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Is royal jelly a sustainable alternative lipid source in aquaculture? Influence of dietary royal jelly levels on fatty acid composition in zebrafish

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ABSTRACT. Royal jelly (RJ) is a bee-derived product widely used as a dietary supplement due to its high potential health benefits. The present study was designed to investigate the effect of feeding RJ on zebrafish fatty acid composition. Zebrafish were fed five distinct diets (D1, D2, D3, D4, and D5) for 56 days supplemented with 0.0, 0.1, 0.4, 1.6, and 6.4% RJ, respectively. Gas chromatography-mass spectrometry and fatty acid methyl ester (FAME) analyses were used to determine FA content in the whole body of zebrafish. The results showed that a feeding regimen that included incrementing RJ doses resulted in statistically significant increases in 16:0, 18:2n-6, and the ratio of eicosapentaenoic acid (EPA) to docosahexaenoic acid (DHA) ($P < 0.05$). In particular, the content of EPA/DHA increased up to 1.3-fold compared to control group D1. Conversely, increasing RJ levels led to significant decreases in DHA, HUFA, and n-3/n-6 PUFA levels in relation to group D1 ($P < 0.05$). In conclusion, considering the EPA/DHA ratio among the analysed fatty acids, the diet with a 1.6% RJ addition was the optimal choice. Therefore, RJ can be recommended as a sustainable alternative lipid source in aquaculture.

Introduction

Fatty acids (FA) are essential nutrients that fish require for their health and survival. While fish oil has traditionally been the primary source of omega-3 fatty acids in fish diets, current priorities in the aquaculture nutrition sector focused on environmental friendliness, and sustainability, there is a need for alternative lipid sources to fish oil (FO) that meet the correct nutritional requirements (FAO, 2022). In this context, many studies over the last two decades have focused on replacing FO with several alternative lipid ingredients such as animal fats, vegetable oils, transgenic oils or algal oils (Bonvini et al., 2018; Zarantoniello et al., 2020; Naylor et al., 2021).

Essential fatty acids are an important parameter of fish quality, but their composition varies depending on a range of factors such as diet, age, season, and genetics (Meyer et al., 2019). Among these factors, diets are particularly important because they are the primary means of ensuring that fish contain high levels of these essential fatty acids. Therefore, differences in the FA composition of fish feeds can have a direct impact on aquaculture (Tocher et al., 2019).

FA and other lipids are widely recognized as essential nutrients due to their roles as energy sources, chemical messengers (including pheromones), hormones, and membrane components. Fish are a rich source of long-chain omega-3 polyunsaturated fatty acids (n-3 PUFA), such as 20:5n-3 (eicosapentaenoic acid, EPA) and 22:6n-3 (docosahexaenoic acid, DHA). However, they cannot synthesize the essential PUFA, 18:2n-6 (linoleic acid, LA) and 18:3n-3 (α-linolenic acid, ALA), which are required for the synthesis of longer-chain PUFA. Instead, fish obtain these essential PUFA through their diet (Tocher et al., 2019; Monroig et al., 2022).

Honeybee (*Apis mellifera* L.) food products, including honey, propolis, bee pollen, and royal jelly (RJ) are increasingly used as alternative ingredients in dietary supplements. Among these products, RJ is a promising candidate due to its unique functional properties in controlling epigenetic modifications in honeybee caste determination. RJ is a gelatinous, white-yellowish, acidic natural secretion produced by young bees in their hypopharyngeal and mandibular glands (Ali and Kunugi, 2020). Fresh RJ is primarily composed of water (60–70%), protein (9–18%), carbohydrates (7–18%), lipids (3–8%), vitamins, and free amino acids. The lipid and fatty acid composition of RJ is a defining trait, consisting mainly of short chain dicarboxylic and hydroxyl fatty acids (80–90%), such as 10-hydroxy-trans-2-desenoic acid (10-HDA), which are essential for production and found only in RJ (Kamakura, 2011). The concentration of 10-HDA is widely recognized as the most reliable indicator of RJ (Sabatini et al., 2009)

The zebrafish (*Danio rerio*) is increasingly used as a vertebrate model organism for lipid metabolism, lipoprotein transport and nutrigenomics research due to its high similarity to humans in this system functioning and processing of dietary fat (Williams and Watts, 2019). Recent research has shown that zebrafish with diet-induced obesity exhibit molecular and physiological changes similar to those found in mammals, making the zebrafish a valuable model for studying the mechanisms underlying human obesity (Oka et al., 2010). To our knowledge, no research has been conducted on the use of RJ as an added lipid source in zebrafish feed. Therefore, the objective of this study was to examine how the properties of RJ influence the fatty acid profile in zebrafish. To this end, zebrafish were fed five experimental diets containing increasing levels of RJ as an added lipid source during a 56-day feeding trial.

Material and methods

Materials and experimental design

Ethical approval for this study was obtained from the Atatürk University Ethics Committee under number 36643897–82/65. The experiment followed the guidelines of the Applied Research Ethics National Association. Zebrafish (*Danio rerio*) were weighed (initial weight – 350 ± 15.0 mg) and then randomly divided into 25 fiberglass aquariums (25 × 30 × 18.5 cm, 10 l, 10 fish per tank) in the Model Organism Unit. Zebrafish for the experiment were obtained from a local vet clinic (Erzurum, Turkey). During the 56-day trial, the optimal pH, temperature, and oxygen saturation levels required for fish were provided and maintained. Measurements were taken hourly using a Smart Oxy Oximeter Probe.

Fish diet production

The formulations of five different experimental diets have been described in detail by Aksakal et al. (2021). Briefly, experimental diets were formulated based on casein and gelatine and RJ was added. Zebrafish were fed in five different experimental groups with an eight-week trial period as follows: group D1 – basal diet as a control without RJ supplementation, and experimental groups D2–5 supplemented with 0.1, 0.4, 1.6 and 6.4% RJ, respectively, by replacing the corresponding amount of wheat meal in the basal diet (Table 1). All fish were starved for 24 h at the end of the 8-week feeding trial. The fish in each aquarium were then exposed to an ice slurry for hypothermic stunning (1:1) for 10 min. A total of three fish were randomly sampled from each tank to analyse FA levels. The lipid profiles of fish from each tank were analysed by freezing the whole fish in liquid nitrogen and storing them at −80 °C.

Lipid and fatty acid composition

Total lipid extraction was carried out using the method of Folch et al. (1957). Whole-body homogenization of zebrafish was carried out (approximately 1 g) in 20 ml of a chloroform/methanol (2:1, v/v) mixture using an Ultra-Turrax device with 0.01% (w/v) boron trifluoride in methanol (BHT). The solution was filtered under a vacuum using Whatman No. 1 filter paper. After filtration, the samples were transferred to clean and dry tubes and 2% of each solution (sample) of magnesium chloride hexahydrate (4 ml) was added.

Tubes were filled with nitrogen and vortexed for 1 min, and subsequently incubated for 24 h for

Table 1. Formulation and approximate composition of five experimental zebrafish diets (g/0.1 kg) (Aksakal et.al., 2021)

Ingredients	Diets				
	D1	D2	D3	D4	D5
Casein (vitamin-free)	40.00	40.00	40.00	40.00	40.00
Gelatin	8.00	8.00	8.00	8.00	8.00
L-Arginine	0.50	0.50	0.50	0.50	0.50
L-Methionine	0.40	0.40	0.40	0.40	0.40
L-Lysine	0.80	0.80	0.80	0.80	0.80
Fish meal	5.00	5.00	5.00	5.00	5.00
Dextrin	9.10	9.10	9.10	9.10	9.10
Wheat meal	14.98	14.88	14.58	13.38	8.58
Royal jelly	0.00	0.10	0.40	1.60	6.40
Cod liver oil	10.00	10.00	10.00	10.00	10.00
Soybean lecithin	4.00	4.00	4.00	4.00	4.00
Vitamin mix[1]	2.00	2.00	2.00	2.00	2.00
Mineral mix[2]	3.00	3.00	3.00	3.00	3.00
Carboximetilecellulose	2.00	2.00	2.00	2.00	2.00
Ascorbic acid[3]	0.06	0.06	0.06	0.06	0.06
Choline chloride	0.17	0.17	0.17	0.17	0.17
Proximate composition					
crude protein	45.68	45.69	45.74	45.92	46.65
crude lipid	14.25	14.25	14.27	14.34	14.63
ash	3.84	3.84	3.84	3.84	3.84
moisture, %	2.30	2.22	2.41	2.45	2.40

D1 – basal diet as a control without RJ supplementation, D2 – diet supplemented with 0.1% RJ, D3 – diet supplemented with 0.4% RJ, D4 – diet supplemented with 1.6% RJ, D5 – diet supplemented with 6.4% RJ; RJ – royal jelly; [1] Roche Performance Premix (Hoffman-La Roche, Inc., Nutley, NJ, USA) per gram: IU: vit. A 2645.50, vit. D_3 220.46, vit. E 44.09; mg: vit. B_{12} 13, riboflavin 13.23, niacin 61.73, d-pantothenic acid 22.05, menadione 1.32, folic acid 1.76, pyridoxine 4.42, thiamine 7.95, d-biotin 0.31; [2] Bernhart Tomarelli salt mixture (ICN Pharmaceuticals, Costa Mesa, CA, USA) g/0.1 kg: calcium carbonate 2.1, calcium phosphate dibasic 73.5, citric acid 0.227, cupric citrate 0.046, ferric citrate (16–17% Fe) 0.558, magnesium oxide 2.5, manganese citrate 0.835, potassium iodide 0.001, potassium phosphate dibasic 8.1, potassium oxide 6.8, sodium chloride 3.06, sodium phosphate 2.14, zinc citrate 0.133, 5 mg of Se in the form of sodium selenite was added per kilogram of the salt mixture; [3] Phosphitan C (Mg-L-ascorbyl-2-phospahte; Sigma-Aldrich, Munich, Germany)

phase formation. The resulting lower phase (containing lipids) was placed in an evaporator system and exposed to heat and nitrogen gas. For extraction of fatty acid methyl esters, 1.5 ml of 2 M NaOH was added to pure oils. Fatty acid methyl esters (FAMEs) were synthesized using the method described by Metcalfe and Schmitz (1961).

FAMEs were analysed using gas chromatography (Hewlett-Packard, 6890 N, Palo Alto, CA, USA) on a DB-23 capillary column (I.D. 60 ml × 0.25 mm, df 0.25 μm). The temperature program on the detector was initially set to 190 °C for 35 min. After that, it was increased to 220 °C at a rate of 30 °C per min, and it maintained there for 5 min. Hydrogen was supplied at a flow rate of 2 ml/min as the carrier gas. Individual fatty acids were identified by comparing the retention time of each fatty acid to that of a reference mixture of fatty acids (Supelco 37-component FAME mix, cat. no.: 47885-U; Sigma-Aldrich, Saint Louis, MO, USA). According to the peak area of the C19:0 internal standard (Sigma-Aldrich, Saint Louis, MO, USA), the quantity of various fatty acids was calculated and reported as a proportion of the total FAME.

Statistical analysis

First, basic statistical parameters such as mean and standard error of the mean were calculated. To reveal differences between treatments, a one-way analysis of variance (ANOVA) and Tukey's multiple range test were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). To obtain more specific data, Pearson's correlation was performed for all data to determine linear relationships between parameters and the correlograms were generated for the obtained data using the "corrplot" package (Wei and Simko, 2021) in the R environment (R Foundation for Statistical Computing, Vienna, Austria). Two-dimensional hierarchical cluster analysis based on Ward's method was conducted using JMP statistical software (version 13.0; SAS Institute Inc., Cary, NC, USA) to classify the diet groups based on the differences for each fatty acid.

Results

Fatty acid profile

The whole-body FA composition of zebrafish fed with varying rates of RJ (0.0, 0.1, 0.4, 1.6 and 6.4%) was summarized. Twenty-nine fatty acid methyl esters (FAME) were extracted from zebrafish and detected using gas chromatography-flame ionization detector-mass spectrometry GC-FID/MS (Table 2). The total FA percentage ranged from 32.9 to 38.1% for monounsaturated fatty acids (MUFA), SFA 27.7–31.2%, and PUFA 15.9–17.1% across all experimental groups.

When the RJ groups were compared with D1 (control group), statistically significant differences were found in the levels of total saturated fatty acids (SFA) in total lipids ($P < 0.05$). Palmitic acid (16:0, PA) was the most dominant SFA among the 29 identified FA. The highest PA level of 19.81 ± 0.16% was determined in fish fed diet D3, while the lowest level was in group D1 – 18.81 ± 0.49% ($P < 0.05$). Oleic acid (18:1n-9, OA) represented the majority of MUFA in all experimental groups. Fish fed diet D2 had the highest level of OA at 36.87 ± 0.08%, while group D4 had the lowest percentage of this FA at 31.90 ± 0.50% ($P < 0.05$).

Table 2. Effects of dietary royal jelly on whole body fatty acid composition of zebrafish (% of total identified fatty acids)

Fatty acid	Diets				
	D1	D2	D3	D4	D5
14:0	2.65 ± 0.09^{c}	2.43 ± 0.08^{c}	4.59 ± 0.44^{a}	3.82 ± 0.09^{b}	2.25 ± 0.27^{c}
16:0	18.81 ± 0.49^{b}	19.39 ± 0.26^{a}	19.81 ± 0.16^{a}	19.54 ± 0.09^{a}	19.74 ± 0.26^{a}
17:0	0.39 ± 0.01^{c}	0.36 ± 0.00^{d}	0.40 ± 0.00^{b}	0.42 ± 0.00^{a}	0.40 ± 0.00^{b}
18:0	4.15 ± 0.09^{c}	4.87 ± 0.05^{b}	3.84 ± 0.02^{d}	5.59 ± 0.12^{a}	5.52 ± 0.09^{a}
20:0	0.45 ± 0.15^{cd}	0.55 ± 0.00^{bc}	0.36 ± 0.11^{d}	0.64 ± 0.00^{ab}	0.66 ± 0.01^{a}
22:0	1.25 ± 0.09	1.05 ± 0.03	1.38 ± 0.02	1.19 ± 0.05	1.20 ± 0.05
SFA	27.71 ± 0.74^{d}	28.65 ± 0.20^{c}	30.38 ± 0.75^{a}	31.20 ± 0.06^{a}	29.77 ± 0.37^{b}
14:1	0.10 ± 0.04^{b}	0.06 ± 0.01^{b}	0.34 ± 0.03^{a}	0.07 ± 0.02^{b}	0.06 ± 0.06^{b}
16:1n-7	0.07 ± 0.01	0.11 ± 0.00	0.08 ± 0.01	0.12 ± 0.04	0.12 ± 0.16
17:1	0.74 ± 0.17^{a}	0.59 ± 0.13^{ab}	0.73 ± 0.24^{a}	0.32 ± 0.11^{b}	0.66 ± 0.09^{a}
18:1n-9	35.32 ± 1.82^{a}	36.87 ± 0.08^{a}	32.10 ± 0.82^{b}	31.90 ± 0.50^{b}	33.39 ± 0.23^{b}
20:1n-11	11.65 ± 0.33^{b}	10.73 ± 0.03^{c}	11.82 ± 0.06^{b}	11.95 ± 0.13^{b}	12.73 ± 0.02^{a}
22:1n-11	0.20 ± 0.00	0.20 ± 0.00	0.19 ± 0.00	0.22 ± 0.00	0.21 ± 0.00
MUFA	36.89 ± 1.70^{a}	38.18 ± 0.24^{a}	33.65 ± 0.55^{bc}	32.96 ± 0.33^{c}	34.83 ± 0.43^{b}
18:2n-6	2.74 ± 0.08^{b}	2.86 ± 0.03^{b}	3.27 ± 0.02^{a}	3.27 ± 0.03^{a}	3.43 ± 0.06^{a}
18:3n-6	0.21 ± 0.01^{bc}	0.18 ± 0.00^{c}	0.47 ± 0.03^{a}	0.18 ± 0.00^{c}	0.23 ± 0.01^{b}
20:2n-6	2.94 ± 0.09^{a}	2.66 ± 0.06^{b}	2.89 ± 0.24^{a}	2.98 ± 0.03^{a}	2.98 ± 0.02^{a}
20:3n-6	0.52 ± 0.01^{a}	0.51 ± 0.00^{b}	0.53 ± 0.00^{a}	0.51 ± 0.00^{b}	0.52 ± 0.01^{ab}
n-6 PUFA	6.42 ± 1.19	6.21 ± 0.09	7.16 ± 0.26	6.94 ± 0.00	7.16 ± 0.04
18:3n-3	2.25 ± 0.08^{c}	2.15 ± 0.02^{d}	2.01 ± 0.00^{e}	2.46 ± 0.05^{b}	2.60 ± 0.03^{a}
18:4n-3	0.46 ± 0.11^{a}	0.34 ± 0.01^{b}	0.21 ± 0.00^{c}	0.33 ± 0.00^{b}	0.37 ± 0.02^{b}
20:3n-3	0.81 ± 0.02^{c}	0.75 ± 0.00^{d}	0.77 ± 0.00^{d}	0.85 ± 0.01^{b}	0.88 ± 0.01^{a}
20:5n-3 (EPA)	4.08 ± 0.11^{b}	3.79 ± 0.02^{d}	3.91 ± 0.01^{c}	4.32 ± 0.01^{a}	3.97 ± 0.04^{c}
22:5n-3	1.48 ± 0.03^{b}	1.26 ± 0.01^{c}	1.73 ± 0.01^{a}	1.45 ± 0.01^{b}	1.48 ± 0.01^{b}
22:6n-3 (DHA)	8.72 ± 0.20^{a}	8.28 ± 0.05^{b}	8.57 ± 0.01^{a}	7.87 ± 0.09^{c}	6.64 ± 0.10^{d}
n-3 PUFA	17.79 ± 0.34^{a}	16.56 ± 0.08^{c}	17.21 ± 0.00^{b}	17.28 ± 0.14^{b}	15.94 ± 0.01^{d}
n-3/n-6 PUFA	2.77 ± 0.03^{a}	2.67 ± 0.05^{b}	2.41 ± 0.09^{c}	2.49 ± 0.02^{c}	2.23 ± 0.01^{d}
HUFA	18.55 ± 0.46^{a}	17.24 ± 0.01^{c}	18.41 ± 0.24^{ab}	17.98 ± 0.12^{b}	16.47 ± 0.06^{d}
EPA/DHA	0.47 ± 0.02^{c}	0.46 ± 0.00^{c}	0.46 ± 0.00^{c}	0.55 ± 0.01^{b}	0.60 ± 0.01^{a}

D1 – basal diet as a control without RJ supplementation, D2 – diet supplemented with 0.1% RJ, D3 – diet supplemented with 0.4% RJ, D4 – diet supplemented with 1.6% RJ, D5 – diet supplemented with 6.4% RJ; RJ – royal jelly, 14:0 – myristic acid, 16:0 – palmitic acid, 17:0 – heptadecanoic acid, 18:0 – stearic acid, 20:0 – arachidic acid, 22:0 – behenic acid, SFA – saturated fatty acids, 14:1 – myristolenic acid, 16:1n-7 – palmitoleic acid, 18:1n-9 – oleic acid, 20:1n-11 – gondoic acid, 22:1n-11 – cetoleic acid, MUFA – monounsaturated fatty acids, 18:2n-6 – linoleic acid, 18:3n-6 – gamma linolenic acid, 20:2n-6 – eicosadienoic acid, 20:3n-6 – dihomo-γ- linoleic acid, n-6 PUFA – omega-6 polyunsaturated fatty acids, 18:3n-3 – linolenic acid, 18:4n-3 – stearidonic acid, 20:3n-3 – eicosatrienoic acid, 20:5n-3 – eicosapentaenoic acid, 22:5n-3 – docosapentaenoic acid, 22:6n-3 – docosahexaenoic acid, n-3 PUFA – omega-3 polyunsaturated fatty acids, HUFA – highly unsaturated fatty acids; data are presented as mean value ± SEM (standard error of the mean); $^{a–d}$ – means within a row with different superscripts are significantly different at $P < 0.05$

The highest levels of n-3 PUFA were observed in fish fed the control diet (17.79 ± 0.34%), whereas the lowest levels were observed in fish fed diet D5 (15.94 ± 0.01%), which included the most RJ ($P < 0.05$). DHA was determined to be the most common n-3 PUFA. We also showed that diet RJ resulted in higher EPA/DHA ratios in total zebrafish lipids. In terms of EPA, group D4 contained the greatest proportion of this acid (4.32 ± 0.01%), whereas group D2 had the lowest level (3.79 ± 0.01). HUFA levels found in the dietary groups varied from 16.47% in group D5 to 18.55% in D1 ($P < 0.05$). The highest and lowest EPA/DHA ratio were 0.60 ± 0.02% in group D5 and 0.47 ± 0.00% in group D1 ($P < 0.05$). With an increase in the amount of RJ in the diet, the EPA/DHA ratio improved significantly ($P < 0.05$).

Consequently, the analysis of FA composition in the whole-body lipids of the fish showed that a dietary regimen with increased RJ supplementation resulted in statistically significant increases in 16:0, 18:2n-6, and EPA /DHA ratio ($P < 0.05$). In particular, the content of EPA and DHA increased up to ~1.3-fold compared to the control group. On the other hand, with increasing RJ supplementation, there was a significant decrease in DHA, HUFA, and n-3/n-6 PUFA levels compared to the D1 group ($P < 0.05$).

Relationships between fatty acid profiles and diet groups

A Pearson correlation analysis (PCA) was carried out to verify the observed distribution of the detected fatty acids. The correlation coefficient values ranged between −1 and 1, with a stronger correlation observed when the absolute value of the correlation coefficient was closer to 1, and a weaker correlation observed when it was closer to 0. DHA was positively correlated with n-3/n-6 PUFA and highly negatively correlated to 18:3n-3, 18:0, 20:0, 20:3n-3, 18:2n-6, 20:1n-11, and EPA/DHA. MUFA were positively correlated with 18:1n-9 and negatively highly correlated with 17:0, SFA, and 18:2n-6. EPA/DHA showed a positive correlation with 20:1n11, 18:2n-6, 20:3n-3, and 18:3n-3. The correlation between n-3/n-6 PUFA and 18:2n-6, 16:0, 20:1n-11, SFA, and EPA/DHA was highly negative.

Positive correlations were recorded between 22:1n-11 and 18:3n-3, 18:0, 20:0, EPA/DHA, 20:3n-3, and EPA. HUFA exhibited a significant positive correlation with n-3 PUFA and DHA, and were negatively correlated with EPA/DHA, 18:0, and 20:0. (Figure 1 and 2). The results of the correlation analysis significantly differed for individual fatty acids ($P < 0.01$).

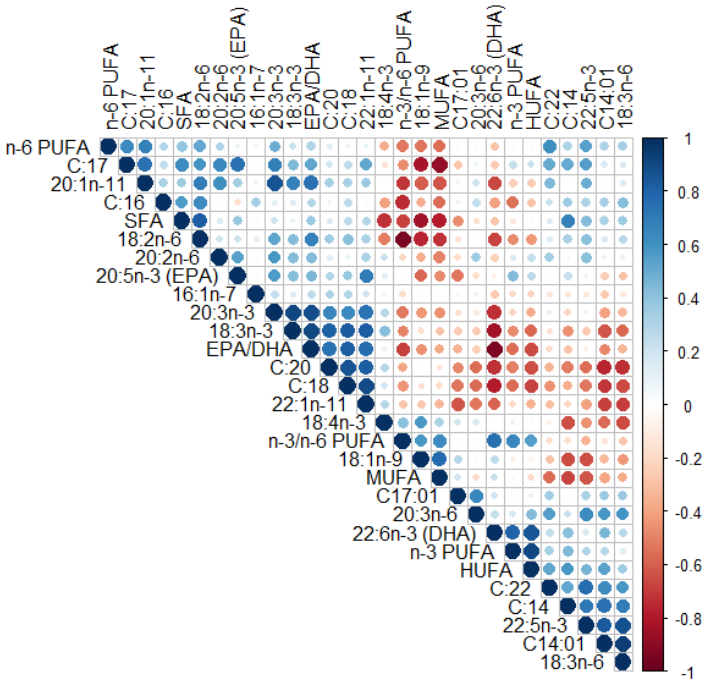

Figure 1. Pearson's correlation coefficients between fatty acids identified in the whole body of zebrafish ($P < 0.05$)

14:0 – myristic acid, 16:0 – palmitic acid, 17:0 – heptadecanoic acid, 18:0 – stearic acid, 20:0 – arachidic acid, 22:0 – behenic acid, SFA – saturated fatty acids, 14:1 – myristolenic acid, 16:1n-7 – palmitoleic acid, 18:1n-9 – oleic acid, 20:1n-11 – gondoic acid, 22:1n-11 – cetoleic acid, MUFA – monounsaturated fatty acids, 18:2n-6 – linoleic acid, 18:3n-6 – gamma linolenic acid, 20:2n-6 – eicosadienoic acid, 20:3n-6 – dihomo-ɣ- linoleic acid, n-6 PUFA – omega-6 polyunsaturated fatty acids, 18:3n-3 – linolenic acid, 18:4n-3 – stearidonic acid, 20:3n-3 – eicosatrienoic acid, 20:5n-3 – eicosapentaenoic acid, 22:5n-3 – docosapentaenoic acid, 22:6n-3 – docosahexaenoic acid, n-3PUFA – omega-3 polyunsaturated fatty acids, HUFA – highly unsaturated fatty acids

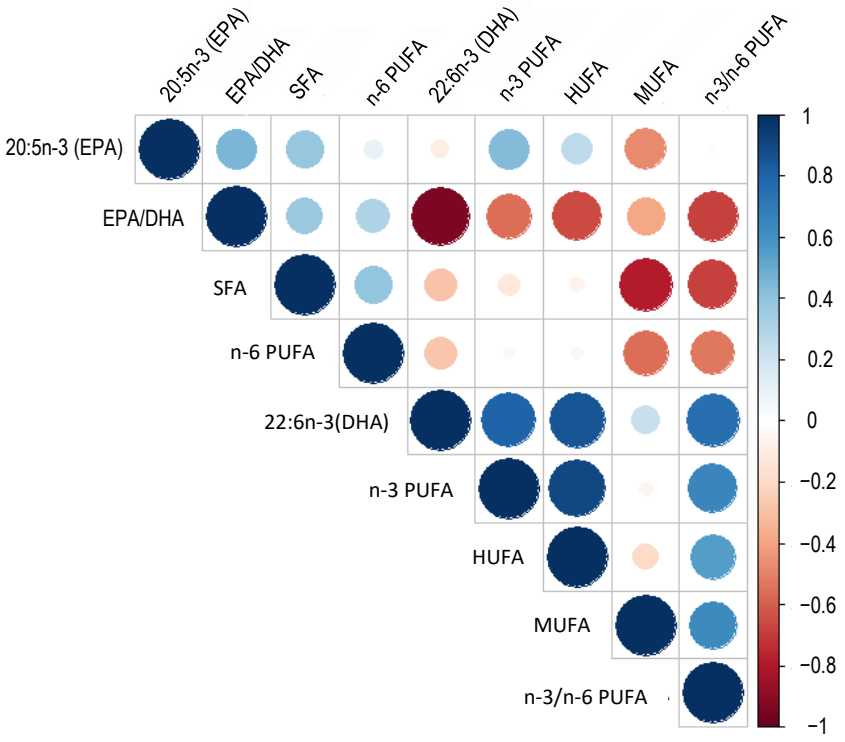

Figure 2. Pearson's correlation coefficients between the main fatty acids identified in the whole body of zebrafish ($P < 0.05$)

SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, n-6 PUFA – omega-6 polyunsaturated fatty acids, n-3PUFA – omega-3 polyunsaturated fatty acids, HUFA – highly unsaturated fatty acids, EPA – eicosapentaenoic acid, DHA – docosapentaenoic acid

At the end of the experiment, hierarchical cluster analysis (HCA) was used to compare the 29 FAs found in the whole body of each group of zebrafish. The results were shown as a dendrogram. All dietary groups were divided into two main clusters: black and red. Zebrafish fed with higher amounts of RJ (D4 and D5) were classified under the red group. The black clusters were further divided into two subclusters: green and blue. The green branch represented only group D3, while the control group and group D2 with the lowest RJ supplementation were clearly separated as blue branches (Figure 3).

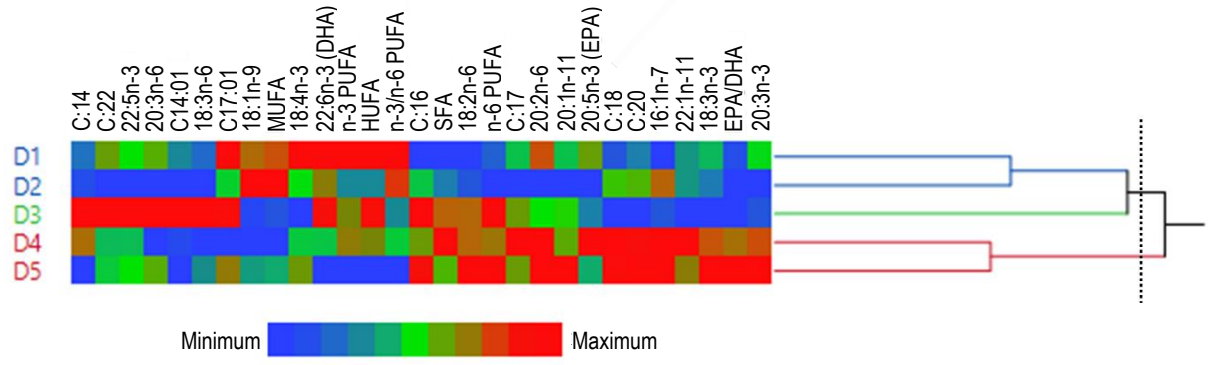

Figure 3. Hierarchical cluster analysis dendrogram of different diets fed to zebrafish, with varying contents of fatty acids

D1 – basal diet as a control without RJ supplementation, D2 – diet supplemented with 0.1% RJ, D3 – diet supplemented with 0.4% RJ, D4 – diet supplemented with 1.6% RJ, D5 – diet supplemented with 6.4% RJ; RJ – royal jelly, 14:0 – myristic acid, 16:0 – palmitic acid, 17:0 – heptadecanoic acid, 18:0 – stearic acid, 20:0 – arachidic acid, 22:0 – behenic acid, SFA – saturated fatty acids, 14:1 – myristolenic acid, 16:1n-7 – palmitoleic acid, 18:1n-9 – oleic acid, 20:1n-11 – gondoic acid, 22:1n-11 – cetoleic acid, MUFA – monounsaturated fatty acids, 18:2n-6 – linoleic acid, 18:3n-6 – gamma linolenic acid, 20:2n-6 – eicosadienoic.acid, 20:3n-6 – dihomo-γ- linoleic acid, n-6 PUFA – omega-6 polyunsaturated fatty acids, 18:3n-3 – linolenic acid, 18:4n-3 – stearidonic acid, EPA – eicosatrienoic acid, DHA – eicosapentaenoic acid, 22:5n-3 – docosapentaenoic acid, 22:6n-3 – docosahexaenoic acid, n-3 PUFA – omega-3 polyunsaturated fatty acids, HUFA – highly unsaturated fatty acids

Discussion

Recent studies have shown that supplementing zebrafish diets with royal jelly can improve growth performance and positively affect the mRNA expression levels of growth hormones (GH-1 and IGF-1) and immune function-related factor TGF-β (Vural et al., 2021). Moreover, it has been found that there is a relationship between nutrition and health of humans, fish, rats, and livestock, and it depends on the optimal feed composition and its utilization (Abdelnour et al., 2020; Aksakal et al., 2021; Aslan et al., 2022). The current investigation is a follow-up of two previous studies by Aksakal et al. (2021) and Vural et al. (2021), which evaluated the relative effectiveness of feeding zebrafish diets with varying RJ supplementation on whole-body FA composition. According to our knowledge, this is the first study to analyse the fatty acid composition of fish fed a diet enriched with RJ. Our findings show that the FA profile of zebrafish fed RJ-supplemented diets had higher MUFA, SFA, and lower PUFA contents. These findings were consistent with the fatty acid profiles of zebrafish fed diets enriched with black soldier fly (Chemello et al., 2022). It is well-established that SFA and MUFA constitute most of the metabolised substrates for *β*-oxidation, metabolic energy production, and especially *de novo* PUFA biosynthesis in fish (Xu et al., 2020; Monroig et al., 2022). In the present study, MUFA (particularly 18:1n-9) showed a negative correlation pattern with SFA (particularly 16:0), with an increased accumulation of MUFA in the whole body relative to dietary intake. This increased concentration relative to the control group suggested that beta-oxidation contributed to the higher SFA concentration in the experimental diets (Mata-Sotres et al., 2021).

Zebrafish fed the RJ-based diets used in this study showed lower n-3 PUFA content than the control group, and different levels of RJ supplementation did not affect the FA profile of zebrafish. It is possible that PUFA are utilised for energy and its excess is stored mainly as MUFA and SFA (Xu et al., 2018). The high MUFA levels in zebrafish may limit the protective effect of LC-PUFA. It is possible that the reduced level of the whole-body fatty acids in zebrafish is associated with a lower beta-oxidation induced by a high lipid diet (Wang et al., 2019). Furthermore, the diminished n-3 synthase activity observed in zebrafish with dietary RJ intake could be due to a mechanism specific to this freshwater fish (Tocher, 2010). It is also noteworthy that biosynthesis of LC-PUFA in brackish water or seawater fish is higher than in freshwater fish (Yu et al., 2021). Our study revealed unequal distributions of the n-3 to n-6 ratios and highly unsaturated fatty acid (HUFA) levels, with the control group exhibiting higher levels of both fatty acids. This observation can be explained by the higher levels of 10-HDA resulting from increased RJ content in the diet (Kamakura, 2011). Zhang et al. (2022) studied the effects of 10-HDA on the efficiency, antioxidant capacity, and resistance of broiler chickens and found that it had the potential to improve the growth performance of broiler chickens as a feed additive. Although the effects of royal jelly on fish were not tested in this study, the results suggest that the RJ-based diet might exert positive effects on animal well-being and productivity. In our study, the levels of LA and ALA increased in fish groups fed a higher RJ ration. Comparable results were observed in previous experiments in which zebrafish and rainbow trout (*Onchorhynchus mykiss*) were fed an insect-based diet (Ewald et al., 2020; Zarantoniello et al., 2020; Chemello et al., 2022).

Omega-3 fatty acid concentrations, especially DHA and EPA, are of great importance in fish farming. Our results demonstrated that RJ-enriched diets negatively affected DHA levels in fish, and 22 carbon fatty acids changed independently of dietary RJ concentration. Specifically, 22:6n-3 (DHA) is a poor substrate, as its incorporation in tissues for *β*-oxidation is less efficient than other fatty acids (Tocher et al., 2019). Consistent with earlier studies, the present study found that the retention of EPA and DHA fatty acids was reduced when high amounts of substitute ingredients were included in fish diets (Ewald et al., 2020). However, in our study, the group fed a diet containing 1.6% RJ (group D4) had the highest participation rate for EPA. We believe that this is due to zebrafish, the animal material used, being used as a model organism in research on lipid metabolism (Williams and Watts, 2019), as it is generally accepted that 8–20% of ALA (the precursor for EPA and DHA) is converted to EPA and 0.5–9% to DHA (Oliver et al., 2020). According to Góra et al. (2022), the differences in the percentage of n-3 PUFA content between small and medium sea bass was mostly attributable to changes in the ratios of 20:1n-11, DHA, and EPA. The HCA results of our study showed that fish fed a diet with the highest RJ level had the highest 20:1n-11 and EPA/DHA ratios. For this reason, we believe that feeding fish with royal jelly could be associated not only with zebrafish weight gain but also their length.

The strong correlations observed between fatty acids can aid in selecting the trait with the highest heritability. In our study, the HCA dendrogram showed that the D4 and D5 groups significantly different from the other groups. Previous studies in juvenile tongue sole (*Cynoglossus semilaevis*) and seabream (*Sparus aurata* L.) have demonstrated that weight gain, feed efficiency and growth performance resulting from diet increased EPA/DHA ratio. Consequently, fish fed a moderate EPA/DHA ratio showed good nutritional status (Xu et al., 2018), which is consistent with the results of our current investigation. The analysis of the fatty acid composition showed a clear correlation between the RJ level and EPA levels, with the correlation becoming stronger with an increased RJ supplementation. Furthermore, supplementation of RJ at 1.6 or 6.4% in zebrafish diet up-regulated the expression of growth hormone genes (GH and IGF-I) of muscle and liver tissue relative to the control group (Vural et al., 2021), indicating its potential in sustainable aquaculture.

Conclusions

In summary, our research demonstrated that a diet containing 1.6% royal jelly was optimal for zebrafish, as it maximized their capacity to produce essential fatty acids (EPA/DHA) in the whole body, and therefore, it can be recommended as a sustainable alternative lipid source in aquaculture. These findings have important implications for the aquaculture industry, highlighting the potential of royal jelly as a valuable feed ingredient for improving fish health and productivity. However, further studies are needed to investigate the effects of royal jelly on other fish species and to improve the overall fatty acid quality of royal jelly-based alternatives. Such research will help ensure the continued success of fish farming and promote sustainable aquaculture practices.

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Conflict of interests

The Authors declare that there is no conflict of interest.

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