 pm for 56 days of maintenance.

Experiment was done to evaluate the most effective source and dosage of synthetic carotenoid in suppressing stress effect due to transportation. Carotenoid dosage was estimated as common and high dosage. Treatment tested was administration of basal diet or without the addition of carotenoid (B); synthetic astaxanthin (Carophyll® Pink 10% Cold Water Soluble); canthaxanthin (Carophyll® Red 10%); and lutein (Carophyll® Yellow 10%) (DSM Nutritional Products Ltd, Basel, Switzerland), with dosage of 100 and 200 mg/kg of each, respectively given code as AS-1; AS-2; CS-1; CS-2; LS-1; LS-2 [10].

Table 1: Composition of experimental diet from several sources and dosages of synthetic carotenoid.

Bahan baku (g kg^{-1})	Test Diet						
	B	**AS-1**	**AS-2**	**CS-1**	**CS-2**	**LS-1**	**LS-2**
Fish meal	637	635.7	634.4	635.7	634.4	635.7	634.4
Gelatin	120	120	120	120	120	120	120
Dextrin	110	110	110	110	110	110	110
Fish oil	63	63	63	63	63	63	63
Vitamin mix	20	20	20	20	20	20	20
Mineral mix	30	30	30	30	30	30	30
CMC[1]	20	20	20	20	20	20	20
Astaxanthin (10%)[2]	0	1.3	2.6	0	0	0	0
Cantaxanthin (10%)[2]	0	0	0	1.3	2.6	0	0
Lutein (10%)[2]	0	0	0	0	0	1.3	2.6
Nutrient composition (% dry matter):							
Crude protein^{NS}	50.98	51.00	50.95	52.33	52.00	52.09	51.99
Crude lipid^{NS}	16.00	17.24	17.54	16.79	16.69	15.80	15.82
Crude fiber^{NS}	2.82	2.47	2.64	2.91	3.05	2.90	2.69
Ash^{NS}	19.96	21.50	21.20	19.62	21.78	20.65	21.09
NFE^{NS}	9.94	7.79	7.68	8.35	6.48	8.56	8.41
Total carotenoids (mg kg^{-1})	15.74	103.50	137.07	106.00	151.09	104.07	142.63

[1]Carboxy Methyl Cellulose
[2]In accordance with the level of carotenoid digestibility [1]
NS = not significant
NFE = Nitrogen Free Extract

Feed raw ingredients used were firstly smoothed and sieved. Pellets were further made using pellet machine and dried using oven at a temperature of 60°C and later stored at a temperature of −20 °C to avoid carotenoid oxidation. Feed were made by formerly tested the nutritional content (proximate analysis) and carotenoid analysis was also conducted. In term of raw ingredients, calculation on formulation according to target demanded was done later. Experimental diets used were artificial feed in the form of pellet (semi pure) with isoprotein of 51-53 %; lipid of 16-18%; ash of 19-22 %; crude fiber of 2-3%; and nitrogen free extract (NFE of 7-10 %) were measured by proximate analysis [11]. The ingredients used and formulation applied are listed in Table 1.

During maintenance, water in the aquarium were siphoned and water quality was monitored regularly. Water quality measured during maintenance was within the normal range for fish, namely dissolved oxygen of 5.00-5.24 mg L^{-1}; temperature of 25-27 ºC; pH of 5.99-6.48; while ammonia and nitrite were not detected. In 56 days of maintenance, three fish were randomly selected in each aquarium to measure their final weight and height, while the remaining fish were used for transportation test. Average final weight and height of fish measured and further used for transportation test were 1.84 ± 0.03 g; 5.2 ± 0.09 cm. Transportation test (simulation) was carried out for 15 hours to determine experimental fish durability against stress response.

Procedure of transportation test was done by putting all fish to be transported into plastic bag filled with fresh water at density of 20 fish/liter. Fish in each plastic bag amounted to 25 fish. Oxygen was filled into the plastic at ratio of 2 part of oxygen volume and 1 part of water volume. Later, plastic bags were placed into styrofoam box and sealed with duct tape as the general transporting process for fish seed. After that, styrofoam box was put into tank which later flowed by water using two pumps placed opposite causing styrofoam to move. After transportation, experimental animals were put back into maintenance container to be observed in accordance with time of observation. Fish blood sampling was done shortly before transportation (H0-); shortly after (H0+); 24 hours post transportation (H24); 48 hours (H48); and 72 hours (H72).

Blood sample was taken from five sample fish to meet the minimum amount of plasma for cortisol and glucose measurement. Before the blood was taken, fish were previously anesthetized using phenoxy ethanol at dose of 0.4 mL/L water. Blood was taken from the blood vessel in dorsal using syringe with size of 26G x 0.5 inch that had been given heparin. Blood sample was placed inside labeled eppendorf tube and put inside container filled with ice to further being moved to laboratory to be centrifuged at a temperature of 4ºC, 18000 rpm for 30 minutes. Supernatant produced was moved

to new eppendorf tubes which were labeled and later stored at a temperature of -20°C for further analysis. Cortisol was measured using commercial kit enzyme-linked Immunosorbent assay (ELISA) Kit EIA Cortisol 1887 (DRG International, Inc.) and read at optical density of 450±10 nm using microplate reader after 10 minutes. Measurement of plasma glucose level was done using commercial kit Glucose liquicolor method of GOD-PAP enzymatic colorimetric test for glucose (Human-Germany) and the result was read using spectrophotometer at wavelength of 500 nm. Data obtained were presented in the form of figure and analyzed descriptively.

Results and Discussions

Cortisol level in Kurumoi rainbowfish plasma given different carotenoid feed indicated different patterns in every observation time. Shortly before transportation, cortisol level was varied between 40-90 ng mL^{-1} plasma. Moreover, a moment after transportation, the level of plasma cortisol in fish that did not given carotenoid (basal) increased sharply from 50 ng mL^{-1} plasma to 143 ng mL^{-1} plasma. In fish given carotenoid feed, cortisol level showed a contrast result that was decreasing shortly after transportation stress was done. In 24 hours post transportation, cortisol of fish fed diet without carotenoid started to decrease, while most fish given feed contained carotenoid experienced increasing phase toward homeostatic point. In fish with basal feed, homeostatic point was achieved in 72 hours post transportation, while in fish with carotenoid, even though homeostatic point has been reached before, cortisol tends to continue decreasing. Fish with AS-2 diet tended to be more constant and did not experience significant change in responding transportation stress (Figure1).

Glucose level in Kurumoi rainbow fish plasma measured in all treatments of experimental diets tended to have mirror-like pattern towards plasma cortisol level. Fish fed basal diet experienced increase in glucose level in 72 hours post transportation, after cortisol in the blood reached homeostatic point. In fish treated carotenoid feed, increase in glucose level commonly occurred when plasma cortisol level was low (Figure 2).

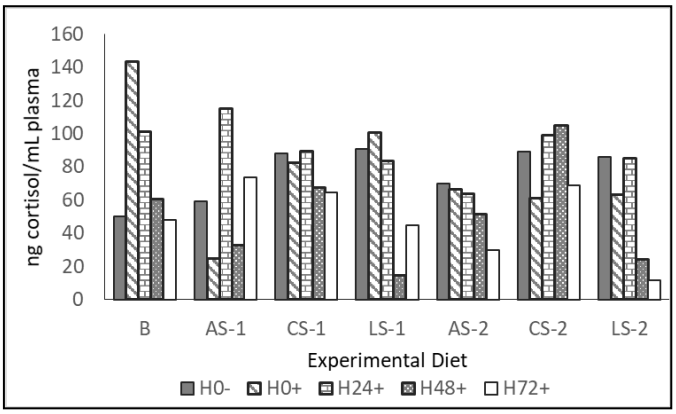

Fig 1: Cortisol level (ng mL^{-1}) in blood plasma of fish *Melanotaenia parva* treated carotenoid diet shortly before transportation (H0-); shortly after transportation (H0+); 24 hours (H24+); 48 hours (H48+); and 72 hours (H72+) post transportation.

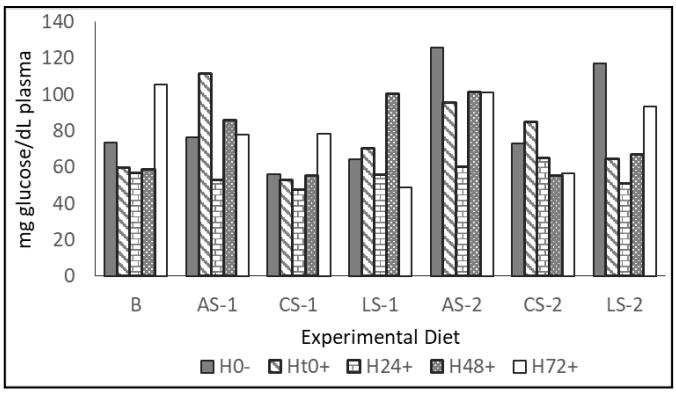

Fig 2: Glucose level (mg dL^{-1}) in blood plasma of fish *Melanotaenia parva* treated carotenoid diet shortly before transportation (H0-); shortly after transportation (H0+); 24 hours (H24+); 48 hours (H48+); and 72 hours (H72+) post transportation.

Stress is a defensive response in animal to stressor. Several sources of stress may be in the form of biotic factor and environment like transportation. Response against stress is considered as adaptive mechanism that allows fish to face the reality or stress to maintain normal status or homeostasis within the body [6]. Blood characteristics is used to evaluate physiological response in fish [3]. Stress response in animal is seen from its changes in the level of cortisol hormone and blood glucose [5].

Research on stress response due to transportation through measurement of plasma cortisol and glucose has been done in salmonid group, such as lake trout *Salvelinus namaycush*, rainbow trout *Oncorhynchus mykiss*, brown trout *Salmo trutta*, and brook trout *Salvelinus fontinalis* [12]. Based on this research, it is known that transportation affected the increase of cortisol hormone in fish body, and the amount of cortisol released was different depends on fish species.

Effect of stress from aquaculture practice in fish and the method to minimize the impact resulted from the activity were performed in several researches like treatment of cold shock and anesthesia, increase in salinity [13], and stocking density [14]. Stress response can also be affected by feed and fish nutrient status [15] since these factors may influence the liver glycogen storage and glucose response [2]. However, study on physiological response with supplementation of certain nutrients in feed to reduce stress level is still very limited, yet some nutrients have the potential to minimize the impact of stress.

Barton *et al.* [6] mentioned that tress response has quite high variability in corticosteroid response between species, with value ranges of 30-300 ng mL^{-1}, yet this variation also refers to the duration or the severity of stress faced. In this study, it is known that plasma cortisol in Kurumoi rainbowfish before transportation stress started was varied with range of 40-90 ng mL^{-1}, indicated normal condition before stress occurred. Increase in plasma cortisol drastically soon after transportation was faced by fish treated feed without carotenoid with an increase of (186%), while fish fed carotenoid tended to have decreasing or constant plasma cortisol (Figure 1).

Cortisol synthesis and its release from interrenal cell has time lag for several minutes [16]. As a result, cortisol circulation level is commonly used as an indicator of stress level

experienced by fish [17]. Result of this study indicated that stress due to transportation can be suppressed early by fish had carotenoid intake since fish. Fish supplied with carotenoid is expected to cope stress faster during transportation duration of 15 hours.

Carotenoid is one of supplementary nutrient with role as antioxidant potential [18, 19, 20], and boosts immune system [21, 22]. Carotenoid is able to minimize stress effect through the increasing immune system [23] and has positive effect in intermediary metabolism in fish [24]. The benefit of carotenoid may occur as a result of protection against oxidative damage. In other words, the relation between carotenoid and glucose metabolism is completely explained through antioxidant effect [25]. Antioxidant and reactive oxygen species influence cell signaling and gene expression [26], biological activity of carotenoid includes induction in communication between cells [27].

In this present study, result showed that carotenoid was able to minimize stress effect and provided positive effect in fish intermediary metabolism, similar with statements by Rehulka [23] and Segner *et al.* [24]. This physiological mechanism causes fish to be able to adapt to balance stress, and in this case, blood chemical feature such as plasma cortisol may look normal [6]. Low plasma cortisol levels when stress is induced because carotenoids can reduce adreno corticotropic (ACTH) hormone which releases cortisol [28].

Recovery from stress, in this case, is measured from plasma cortisol level return into the rest condition that occurs in period range of hours or minutes. The rate of decline will be slow if stressor still exists in the recovery environment [29]. Recovery from acute stress exposure was reported to occur immediately, namely 2-6 hours [30, 31] or 24-48 hours [32, 33]. In wild brown trout, cortisol level of *Salmo trutta* were back to normal after 6 hours [9] and in the next study, the earliest post-stress time was 24 hours [34]. In rainbow trout *Oncorhynchus mykiss* put in cage in a river post catch, plasma cortisol recovery occurred in 24 hours [35]. In this study, stress recovery period was faster in fish that had carotenoid intake, where in observation time of 24 hours post transportation, fish experienced recovery period to homeostasis level that was the same as the initial condition before transportation, while fish did not fed carotenoid (control) required longer time.

In general, increase in plasma cortisol due to stress response will increase plasma glucose level. Increasing plasma glucose is started by glycogenesis mediated by catecholamine and in advanced stadia and gluconeogenesis mediated by cortisol is formed during anaerobiosis released towards plasma [7] with limited liver glycogen storage [36, 37]. This statement is contradictive considering this study in which it is known that plasma glucose level of Kurumoi rainbowfish, before and after transportation stress, was inversely proportional to cortisol level. Both profiles are like mirror, where decrease in plasma cortisol level will increase glucose level, vice versa. This research reflected that plasma glucose showed different recovery profile and increase from cortisol [38, 9].

Stress hormone together with cortisol mobilize and increase glucose production in fish through glycogenesis and glycogenolysis path [39] to meet the need of energy that is caused by stressor. Glucose production is mostly mediated by cortisol action that stimulates liver gluconeogenesis and also stops peripheral glucose intake [17]. Knowledge regarding biological mechanism related to carotenoid on glucose metabolism is still limited [40]. In this study, transportation stress in treatment AS-2 did not affect the increasing plasma cortisol level, this finding may be related to the high level of glucose in normal condition or shortly before transportation performed. Higher glucose level compared with other treatment is expected to supply energy required by rainbow fish when induced with stress. This caused fish to not experience drastic cortisol increase or glucose sensitivity of rainbow fish was higher than cortisol in coping stress.

It causes fish do not face a drastic increase in cortisol due to obstacles that occur due to carotenoid ability in reducing ACTH. Stress is mostly responded to by glucose as a supplier of energy to produce homeostatic conditions. In this study, the increasing of blood glucose can be derived from the adrenaline hormone released when there is a stress trigger, which causes the glucagon hormone to be released to convert glycogen to glucose. The glucose product then becomes a source of energy for Kurumoi rainbowfish to reach the homeostatic phase [41].

Conclusion

Cortisol and glucose profiles of rainbowfish blood in coping transportation stress were varied depend on the presence of carotenoid or the type or dosage of carotenoid given. Plasma cortisol and glucose as stress response mechanism showed contrast result one another. Fish sensitivity in releasing glucose was higher than that in releasing cortisol when fish respond to stress. Fish given feed contained carotenoid was better in suppressing stress compared to feed without carotenoid.

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