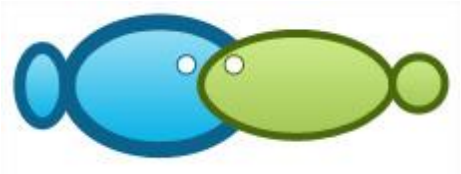

Biochemical profiles of hybrid grouper (*Epinephelus fuscoguttatus* x *E. lanceolatus*) larvae fed with enriched live feeds and commercial diets

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Abstract. The reliance on live feed for early larval rearing in fish has increased the production cost. Furthermore, live prey feeding without enrichment have been reported to be lacking nutritional value and insufficient for larval growth. This study aimed to determine biochemical profiles of hybrid grouper larvae after being fed with enriched live feeds and micropellets. Hybrid grouper larvae were divided into three groups of 45 days different feeding treatments: diet A (fed with unenriched live feeds – OTO), diet B (fed with enriched live feeds – NTR) and diet C (fed with enriched live feed – ORG). The results showed that protein and lipid deposition in soft tissue of fish was not different among feeding treatments (ranged from 519 to 600 mg g dry weight^{-1} for protein content and from 222 to 245 mg g dry weight^{-1} for lipid content). Fish fed with enriched live feed in diet B and diet C showed a higher proportion content of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) than fish fed with un-enriched live feed in diet A. The finding of this study suggests that live feed enrichments and different micropellet diets did not alter protein and lipid deposition. Live feed enrichment increased the proportion content of MUFA and PUFA in fish.
Key Words: larval rearing, micropellet, proximate, replacement diet.

Introduction. The recent global aquaculture production of grouper has been dominated by Asian countries i.e. China, Taiwan, Indonesia and Malaysia. These countries contributed up to 93% of the global production, consisting of 48 species and 16 hybrid groupers (Rimmer & Glamuzina 2019; Ybanez Jr. & Gonzales 2023). Indonesia was the third largest grouper producer, contributing 11% of global production (Ybanez Jr. & Gonzales 2023). This production is estimated to continue to increase as Indonesian government has targeted fishery products as one of the commodities to support national food securities (Oktopura et al 2020). Therefore, aquaculture industries have now focused on improving the production through farming practice and innovation (Henriksson et al 2019).

Several technologies have been recently applied in aquaculture industry in attempt to produce high quality offspring relative to their parental species e.g., selective breeding, gynogenesis, androgenesis, transgenesis, gene editing and hybridization (Wang et al 2019; Regan et al 2021). Of these techniques, hybridization is considered to be one

of the most common, inexpensive and effective technique for breeding in fish, crustaceans and molluscs (Bartley et al 2000; Mbiru et al 2016; Payet et al 2016; Wang et al 2019; Lai et al 2023). Aquaculture development of hybrid grouper, between tiger grouper *Ephinephelus fuscoguttatus* and giant grouper *E. lanceolatus* has increased significantly since it was introduced in 2006 (Ch'ng & Senoo 2008). In 2021, grouper aquaculture production in Indonesia reached ~2,200 t, of which hybrid grouper, tiger grouper *E. fuscoguttatus* x giant grouper *E. lanceolatus,* hereafter known as TGGG hybrid grouper was reported to contribute major proportion (Ismi & Budi 2020). Despite the increasing trend of production, the efficiency production, particularly larval rearing stage of hybrid grouper is still facing major challenges.

One of the major bottlenecks in mass production of grouper is high mortality at the larval stages (Ma et al 2013; Rimmer & Glamuzina 2019). It has been reported that grouper larvae are naturally sensitive to physical disturbance during larval rearing e.g., light intensity, aeration, rearing tank colour (Toledo et al 2002; Ma et al 2013; Ren et al 2021; Lukas et al 2023). Moreover, the reliance of grouper larvae on live feed in their early larval stage has limit the application of wide range of feeding. Larval rearing of TGGG hybrid groupers is commonly using a combination of rotifers *Brachionus* sp. and algae *Nannochloropsis* sp. for the first 20 days of larval feeding. Brine shrimps, *Artemia* sp. are applied at day 20-40 with the introduction of micro pellet to proportionally replace *Artemia* around day 30 of larval rearing (Ch'ng & Senoo 2008; Ching et al 2018).

The use of live feeds for fish larval feeding is thought to be non-cost effective practice. For example, the cost of live feed feeding to grow up juvenile seabass (*Lates calcarifer*) to 45 days old and early weaned smallmouth bass (*Micropterus dolomieu*) accounted for up to 79% of production cost in early larval rearing stage (Ehrlich et al 1989; People Le Ruyet et al 1993). This cost is commonly associated with live feed cost, intensive labour work for mass culture of live feeds and microalgae *Nannochloropsis* sp. (Bentley et al 2008; Watanabe et al 2016). In terms of nutritional contents, rotifers and *Artemia* are lacking of some essential nutrients e.g., long chain polyunsaturated fatty acids (PUFA), taurine, selenium, iodium (Conceição et al 2010; Pratiwy et al 2021). Therefore, the use of enrichment in live feeds to ensure adequate nutritional value of feeding and early introduction of micro diets to reduce the reliance of live feed is crucial to improve the overall production of fish including TGGG hybrid groupers.

Several enrichment techniques and products have been previously studied on wide range species of fish larvae. The inability of rotifers and *Artemia* to provide adequate essential fatty acids e.g., docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) can be manipulated by short- or long-term feeding using lipid emulsion, lipid microcapsules, selected microalgae in various forms (e.g., spray, paste, freeze-dried, concentrate) and several commercial enrichment products (Watanabe et al 2016; Betancor et al 2019). The use of microdiets has also been used to replace live feed proportionally at early larval rearing, but the success varies depending on species, size, frequency of feeding and rearing system (Blair et al 2003; Andrade et al 2012; Kolkovski 2013; Alam et al 2015; Watanabe et al 2016; Sedki et al 2019). Despite nutritional improvement in enriched live feed and microdiets, food particle utilization by fish larvae is affected by feeding process depending on rearing system to deal with dispersion of small particles of microdiets in water column. Therefore, the successful interaction of larvae and microdiet particles will support larval growth and performance. This study aims to examine the biochemical profiles of TGGG hybrid grouper larvae fed with enriched live feeds and microdiets.

Material and Method

Live feed culture and enrichment*. Nannochloropsis* sp. used for rotifer feeding was cultured at 20,000 L in concrete tank using agricultural fertilizer following the method of Tugiyono et al (2018). At day 5 of culture, *Nannochloropsis* sp. were harvested and transfered to 20,000 L of rotifer culture tank to feed rotifer at density of 250 cells per individual rotifer. *Artemia* was cultured using EG Artemia cyst (SepArt-D-Fence®) (INVE, Belgium) which was hatched in 700 L tank with the water volume corresponds to required

density. Water was chlorinated and neutralized before the starting of the process and ACE Pro was used for bacterial control. For *Artemia* hatching, water temperature was maintained at 29-30°C using a water heater and pH of 8.0-8.5.

Rotifer enrichment was done in 1,000 L tank, where the water has been previously chlorinated overnight 50 ppm and neutralized using sodium thiosulphate at 4 g 1000 L^{-1}. Enrichment tank was maintained at 25-30°C water temperature and 30 ppm water salinity. Two enrichment dosages of 160 ppm DHA protein selco® (INVE, Belgium) was given to rotifer by three hours apart. After a total of 6 hours enrichment, the enriched rotifer was harvested and used for TGGG larval feeding.

Artemia enrichment was done using *Artemia* nauplii harvested after 22 h of hatching. Two dosages of enrichment of 200 ppm A1 DHA Selco® (INVE, Belgium) were given by 10 h apart from each other. Water temperature and pH during enrichment were maintained at 26°C and 7.5-8.0 respectively. Sodium bicarbonate and NaOH were applied to maintain a minimum pH of 7.5. Enriched *Artemia* for larval feeding was harvested after 22 h of enrichment period.

Larval source, rearing and experimental design. The experimental preparation, feeding treatments and laboratory analysis was conducted from June 2023 to February 2024. Eggs were sourced from Bali's broodstock center and transferred to the incubation tank in BRIN Gondol hatchery facilities, Research Center for Fishery, Indonesia. Eggs were disinfected using 10 ppm Iodine for 10 minutes prior to being stocked in experimental tanks. Eggs were then transfer to six of 6000 L experimental tanks (two replicate tanks of each feeding treatments; diet A (fed with unenriched live feeds – OTO), diet B (fed with enriched live feeds – NTR) and diet C (fed with enriched live feed – ORG) at density of 10 eggs L^{-1} for each tank. Larval rearing was maintained under 12:12 photoperiod at water temperature of ~28°C in all experimental tanks. The diets used in this study were formulated diets that have not been all commercially established and therefore they were presented in specific code with the nutritional profiles presented in Table 1.

Larval from diet A (control group) were fed with un-enriched rotifer from D2 to D25 of larval rearing at initial concentration of 5 individual mL^{-1} and increased to 15 individual mL^{-1} in D25, where fresh algae, *Nannochloropsis* sp. were given during this period to support rotifer feeding. Un-enriched artemia (instar I) feeding was introduced from D14 to D36. Commercial pellet A at micropellet size corresponding to larval size was given proportionally in the morning and afternoon at a total of 7.1 g m^{-3} day^{-1} in D8 .

Larval fish from diet B and diet C groups were fed with enriched rotifer using DHA protein selco® (INVE, Belgium) from D2 to D25 at initially 5 individual mL^{-1} and gradually increased to 15 and 12 individual mL^{-1} in day 23 respectively. This concentration was decreased to 6 individual mL^{-1} in D25 prior to complete pellet feeding. Green water system, Sanolife® GWS (INVE, Belgium) as a replacement for fresh algae feeding were introduced from D2 to D25 at 8-12 g day^{-1} $tank^{-1}$. *Artemia* was given from the third week to the last day of experiment. Micropellets in diet B were introduce from D3 in the morning and afternoon at a total of 20 g m^{-3} day^{-1}, while micropellets in diet C were used from D8 of larval rearing at a relative similar concentration. The diet A has a relative high proportional content of protein, while diet B and diet C was high in proportional content of lipid (Table 1).

Table 1

Proximate composition of pellet used for feeding treatment

Proximate content (%)	*Diet A*	*Diet B*	*Diet C*
Moisture	6.3-8.0	8.0-10.0	8.0-10.0
Protein	55.1-60.5	51.0-54.0	53.0-55.0
Lipid	11.1-14.9	18.0-19.0	12.0-13.5
Ash	15.0-16.4	10.0-12.0	10.0-11.5

Water quality parameters were checked the morning prior to tank siphoning. The daily water temperature in all thanks ranged from 27.7 to 29.1ºC. The daily dissolved oxygen (DO) and pH of water in all treatment tanks ranged from 3.7 to 5.4 mg L^{-1} and from 7.4 to 8.2 respectively. Water exchange in all treatment tanks was done starting from D7 by 7% and it increased to 50% in D39 followed by flow through water system until D45. Water siphoning was done daily starting at the second week. Pellet feeding was then given after siphoning and water exchange corresponding to feeding treatment tanks.

Biochemical analysis. A total of 150 fish sampled from each experimental tank were stored in a freezer at -20°C for later biochemical analysis. Fish samples were then freeze dried in a freeze dryer (Labconco, USA) for 48 h and were ground using a chopper. Two replicates of ~50 mg from ground sample from each feeding treatment were used for both lipid and protein analysis. One replicate of 15 g samples from each feeding treatment were used for fatty acid analysis. Meanwhile, two replicates of ~25 mg samples from each treatment were used for determining the ash free dry weight (AFDW) and were ashed in a muffle furnace (Nabertherm, Germany) at 550°C for 4 h. Lipid extraction was done using chloroform-methanol method with modification (Wang et al 2014; Supono et al 2023). Crude protein content was determined using micro-bicinchoninic acid (BCA) protein assay kit (ThermoFisher Scientific, USA) by reading against bovine serum albumin (BSA) standard at 540 nm (Wang et al 2014). Fatty acid analysis was done following the method of AOAC (2000).

Data analysis. Data of lipid and protein content were checked for the normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's test, respectively. One-way ANOVA was used to compare the mean value of all parameters observed when the data met normality and homogeneity of variances. When the data violated the assumption of normality and homogeneity of variances, a Kruskal-Wallis test was used to compare mean values of all parameters. A Tukey's posthoc test at a significance level of 0.05 was used where the analysis indicated an overall significant different among treatment.

Results

Protein content. There was no statistical differences on the protein content of fish from different feeding treatments ($F_{(2,5)}$ = 0.94, p > 0.05). The average protein content of fish ranged from 519 to 600 mg g dry $weight^{-1}$. Although there were no statistical differences, fish fed with diet C appeared to have the lowest body protein content (519.30±58.68 mg g dry $weight^{-1}$) among fish from different feeding treatment (600.50±44.38 mg g dry $weight^{-1}$ for diet A and 586.68±24.79 mg g dry $weight^{-1}$ for diet B) (Figure 1).

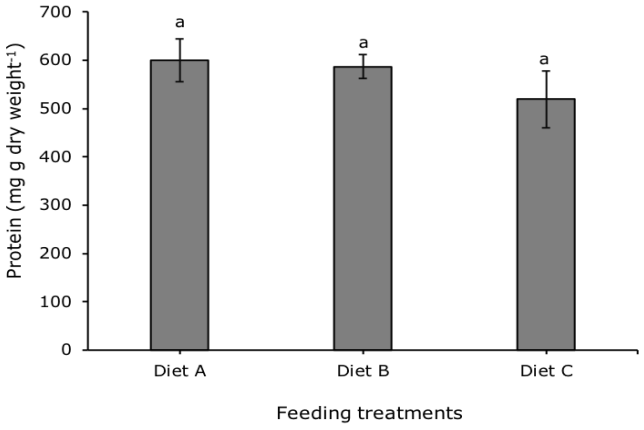

Figure 1. Mean (±SE) total protein content following 45 days of larval rearing using different feeding treatments.

Lipid content. Total lipid content of fish was not statistically different among feeding treatments ($F_{(2,5)}$ = 2.82, p > 0.05). Total lipid content was 225.17±0.22 mg g dry weight^{-1} for fish from control group, 245.46±11.36 mg g dry weight^{-1} for fish fed with diet B and 222.28±6.37 mg g dry weight^{-1} for fish fed with diet C (Figure 2).

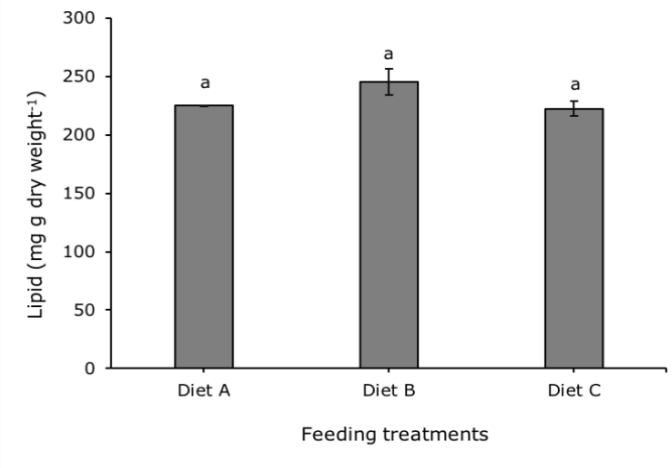

Figure 2. Mean (±SE) total lipid content following 45 days of larval rearing using different feeding treatments.

Fatty acids. Overall, there was 19 fatty acids from fish of all feeding treatments. The fatty acids from mono-unsaturated fatty acids (MUFA) group composed major proportion by 22 to 35% of total fatty acid content, while poly-unsaturated fatty acids (PUFA) contributed up to 8% of total fatty acid content. Saturated fatty acids (SFA) contributed small proportions of total fatty acid content by only up to 2%. Fish fed with diet B tended to have a higher proportion of MUFA and PUFA, by 35.42% and 8.52% respectively (Table 2).

Table 2

Fatty acid composition of fish fed with different feeding treatments

No	*Fatty acids*	*Content (%)*		
		Diet A (Control)	*Diet B*	*Diet C*
	SFA			
1	C4:0	0.01	0.02	0.01
2	C6:0	0.03	0.02	0.02
3	C12:0	0.02	0.30	0.02
4	C14:0	1.87	0.35	0.39
5	C17:0	0.15	0.28	0.30
6	C18:0	0.05	0.06	0.06
		2.13	1.03	0.80
	MUFA			
7	C14:1	0.11	0.10	0.11
8	C15:1	6.66	4.74	5.36
9	C16:1	2.24	1.55	1.67
10	C17:1	2.07	2.78	3.09
11	C18:1n-9c	1.91	2.54	2.68
12	C18:1n-9t	6.35	11.4	12.28
13	C20:1	2.47	9.54	10.05
14	C22:1n-9	0.65	0.10	0.18
		22.46	32.75	35.42
	PUFA			
15	C18:2n-6c	2.39	3.41	3.80
16	C18:2n-9t	0.03	0.06	0.07
17	C18:3n-6	0.63	0.51	0.60
18	C20:3n-3	0.23	0.40	0.50
19	C22:6n-3	4.06	2.49	3.55
		7.34	6.87	8.52

Discussion. Biochemical profiles of wide range aquaculture species have been reported to be dependent on the composition of feedings (Ahmed et al 2022; Supono et al 2022; Supono et al 2023). The proximate compositions are varied within and among species depending on several factors. For example, among different species of fish, their proximate compositions are influenced by geographical areas, seasons and feeding behaviours (Di Lena et al 2016; Shija et al 2019; Sharma et al 2020). Meanwhile, proximate compositions within the same species of fish are influenced by factors such as age, reproductive status, local environmental condition, maturation (Ahmed et al 2022). In TGGP hybrid grouper, biochemical composition of larvae was reported to be varied during early larval development where lipid content decreased gradually while protein content remained stable (Gao et al 2023).

In the present study, the biochemical composition of soft organic tissue from fish following different treatments has been dominated by protein followed by lipid content. In general, protein has been reported to be the most dominant component from soft organic tissue of wide range species from fresh water, brackish and marine fish (Venugopal & Shahidi 1996; Ahmed et al 2022). Total protein content of TGGP larvae in the present study (ranging from 519 to 600 mg g dry weigth^{-1} or 51 to 60% from a total of 1 g dry weight of organic tissue) was not significantly different following different treatments of live feed enrichment and micropellet. This could be due to all the pellets used for feeding in three feeding treatments had high proportion of protein (> 50%). It has been reported high that dietary protein of ~50% are required to support optimum growth of marine fish larvae including hybrid groupers (Rønnestad et al 2003; Yong et al 2019; Yan et al 2021). At larval stages, the growth of fish occurred rapidly and therefore they required high input of protein in a form of amino acids to accumulate muscle biomass deposition. It has been reported that amino acids is primary component for early larval structural growth and development (Rønnestad et al 2003).

Similar to protein content, lipid content of fish was not significantly different among feeding treatments. The lipid composition of early stages of fish larvae has been reported to rely on the yolk sac and it decreased gradually until they are at mouth opening stage (Gao et al 2023). Therefore, live feed enrichment aimed to provide sufficient lipid, particularly essential fatty acids, at early larval stages (Watanabe et al 2016; Joshua et al 2022). Fish fed with enriched live feed in this study demonstrated a higher proportion of mono-unsaturated fatty acids compared to fish fed with unenriched live feed. Following the opening mouth stages, a success effort to replace totally live feed with pellet for early larval feeding has not been reported in marine fish (Cahu & Infante 2001). In contrast, partial replacement of live feed with pellet has been reported to result in better growth and survival in some species of marine fish, including grouper fish compared to single feeding either live feed or pellet only (Wang et al 2009). The co-feeding (partial replacement of live feed with formulated compound diets) resulted in a similar range of lipid deposition in soft tissue of fish (22-24%). It appears that lipid proportion in three different pellets was within the optimum requirement for hybrid grouper larval growth and lipid deposition in tissues. It has been reported that lipid content of 16.5% in feeds resulted in optimum growth in hybrid grouper larvae and lipid retention in organic soft tissue of fish increased with lipid proportion in diets within a range of 8 to 20% (Yong et al 2019).

Conclusions. The findings of this study suggests that the application of live enrichment and early introduction of micropellet with different proximate composition did not show improvement on protein and lipid deposition on soft tissue of TGGG hybrid grouper larvae. There was an indication that the proportion of some essential fatty acids, particularly monosaturated fatty acids, was higher in fish fed with enriched live feed. Further studies on energy reserves deposition and utilisation, particularly in response to stress, need to be investigated.

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Conflict of interest. The authors declare that there is no conflict of interest.

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