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The effect of royal jelly dietary on growth performance and expression of genes related to growth and immunity of zebrafish, *Danio rerio*

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ABSTRACT

Royal jelly (RJ) is a natural product that is fed to bee queens during their entire life, and it contributes to their growth and development, fertility, and long lifespan. Although it is known that these unique effects of RJ, it has not been evaluated fed diet studies in fish until now. The study was carried out to determine the influence of RJ diets with different rates (0.0; 0.1; 0.4; 1.6 and 6.4 %) on expressions of growth (GH-I, IGF-I) and immune system (TGF-β) hormones of zebrafish (*Danio rerio*). Growth parameters was also studied and found to be higher in D4 group containing 1.6 % additives compared with other groups. RJ dietary supplementation showed significantly up-regulated in GH-1 gene expression in the liver tissues of fish fed D5 diet respect to the control group. Conversely, expression level was down-regulated in kidney tissues of group D4 compared with the control group ($p < 0.05$). IGF-1 gene expression noticeably increased in the muscle tissues of fish fed the D5 diet containing 6.4 % RJ compared to the control group. The greatest decrease was found in the muscle tissues of group D3 diet ($p < 0.05$). Although among the diet groups the highest amount of TGF-β expression was found in the muscle tissue of fish that were fed the D4 diet. It was seen that the mRNA level amounts obtained in all applications were below the level of the control group. These results indicate that the high-dose RJ included in the feed positively influenced the growth hormones of the zebrafish at transcriptomic level. To sum up, this study highlights the importance that 1.6 % or 6.4 % RJ included in the diet of zebrafish positively influenced their growth performance and growth hormone gene expression and may be considered a potential to be sustainable alternative for growth promoter in aquaculture practice.

1. Introduction

Zebrafish has commonly used in development and molecular genetics due to its easy and low-cost breeding, their capacity to produce numerous offspring, short generation time interval (12–14 weeks), its fully sequenced and high-quality genome sequence (Driever et al., 1994; Roush, 1996; Clark, 2003; Williams and Watts, 2019). In addition, it has recently been proposed as a possible model organism for nutrition and growth studies in fish (Dahm and Geisler, 2006; Johnston et al., 2008; Siccardi et al., 2009). Within the exisiting variability of *D. rerio* feding protocols, few studies have evaluated the effect of diet on adult *D. rerio* growth and survival. For instance, Jaya-Ram et al. (2008) showed that the level of dietary highly unsaturated fatty acid affected *D.rerio* egg production and hatchability. Meinelt et al. (1999) determined that growth and fertilization rates of adults *D. rerio* were affected by the dietary level of n-6-polyunsaturated fatty acids. Growth is an important trait because it is linked with the productivity and profitability of aquaculture production (De-Santis and Jerry, 2007). Phenotypic expression of its under genetic control, but also depends upon different environmental effects, mainly nutrition (Moriyama et al., 2000). Food sources in aquacultured fish became important due to the change of fish diets in the aquafeed sector (Hardy, 2010).

Age-dependent division of labor is one of the most typical features of honeybees (Robinson et al., 2005). In a colony of honeybee (*Apis mellifera* L.) queens live and reproduce 1–4 years, yet worker bees, which are derived from the same diploid genome, live only 3–6 weeks in temperate climates (Page and Peng, 2001; Remolina and Hughes, 2008). In addition, queen is fed throughout their lives with RJ, which is produced by the hypopharyngeal, postcerebral and mandibular glands of the worker honeybees. However, young worker honeybees are fed with

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this RJ during only larval stages. This situation causes the queen and worker bees developing from the same fertilized egg to possess different anatomic and physiological characteristics. For example, a queen does not possess a corbicula and a strong mandible to collect pollens while a worker bee is infertile. This unique function can be played an important role in caste determination of honeybess, and this hypotesis was supported by Zheng et al. (2011) that, the level of caste difference, and the amount and composition of the RJ produced can change depending on the size of the colony population, harvest season, method of feeding, geographical conditions where the bees are located, climate, nectar sources and bee genotype. RJ contains proteins, sugars, lipids, vitamins, and free amino acids, together with a variety of bioactive subtances, including peptides, acethylcoline and fatty acids such as 10 HDA. Several studies have reported that RJ has antioxidant activity against oxygen free radicals (Silici et al., 2009, 2011). But the mechanism by which RJ exerts its growing and differentiating effects on queen bee is largely unknown.

In nutrigenomic studies, GH, IGH-I and TGF are one of the most researched topics (Asaduzzaman et al., 2017; Hoseinifar et al., 2018; Ahmadifar et al., 2019a, 2019b; Zemheri-Navruz et al., 2020). In fish, GH is involved in almost all primary physiological processes in the body including the adjustment of ionic and osmotic balance, lipid, protein and carbohydrate metabolism, skeleton, soft tissue growth, reproduction and immune function. The insulin-like growth factor I (IGF-I) is a component which stimulates the growth, development and differentiation of fish and witches most expressed in the liver among fish tissues (Duan, 1998; Reinecke, 2010; Siharath et al., 1995). TGF-β is a multifunctional gene involved in the adjustment of various cellular activities. There are some studies determine the GH and IGF levels in various fish to which GH-treatment and GH-transgenic applications are made (Devlin et al., 2006; Overturf et al., 2010).

The current study aimed to determine the influence of RJ, which was included in zebrafish diets in different rates (0.0 %; 0.1 %; 0.4 %; 1.6 % and 6.4 %), on hepatosomatic index, specific growth rate, relative growth, feed conversion rate and survival rate as well as expression levels on some growth (GH-I, IGF-I) and immune system (TGF-β) hormones.

2. Materials and methods

2.1. Fish material and experimental design

A total of 250 zebrafish (1♂:1♀) which are two-month-old and weighing 0.35 ± 0.15 g were used in the study. The zebrafish used in the trial were supplied from a pet shop. Fish were placed into the 25 research tanks in the ARS-1500-RAS Model Organism Unit (Akuamaks Company, Ankara, TURKEY). Five experimental groups with 10 fish in each tank were designed for each group. During the trial, the fish were subjected to a photoperiod of 14 h of light and 10 h of darkness. Water temperature, pH, dissolved O_2 and oxygen saturation level were measured daily in the per minute by using a Smartoxy Oxymeter (Technos Company, Chioggia, ITALY). During the experiment period, temperature, dissolved O_2, oxygen saturation level and pH of the freshwater were measured 26 ± 1 °C, 9 ± 0.5 mg L^{-1}, 70–80 % and 7.3 ± 0.3 respectively.

The fish were first fed with basal diet (control group, 0% RJ) during acclimation period for one week After acclimation, five zebrafish trial groups were fed at 1%, 2%, 5%, 8% and 10 % of daily live weights to determine the survival rate. The most successful diet was observed with feed at 5% of daily live weights. Feedings were made for four times per day including at 09.00, 12.00, 15.00 and 18.00 h. Fish treatments were conducted according to the Applied Research Ethics National Association.

2.2. Diet preparation

In the trial, 5 different feeds were prepared based on casein and gelatine so as to contain RJ including Diet 1 (D1; control 0.00 %), Diet 2 (D2; 0.10 %), Diet 3 (D3; 0.40 %), Diet 4 (D4; 1.60 %) and Diet 5 (D5; 6.40 %). The RJ used in the study was supplied from the company Civan Arıcılık in the city of Bursa, Turkey. The materials used in the diets included gelatine (G2500-1KG), casein (C3400-1KG SIGMA), dextrin (MP-901520), L-arginine (MP-194626), L-methionine (MP-194707), L-lysine (MP-194696), choline chloride (MP-101386), carboxymethyl cellulose (CMC), mineral mixture (Bernhart Tomarelli, MP-902843) (Mp-Bio Company), and vitamin mixture (Roche Company) (Table 1).

All the feed additives except for royal jelly, choline chloride and liver oil were combined well into a homogenous mixture to produce the experimental diets. As ingredients, casein (40 %), gelatin (8%), L-arginine (0.5 %), L-methionine (0.4 %), L-lysine (0.8 %), fish meal (5%), dextrin (9.1 %), cod liver oil (10 %), soybean lecithin (4%) vitamin mixtures (2%), mineral mix (3%), α-cellulose (2%) and vitamin C ester (0.06 %) were used to formulate the feed. After stirring them in food mixer, the choline chloride, the RJ and liver oil were slowly solved in pure water and then added into the mixture. The experimantel diets were formulated by supplementing 0.1 %, 0.4 %, 1.6 % and 6.4 % RJ by substitution of the amount of wheat meal in the basal diet. All the feed additives were thoroughly mixed and pellet to achieve a uniform texture, through a meat chopper machine at 1–2 mm diameter. The diets were stored at −20 °C for 2 days. The pelletized feeds were dried in lyophilizer for 24 h.

Table 1
Composition of the five experimental diets fed to zebrafish (g 0.1 kg^{-1}).

	Diets				
Ingredients	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

[1] Roche Performance Premix (Hoffman-La Roche, Inc., Nutley, NJ) per gram: vitamin A, 2645.50 IU; vitamin D3, 220.46 IU; vitamin E, 44.09 IU; vitamin B12, 13 mg; riboflavin, 13.23 mg; niacin, 61.73 mg; D-pantothenic acid, 22.05 mg; menadione, 1.32 mg; folic acid, 1.76 mg; pyridoxine, 4.42 mg; thiamin, 7.95 mg; D-biotin, 0.31 mg.

[2] Bernhart Tomarelli salt mixture (ICN Pharmaceuticals, Costa Mesa, CA) g 0.1kg-1: 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. Five milligrams 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, Germany.

2.3. Determination of growth performance

During the trial period, the live weights of the fish on days 0, 15, 30, 45 and 56 were measured. The measurements made on days 15, 30 and 45 were made in order to increase the reliability of the rates of feeds daily given to the fish.

At the end of the experiment, survival rate (SR), weight gain (WG), specific growth rates (SGR), hepatosomatic index (HSI) and feed conversion ratio (FCR) were calculated according to the following formulas: SR= (final number of fish/initial number of fish)x100, WG=(final weight–initial weight)×100/initial weight, SGR=(ln final weight–ln initial weight)×100/duration of the experiment in days, HSI=(Liver weight of fish (g)/Live weight of fish (g))×100, and FCR = feed consumed/weight gain.

2.4. Fish tissue sampling

Following the 56-day feeding activities, the zebrafish were subjected to hypothermia (ice slurry (1:1) for hypothermia stunning (10 min)) and their tissues were sampled. In the beginning and the end of the trial, 5 zebrafish were randomly selected for use in total RNA isolation for gene expression analyses. Muscle, liver and kidney samples in RNA Later Stabilization Regent solution (Qiagen, cat. no. 76106) were kept at −80 °C until use.

2.5. RNA isolation and cDNA synthesis

Total RNA was isolated from 50 mg frozen muscle, liver and kidney tissues with a RNeasy Lipid Tissue Mini Kit (Qiagen, cat. no. 74804) using the Qiacube robot (Qiagen, Hilden, Germany). RNA was treated with DNase I in order to avoid genomic contamination. RNA concentrations and quality were verified by means of nanodrop spectrophotometer (μDrop™ Plate, Thermo Scientific, cat. no. 12391) and RNA gel electrophoresis, respectively. Following isolation, cDNA synthesis was performed using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis Kit (Invitrogen, cat. no. 11146-016) according to the manufacturer's protocol. All cDNA was stored at −20 °C until use.

2.6. TaqMan probe and primer design

Primers and TaqMan probes were designed in Primer3 software (www.bioinfo.ut.ee/primer3) using zebrafish GH-I, IGF-I and TGF-β BLASTed (www.blast.ncbi.nlm.nih.gov) to ensure correct mRNA sequences. β-actin gene was also used as a house keeping gene to normalize the expression of the target genes. In order to perform Real Time PCR, TaqMan probe of the target and reference gene were conjugated with FAM/TAMRA and CY5/BQ2 respectively. Primer and prob sequences, amplification length and accession numbers are given in Table 2

2.7. Quantitative real time PCR

Quantification of gene expression by real-time PCR analysis was performed using a thermal cycler RotorQ-Gene (Qiagen, Germany). The real-time PCR was carried out in a reaction volume of 50 μl containing cDNA, 900 nM of both target and reference forward and reverse primers, 250 nM both target and reference TaqMan probes, and 25 μl FastStart TaqMan Probe Master (Applied Biosystems) which consists of AmpliTaq Gold DNA Polymerase, AmpErase uracil N-glycosylase (UNG), dNTP with dUTP, and optimized buffer component. The amplification and detection of the samples and the standards were performed using the following thermal cycling conditions: 50 °C for 2 min for activation of optical AmpErase UNG enzyme, 95 °C for 10 min as hot start to activate AmpliTaq Gold DNA polymerase followed by 45 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Real-Time PCR data were analysed using the efficiency $(e)^{(-\Delta Ct)}$ method, which is used to determine mRNA levels in gene expression against control group and housekeeping genes (β-Actin). Analytical sensitivity was confirmed by running standard curves. Amplification efficiency (e) was calculated based on the slopes of the curves (slope) using the formula $e = 10^{(-1/slope)}$ (Pfaffl, 2001), and the slope value via Rotor-Gene software.

2.8. Statistical analysis

Results were expressed as mean ± SD (n = 5). Each experimental unit (aquarium) was considered a replicate. Data were subjected to one-way analysis of variance (ANOVA) to test the effect of dietary lipid sources on expression level and subsequent comparison of means by Duncan's multiple range tests. Differences were considered statistically significant at $p < 0.05$.

3. Results

The average live weight values belonging to each period and analysis results are given in Table 3. A statistically significant difference was found between the average live weight values of the trial group fish and the diet groups during the trial period ($p < 0.05$). In the end of the trial, the highest growth rate was found in the D4 diet group and the lowest individual relative growth with 0.890 ± 0.07 g was determined in the D3 group ($p < 0.05$). The percentages of the relative growth in the feed groups as calculated in the study are given in Table 3. It was found that, except for the D3 group, there was no statistically significant difference among the groups D1, D2, D4 and D5 ($p > 0.05$). The highest value was found in the D2 feed group with 237.04 ± 11.85 and the lowest growth rate was found in the D3 with 182.04 ± 8.80. The specific growth rate and percentage values of the zebrafish in all the groups were similar as shown in Table 3. In the end of the research, the highest value was found in the group which was fed the SGR D4 diet with 1.63 ± 0.04 % while the lowest value was found in the D3 with 1.07 ± 0.09 %. In terms of SGR

Table 2
Gene spesific primer and prob sequence, amplification length (bp), GenBank accession number of zebrafish.

Genes	Primer/ Prob	Sequence (5′-3′)	Amplification length (bp)	Gen Bank Accession Number
GH	Forward	GCATCAGCGTGCTCATCAAG	108	AJ937858.1
	Reverse	TGGTCTCCCCTACGGTCAG		
	Prob	$^{FAM-}$TGGATGACAACGACTCCCTGCCGTT^{-TAMRA}		
IGF-I	Forward	AGTACCCACACCCTCTCACT	132	NM_131825.2
	Reverse	AAAGCCCCTGTCTCCACAC		
	Prob	$^{FAM-}$ GCGTTGACTCCCGCGACTCTGGA $^{-TAMRA}$		
TGF-β	Forward	GACCACCCATTCCTTGCTGA	144	NM_001037683.2
	Reverse	GCCCTTCCCAATGCTCTCTT		
	Prob	$^{FAM-}$ AGTGCAACGGACCATCGCCAGGA $^{-TAMRA}$		
β-Actin	Forward	CCTCTCTTGCTCCTTCCACC	150	AF057040.1
	Reverse	TACTCCTGCTTGCTGATCCAC		
	Prob	$^{CY5-}$ GGCCTCCCTGTCCACCTTCCAGC-^{BQ2}		

Table 3
Growth performance and survival of zebrafish fed diets with different level royal jelly for 8 weeks. (means ± SEM; n = 5). The data are expressed as means ± SEM (n = 5). a, b, c, d: statistical signifigance between each values in the lines. $p < 0.05$ was considered as significant.

	D1	D2	D3	D4	D5
Initial weight (g)	0.479 ± 0.07	0.515 ± 0.04	0.489 ± 0.05	0.533 ± 0.02	0.493 ± 0.01
Final weight (g)	1.103 ± 0.05^{c}	1.216 ± 0.03^{bc}	0.890 ± 0.07^{d}	1.278 ± 0.05^{a}	1.168 ± 0.01^{bc}
Weight gain (%)	230.14 ± 6.90^{a}	237.04 ± 11.85^{a}	182.04 ± 8.80^{b}	249.11 ± 5.45^{a}	237.01 ± 4.12^{a}
SGR (%)	1.49 ± 0.05^{a}	1.54 ± 0.09^{a}	1.07 ± 0.09^{b}	1.63 ± 0.04^{a}	1.54 ± 0.03^{a}
FCR	2.28 ± 0.26	2.61 ± 0.10	2.41 ± 0.29	2.81 ± 0.44	2.45 ± 0.01
HSI (%)	1.04 ± 0.06^{bc}	0.48 ± 0.13^{c}	1.95 ± 0.24^{ab}	2.39 ± 0.65^{a}	1.84 ± 0.07^{ab}
Survival (%)	79.33 ± 6.77	82.67 ± 5.81	88.00 ± 1.15	78.67 ± 10.48	84.00 ± 1.15

values, it was determined that the D3 group was statistically different from the other four groups ($p < 0.05$) but that the difference among the values of the feed groups D1, D2, D4 and D5 was not statistically significant ($p > 0.05$). It was determined that in the diets to which different concentrations of RJ were added, this made no influence on the feed conversion and survival rates of the fish and the findings relating to both parameters are given in Table 3 ($p > 0.05$).

The hepatosomatic index values of the zebrafish which were fed four different feeds in the end of the eight weeks trial period are given in Table 3. Based on these data, the highest HSI value was found in the group which was fed the D4 feed (2.39 ± 0.65), while the lowest value was found in the fish which were fed the D2 feed (0.48 ± 0.13). No statistically significant difference was found between the group D4 and the groups D3 and D5, between D1 and D2 or between the group D1 and the groups D3 and D5 ($p > 0.05$).

Expression profiles of growth-related and immune system genes (GH, IGF-I and TGF- β) in the muscle, liver and kidney tissue after 56-day feeding experiment are shown in Fig. 1. Growth related genes such as GH-I and IGF-I significantly up-regulated in liver and muscle tissue of fish fed with the highest dose compared to the control group ($p < 0.05$). While a greater decrease in GH-I gene expression levels was found in kidney tissues of fish fed with D4 diet. The GH-I gene expression in the liver tissues for all diets was higher and statistically significant ($p < 0.05$) in comparison to other muscle and kidney tissues. The lowest expression value in this tissue is 1.02 ± 0.13 with D2 diet. In the muscle tissue the highest level of GH-I gene expression was found in the fish fed by the D4 diet with 2.22 ± 0.42 while the lowest level of expression was found in the D3 group with 1.19 ± 0.15. In the kidney tissue among D2, D3 and D5 diet group fish, the GH-I gene expression was no statistically significant increase when compared ($p > 0.05$).

The muscle, liver and kidney tissues of the fish samples belonging to the diet groups were analyzed in terms of the level of IGF-I mRNA, which is another gene involved in the growth metabolism and the findings obtained are shown in Fig. 1. As a result of the Real Time PCR analyses made with the mRNA obtained from muscle tissues, the highest level of expression was found in the D5 group, which contained 6.4 % RJ, with 2.33 ± 0,55, while the lowest value was found in the D3 diet group as in the case of GH-I hormone with 0.92 ± 0,46. Besides, it was found that the expression level of the D5 group was higher when compared with other diet groups ($p < 0.05$). In liver tissues, as in muscle tissues, the highest IGF-I expression level was recorded in the D5 group fish with 1.28 ± 0.33 while the lowest value was found in the D4 group as a result of the analyses. According to the study findings, there was no statistical difference between the diet groups in terms of the IGF-I mRNA expression level in the kidney tissue ($p > 0.05$).

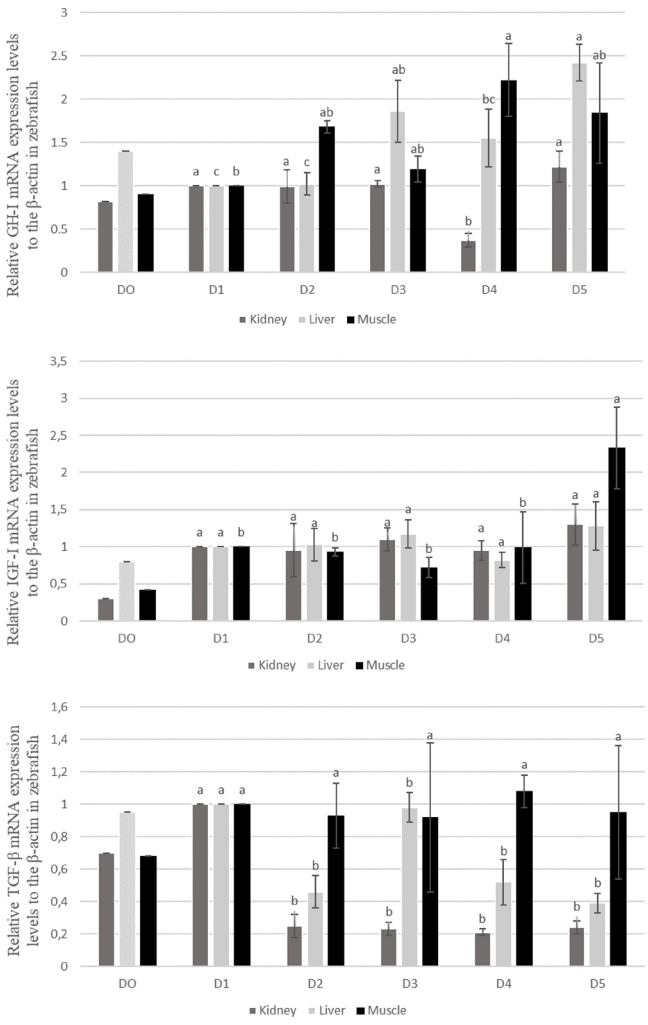


Fig. 1. Relative GH-I, IGF-I and TGF-β mRNA expression levels to the β-actin in zebrafish kidney, liver and muscle tissues. The data are expressed as means ± SEM(n = 5). a, b, c, d: statistical significance between each values in the lines. $p < 0.05$ was considered as significant.

TGF-β gene, which controls the expression of some genes like GH-I and IGF-I, which play an important role in the immune system and which are also involved in the growth metabolism. It was seen that the mRNA level amounts obtained in all applications were below the level of the control group, that is, 1. In the muscle tissues, among the diet groups, the highest amount of TGF-β mRNA expression was found in the fish that were fed the D4 diet (1.08 ± 0.10) while the lowest amount was found in the D3 group fish (0.92 ± 0.46) ($p > 0.05$). In the liver tissues, among the diet groups, the highest amount of TGF-β mRNA expression was found in the fish that were fed the D3 diet (0.98 ± 0.09) while the lowest amount was found in the D5 group fish (0.39 ± 0.06). In the kidney tissue, in all the treatments groups, the TGF-β mRNA level were found to be below 1, the control level, which was used as the calibrator. No statistical difference was found among the TGF-β mRNA levels of the diet groups (D2, D3, D4 and D5) in the kidney tissue ($p > 0.05$). Insert Fig. 1

4. Discussion

In this study, which examined the influence of RJ that was included in zebrafish diets in different rates on growth and development, the highest growth, specific growth rate and hepatosomatic index value was found in the D4 group, whereby 1.6 % RJ was included in the diet.

Adding 0.4 % RJ in the zebrafish diet (the D3 group) resulted in the least growth, relative growth percentage and specific growth. In the end of the experiment, although no dose-dependent increase was observed, it was determined that not all the doses applied adversely influenced the growth rates of the fish. Besides, the hepatosomatic index, which is reported by various studies to increase based on the amount of fat taken through diet, displayed a similar tendency with relative growth parameters (Rosenlund et al., 2001; Torstensen et al., 2000).

According to our results, higher GH expression occurred in muscle and liver tissues compared with kidney tissues. This situation might be explained by the fact that the zebrafish is a freshwater fish as kidney development is improved in freshwater fish compared with saltwater fish. That is to say, synthesis of IGF-I starts in the liver with the secretion of GH hormones from the hypothalamus according to the system of the growth hormone and the IGF metabolic system. In the subsequent stages of this mechanism, depending on the amount of the IGF-1 synthesized in the liver, the IGF-II synthesis begins in the kidney and in other tissues and thus tissue development occurs (Duan, 1997; Reinecke, 2010; Sheridan and Hagemeister, 2010).

Recently, some data are obtained regarding the molecular mechanism created by IGFs that stimulate muscle development in fish. In our study, the highest IGF-I mRNA level was observed in the fish that were fat RJ containing diets by 6.4 %. In terms of GH-I, the mRNA level was high in the D4 group, but the IGF-I level was lower. And individual variation or stress factor among fish might account for this situation. In fish, the IGFI makes dramatic and direct effects on the muscle system (Fuentes et al., 2013). It was shown in some fish types that IGFs stimulate the intake of glucose and alanine, protein synthesis and myoblasts proliferation (Castillo et al., 2004; Degger et al., 2000). Recent studies have shown that IGFs regulate the expression of various genes involved in myogenesis. In addition to the fact that IGFI is a strong promoter of muscle development and promyogenic molecules in fish by enhancing protein synthesis, this factor has a major role with the inhibiting molecules involved in protein degradation and muscle atrophy. Both systemic and local resources of IGFI are responsible for the stimulation of muscle development in fish (Fuentes et al., 2013). IGF-I is considered to be a key molecule in the prevention of muscle atrophy in fish while it also prevents protein degradation (Cleveland and Weber, 2010).

In our study, the levels of expression of the related gene in the liver tissue differed with the muscle tissue. GH was expressed in higher levels in the muscle tissues of the fish that were fed the D4 diet compared with other applications although it was expressed in lower levels in this group in the liver tissue. As a growth hormone is secreted from the pituitary gland, the variability of this hormone's gene expression is a natural result of the growth mechanism (Picha et al., 2008). It was reported in a study by Benedito-Palos et al. (2007) that when fish are fed fat sources which do not contain sufficient fatty acid composition needed by that fish, a decrease occurs in the amount of the mRNA level of the growth genes. IGF-1 is expressed in higher rates in the liver and the rate of its expression in the liver is stimulated by GH (Duan et al., 1993). It is an expected result that the genes of the IGF I hormone are expressed more compared with the liver tissue because, in the operation mechanism of GH-IGF system, the IGF secretion starts depending on GH amount secreted from the hypothalamus. Although this hormone is secreted in the highest amount in the liver, it is also secreted in certain amounts in all the tissues where growth occurs (Reinecke, 2010). GH on the axis of liver is indirectly influenced by hunger and insufficient nutrition, lack of specific nutrients or modifications in the hormonal status stimulated nutritionally (Perez-Sanchez and Le Bail, 1999). Alike the direct influence of GH, the systemic influence through liver IGFs is affected by nutritional manipulations.

Cytokines preventing cellular division inhibit the mechanisms activated by growth factors for cellular division. They can prevent the "down regulation" of growth factor receptors like TGF-beta, inactivation of receptors or transfer of the signals which normally enable cellular division. In fact, they can lead to irregularities in the production of autocrine growth factors. TGF-β is a hormone which controls cellular growth and differentiation in living beings. It functions as a morphogen in the regulation of the immune system and the differentiation of developing cells. This gene is synthesized in almost all cells and it is involved in the intracellular signal mechanism (Clark and Coker, 1998; Nagaraj and Datta, 2010). It also controls the expression of GH-1 and IGF-1 genes. No difference was found when the influence of the diets containing different rates of RJ on the TGF-β mRNA level and muscle tissues. It was found that the relative growth values of those diet groups with low level of TGF-β mRNA in the liver are higher compared with other groups but that the difference is not significant. Besides, the finding that the gene of the IGF-1 hormone, which initiates the growth in liver tissues, is expressed in higher rates compared with these groups is compatible with the findings that TGF-β controls the expression of the growth genes (GH-1 and IGF-1) (Ding et al., 2004; Xu et al., 2003). It was determined that diet groups displayed less expression in the kidney tissue compared with the liver and that this rate is lower by about 80 % compared with the control. These data show that IGF-I is inversely related with kidney tissue mRNA levels. That is, the increase of IGF-I in the tissue leads to a decrease of TGF-β. In recent years, varies studies are made about of the influence of various factors on the amount of growth hormones in kidney tissues and also on the mRNA levels of the genes belonging to these hormones using model organisms. The amount of growth hormones both in plasma and also in tissues influences the development of the kidney and the regulation of its functions (Roelfsema and Clark, 2001).

In summary, dietary ingredient can act to modify gene expression or act as cofactors within metabolic structure (Williams and Watts, 2019). It can be said according to the research findings that high doses of RJ make a positive effect on GH expression. These data are also compatible with classic growth parameters. While IGF is expressed in high levels in muscle tissues, addition of different rates of RJ in other tissues did not produce a difference. In the case of TGF-β expression, no significant difference was observed especially in muscles and it was determined on the contrary that GH was expressed in higher levels in the liver and kidney in lower doses.

To the best of our knowledge, any study has not been conducted to examine the effects of royal jelly on growth and development of zebrafish using classical and molecular methods. In our study, the royal jelly supplementation in the diet of zebrafish positively influenced their growth and development, GH and IGF-I mRNA expression levels in muscle and liver tissues. As a result, royal jelly is a natural product without any reported side effects for fish and has a potential to be sustainable alternative for growth promoter in aquaculture practice.

CRediT authorship contribution statement

Conceived and designed the experiments: E.A. (group leader) and S. S. Performed the experiments: E.A., S.S. and O.V. Analyzed the data: E. A, S.S., and O.V. Contributed reagents/materials/analysis tools: E.A., O. V. and S.S. Writing- Reviewing and Editing: E.A., S.S. and O.V. All authors read and approved the final and revised version of the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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