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Ph.D. THESIS

Effects of Using Emulsifier as Feed Additive on Growth and Reproductive Performance of Zebrafish (*Danio rerio* Hamilton, 1822)

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PREFACE

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TABLE OF CONTENTS

Page Number

LIST OF FIGURES

Page Number

LIST OF TABLES

Page Number

LIST OF SYMBOLS AND ABBREVIATIONS

Symbols	Explanations
°C	: sentagrate degree
%	: Percentage
µm	: Micrometre
s	: Second
♀	: Female
♂	: Male
∑	: Total

Abbreviations	Explanations
Control	: Diet content only fish oil 9% without sunflower and linseed oil
S6%L0%	: Diet content 6% of sunflower oil without linseed oil
S0%L6%	: Diet content 6% of linseed oil without sunflower oil
PGPR	: Polyglycerol polyricinoleate
WG	: Weight gain
FBW	: Final live body weight
IBW	: Initial live body weight SBW
D	: Experiment days
SGR	: Specific growth rate
FCR	: Feed conversion ratio
FI	: Feed intake
PER	: Protein efficiency ratio
CPFI	: Crude protein fed intake
AD	: Apparent digestibility
NI	: Nutreint intake
NF	: Nutrient in faeces
WBD	: Weight before drying
WAD	: Weight after drying
WBB	: Weight before burning
WAB	: Weight after burning
V_a	: Volume of acid used for sample titration
V_b	: Volume of acid used for the blank
N	: Normality of acid
SW	: Sample weight by grams

W_a	: Beaker weight with oil after extraction
W_b	: Empty Beaker weight before extraction
W_0	: Sintered glass crucible weight
W_A	: Sintered glass crucible, cellulose and ash weight
W_B	: Sintered glass crucible and ash weight
GCMS	: Gas Chromatograph Mass Spectrometer
MS-222	: tricaine methane sulfonate
CASA	: Computer-Aided Sperm Analysis
SKPs	: Sperm Kinemetic Parameters
VAP	: Average Path Velocity
VSL	: Straight Line Velocity
VCL	: Curvilinear Velocity
LAS	: Leica Application Suite
TEN	: Total Eggs Number
UEN	: Unfertilized Eggs Number
HLN	: Hatched Larvae Number
hpf	: Hours of post-fertilization
TFEN	: Total Fertilized Eggs Number
($p<0.05$)	:Significant Different
($p>0.05$)	: No Significant Different
FAs	: Fatty Acids
ARA	: Arachidic acid
EPA	: Eicosapentaenoic acid
DPA	: Docosapentaenoic acid
DHA	: Docosahexaenoic acid
FA	: Fatty acid
SFAs	: Saturated fatty acids
MUFAs	: Monounsaturated fatty acids
PUFAs	: Polyunsaturated fatty acids
C8:0	: Caprylic acid
C10:0	: Capric acid
C12:0	: Lauric acid
C13:0	:Tridecylic acid
C14:0	: Myristic acid

C16:0	:Palmitic acid
C16:1(n-7)	:Palmitoleic acid
C17:0	:Margaric acid
C18:0	:Stearic acid
C18:1(n-9)	:Elaidic acid
C18:2(n-6)	:Linoleic acid
C20:0	:Arachidic acid
C18:3(n-3)	:Linolenic acid
C20:1	:Eicosenoic acid
C21:0	:Heneicosylic acid
C22:0	:Behenic acid
C20:3(n-3)	:Eicosatrienoic acid
C20:4(n-6)	:Arachidonic acid
C23:0	:Tricosylic acid
C20:5(n-3)	:Eicosapentaenoic acid
C24:0	:Lignoceric acid
C24:1 (n-9)	:Nervonic acid
C22:5(n-3)	:Docosapentaenoic acid
C22:6 (n-3)	:Docosahexaenoic acid

ABSTRACT

Effects of Using Emulsifier as Feed Additive on Growth and Reproductive Performance of Zebrafish (*Danio rerio* Hamilton, 1822)

THESIS Ph.D.

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Title

This thesis study substantate out to evaluate the influence of feed prepared by using fish oil, sunflower or linseed oil together with emulsifier (E-476) on the growth performance, nutritional benefit and spawning executive of zebrafish and to determine the fatty acids profile. Six diet groups were formed in the 146-day experiment, and 30 zebrafish were used in each group, and the experiment was designed as three replications. Fish, sunflower (S) and linseed (L) oil and PGPR (0.06%) were used to form the control and five experimental diet groups. Control, S6%L0% and S0%L6% feed groups do not contain PGPR, while the remaining Control+PGPR, S6%L0%+PGPR and S0%L6%+PGPR groups contain PGPR. The fish were fed diets that contained PGPR had higher values ($p<0.05$) in final live body weight, spesific growth rate, feed conversion ratio, protein efficiency ratio, digested lipids amount, whole body lipid percentage and fatty acid percentages. Sunflower oil diet fatty acid digestibility enhanced by the presence of PGPR over other diets. While sperm kinematik parameters (VAP, VCL and VSL) of male fish fed with PGPR-free sunflower oil-containing feed (S6%L0%) were higher values ($p<0.05$) matched to other groups, the percentage of fertilized eggs and hatching percentages ($p<0.05$) getted. Sperm progressive motility parameters (VAP, VCL, and VSL) of S0%L6% and S0%L0+PGPR groups ($p<0.05$) than

other groups. Fertilized egg chorion diameter, yolk diameter, and larval length from fish fed PGPR-containing feed groups were significantly greater than the other groups ($p<0.05$). In addition, embryos belonging to these groups completed the embryogenesis process in a shorter time compared to other groups.

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Keywords: Polyglycerol polyricinoleate, model fish, aquaculture, feeding

SUMMARY

Yem Katkı Maddesi Olarak Emülgatör Kullanımının Zebra Balığı'nın (*Danio rerio* Hamilton, 1822) Büyüme ve Üreme Performansı Üzerine Etkisi

Ph.D. THESIS

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Bu tez çalışması, balık yağı, ayçiçeği veya keten tohumu yağı ile birlikte emülgatör (E-476) kullanılarak hazırlanan yemlerin zebra balığının büyüme performansı, yemden yararlanma, gamet ve embriyo kalitesi, yumurtlama performansı üzerindeki etkisini değerlendirmek ve yağ asit profilini belirlemek amacıyla yapılmıştır. 146 gün süren denemede 6 adet diyet grubu oluşturulmuş ve bu gruplarda balık, ayçiçeği (S) ve keten tohumu (L) yağları ve PGPR (%0.06) kullanılmıştır. Her grupta 30 adet zebra balığı kullanılmış olup, denemeler 3 tekerrürlü olacak şekilde tasarlanmıştır. Kontrol, S6%L0% ve S0%L6% besleme grupları PGPR içermezken, geri kalan Kontrol+PGPR, S6%L0%+PGPR ve S0%L6%+PGPR grupları PGPR içermektedir. PGPR içeren yemlerle beslenen balıklar, final canlı vücut ağırlığı, kazanılan ağırlık, spesifik büyüme oranı, yem dönüşüm oranı, protein etkinlik oranı,

sindirilmiş lipid miktarı, tüm vücut lipid yüzdesi ve yağ asidi yüzdelerinde daha yüksek değerlere ($p<0.05$) sahipti. Ayçiçek yağı içeren diyetin yağ asidi sindirilebilirliği, diğer diyetlere göre PGPR'nin varlığıyla artış göstermiştir. PGPR içermeyen ayçiçek yağı içeren yemle (S6%L0%) beslenen erkek balıkların sperm kinematik parametreleri (VAP, VCL ve VSL), döllenmiş yumurta yüzdesi ve kuluçka yüzdeleri diğer gruplara göre daha yüksek değerlerde ($p<0.05$) olduğu tespit edilmiştir ($p<0.05$). Diğer gruplara göre S0%L6% ve S0%L0%+PGPR gruplarının ($p<0.05$) sperm progresif motilite parametreleri (VAP, VCL ve VSL), döllenmiş yumurta koryon çapı, yumurta sarısı çapı ve PGPR içeren yem gruplarının larva uzunlukları diğer gruplara göre anlamlı olarak daha fazlaydı ($p<0,05$). Ayrıca bu gruplara ait embriyolar diğer gruplara göre embriyogenez sürecini daha kısa sürede tamamladığı tespit edilmiştir.

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Anahtar Kelimeler: Polyglycerol polyricinoleate, model balık, akuakültür, besleme

1. INTRODUCTION

Aquaculture and capture fisheries are both concerned as necessary universal providers of animal protein supply, while aquaculture is growing in its contribution to the supply, which raises the need for fish meal and fish oil and thus led to an increase in their prices. The serious search for non-traditional alternative ingredients, push significant researches to concentrate on alternative fishmeal and fish oil with inexpensive and possibly less environmentally cumbersome ingredients, such as plant by-products (Boyd *et al*., 2022; FAO, 2022).

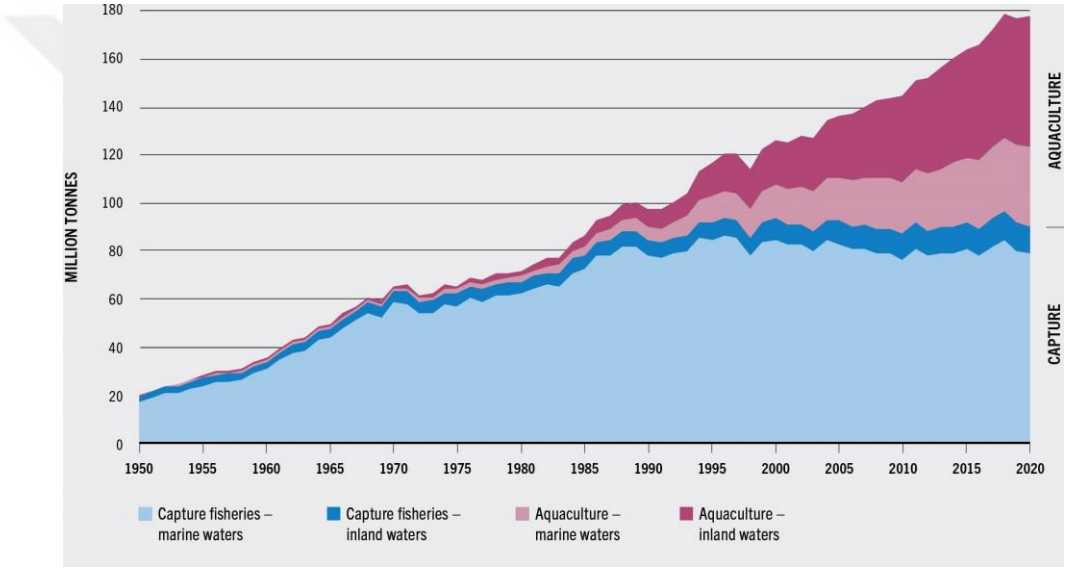

Figure1.1: Compare global production changes between capture fisheries and aquaculture sectors during the last 70 year (FAO, 2022).

Lipids, including fatty acids, represent an important source of metabolic energy and as synthetic molecules and steroids for composing the cells and tissues to drive the growth and spawning process in fish (Tocher, 2003; Tulasi *et al*., 1992). The amount of energy required to drive metabolic processes, the daily percentage of amino acids, and the amount of stored energy in the form of adipose tissue in the fish body are greatly affected by the level of dietary lipids and their composition of fatty acids (Gélineau *et al*., 2001; Martino *et al*., 2002). The changes in the values of the measurements; BW, SGR, FU and other growth performance parameters of fish are redound on nutritional lipids grades, and there is a

positive relationship emong the FAs composition of fish fillets and liver, and dietary FAs profile (Xue *et al*., 2006; Chatzifotis *et al*., 2010). In the spawning season, there is a high need for energy to create many temporary tissues that play a main role in the reproduction process; egg, sperm, and other cells and fluids (mucus) related to reproduction, and need energy for swimming and movements mating behaviors between males and females, also the importance of energy for migration species as Atlantic salmon (*Salmo salar*) that consume 60–70% of the total stored body energy to transfer headwaters of rivers and streams for spawning (Jonsson *et al*., 1997, Kaushik and M´edale, 1994, John and Ronald, 2002). In common the (n-3) HUFA plentiful in fish eggs than other FAs types especially in marine fish, and the (n-6) PUFA are high in freshwater fish roes chiefly 20:4n-6 and 18:2n-6 (Tocher, 2003).

Plant oils contain most of the essential fatty acids, including all groups of SFAs, MUFAs , and PUFAs but particularly a higher percentage of n-6 PUFAs, and the profile of fatty acids varies depending on the type of plant oils (Orsavova *et al*., 2015). It has been determined that the growth rate and feed conversion rate of fish fed with diets containing vegetable oil do not adversely affect, adequate growth performance is provided, and there is no negative effect on reproductive performance (Izquierdo *et al*., 2005; Xu *et al*., 2015; Agh *et al*., 2019).

Sunflower and linseed oils are considered good sources of arachidonic acid and linolenic acid, respectively, with a low percentage of remaining lipids from the fish meal diet is a good way to provide an adequate amount of essential fatty acids requirements for the successful spawning of O. *mykiss* broodstock (Agh *et al*., 2020). The introduction of crude palm oil as a vegetable oil in the diet of female Nile tilapia (*Oreochromis niloticus*) lead to; premature first spawning activity, extended duration of broodfish fecundity, enlargement of the gonads, increased number released of eggs, raise hatching rate, and reduce cases of larval distortions (Ng and Wang, 2011).

Emulsifiers are amphiphilic molecules that contain polar and non-polar sides. They are considered to function as consolidators between the water and oil interface by reduce the surface tension and work as decomposers to increment the active surface of lipids to improve the function of lipase, which disassemble triglyceride molecules into free fatty acids and monoglycerides and compose micelles from digested lipids (Hasenhuettl and Hartel, 2008; Roy et al., 2010; Zhang et al., 2011; Göksel, 2018). The secretion of bile salts is insufficient in neanic periods of fish due to incomplete development of the liver (Iijima *et al*. 1998;

Hofmann *et al*. 2010; Sadeghpour *et al*., 2018; Yúfera, 2018). Many studies on fish feeds investigated that; using different types of emulsifier materials (supplement bile salts, Hydrogenated soybean oil, Lysophospholipids, Cholic acid, and Tween-80) as feed additives in the fish meal has positive effects on feed efficiency, digestibility, and growth performance (Yamamoto *et al*., 2007; Adhami *et al*., 2017; Bergman *et al*., 2018; Taghavizadeh *et al*., 2020).

Zebrafish is play basic role as research model as a teleost fish due to rapid growth, high offspring ability , and easy of reproduction management under lab condition. The nature of its feeding on different feed sources led to its similarity in feeding behavior with other types of fish, whether carnivores, herbivores, or omnivores. This encouraged researchers to use zebrafish as experimental units to study different feed ingredients and additives before applying it at the field level on cultured fish species. (Linbo, 2009; Tocher *et al*., 2001; Ulloa *et al*., 2011; Tye *et al*., 2015). This study aims to investigate the swaying of polyglycerol polyricinoleate, PGPR with vegetable oils on dietary lipids utilization, tissues fatty acid composition, growth and reproductive performance of zebrafish as a model research for farmed fish species.

2. GENERAL PARTS

2.1. FISH NUTRITION

Nutrition science is largely based on concepts of mathematics, physics, chemistry, biochemistry, cytology, genetics, microbiology, physiology, endocrinology, pharmacology, and animal behavior science. Therefore, nutrition is the science of the interaction of food elements with the parts of the living body, including the formation of feed, the different stages of digestion, starting with the process of eating food until the excretion of undigested waste, and directing the digested and absorbed nutrients to the processes of catabolism and anabolism for the synthesis of biomolecules in order to compensate and renew cells and tissues (D'Mello, 2000; Jobling, 2016; Prabu *et al*., 2017). Different types of feeds and feed ingredients considered an important supply with energy and nutrient elements (National Research Council,1993). Feed items and feed additives compose 40- 60% of overall aqueous production cost, for this reason, several studies have been encouraged conducted to search for unconventional alternatives to fish powder and fish lipids that are plenty with lower price values (Bais, 2018; Bandara, 2018; Turchini, 2019). All manufactured diets includes necessary nutrients involving protein18-50%, lipid10-25% , carbohydrate15-20, ash<8.5%, and vitamins 0.5, for better cultured aquatic organisms growth and health (Craig *et al*., 2017). Fish can be divided according to the nature of their feeding behavior into three main section; herbivorous, carnivorous, and omnivorous (Sazima,1986; Lazzaro, 1987; Mariani and Alcoverro,1999 ; Ranjan *et al*., 2009). The crude protein in herbivorous and omnivorous fish feeds ranges from 25 to 35%, while carnivorous fish feeds between 40 to 55% (Hasan, 2001; Hashim, 2005; Sales and Janssens, 2003). Omnivorous fish can consume diets with a higher level of nitrogen-free extract (NFE) than carnivorous, which that; increasing the soluble carbohydrate in their diet decreasing the feed efficiency (Hemre, 1993; Helland and Grisdale-Helland, 1998; Ming,1993; Lall and Tibbetts, 2009). Zebrafish are diverse feeds consumer a multi types of microscopic aquatic plants Invertebrates and crustaceans and even commercial synthetic feed (Dutta, 1993; Spence *et al*., 2007; Vargesson, 2007; Linbo, 2009; Watts *et al*., 2016).

2.2. LIPIDS IN FISH NUTRITION

2.2.1. Lipids as a Source of Energy and Fatty Acids

Lipids involved a wide board of compounds that insoluble in aquatic mediums and soluble in nonpolar solutions (Dowhan and Bogdanov, 2002; Kerr *et al*., 2015; Kiernan, 2015). Lipids are utilized for energy generation mostly from glycerophospholipids, concerned as important structural units inside live cell membranes, play role as transfers for lipophilic compounds, and are pro- substances to synthesis eicosanoids (Henderson and Tocher,1987; Tocher, 2003). Neutral lipids include several molecules; triacyl-, diacyl- and monoacylglycerols, sterols, sterol esters, free fatty acids and wax esters (Gunstone and Harwood, 2007). Fatty acids are characterized by the presence of a methyl and a carboxyl group as a functional group on the carbon chain. The number of carbon atom and unsaturated bounds it distinguishing the properties of fatty acids in terms of classification and importance. The absence or presence of a double bonds determines the fatty acid's classification from saturated fatty acids (SFAs), mono unsaturated fatty acids (MUFAs), until high unsaturated fatty acids (HUFAs), and poly high unsaturated fatty acids (PUFAs), and also the number of carbon atom after methyl group that have first double bond it divide the PUFAs to n-3 or n-6 (Brenna,2002; Burdge and Wootton, 2002; Burdge and Calder, 2005; Brenna *et al*., 2009; Mišurcová *et al*., 2011; Proust *et al*., 2014).

2.2.2. Lipids Digestion and Absorption Mechanism in Fish

The dietary lipids enter the digestive system, then gastric glands release lipase enzymes inside the lumen of the stomach, intestine, and caecum to hydrolysis lipids. The brush border of the enterocytes absorbed the shorter fatty acid of less than 10 carbon atoms and glycerol. However, bile salt is mixed with a longer fatty acid of more than 12 carbon atoms to cleave and emulsified then compose micelles. The fatty acids transferred by diffusion from lumen to inside epithelial cells on brush border of intestinal wall. The Absorption of dietary lipids intestines of salmon fish are characterized by the presence of scavenger receptor class B, type1 (Kleveland *et al*., 2006; Olsen and Ringø, 1997). Absorbed fatty acids are formed in lipoproteins before released in to lymphatic vessels. In freshwater species the endoplasmic reticulum of epithelial cells chylomicrons is configured with reactions of re-esterification and

very low density lipoprotein (VLDL) such as happened in lumen during absorption. The classification of fatty acids determines the size of lipoprotein molecules; the saturated fatty acids composed the smaller particles of VLDL, while PUFAs produced larger chylomicrons. The fish similar to mammals, the absorbed lipids transported from intestinal wall to liver through lymphatic vessels (Caballero *et al*., 2003; Sheridan *et al*., 1985).

2.2.3. Oil Sources Used in Feed Fish

The fish oil content a highly unsaturated fatty (n-3) with high transformative efficiency therefore; it considered as major item in processed fish feeds for freshwater fish and more important in marine fish diets (Kim *et al*., 2002; Izquierdo *et al*., 2003). The world production of fish oil arrived at maximum, then continued to descend annually, while the requirements for fish feed production are raised sequentially. This created a real need to introduce alternative lipids sources of fish oil to support the aquaculture sector. (Jordal *et al*., 2007; Bouraoui *et al*., 2011). Many types of research results reflected that; using plant oils in freshwater fish feed alternative to fish oil had no adverse impact on growth performance (Drew *et al*., 2007; Yıldız *et al*., 2010). The level of linoleic and linolenic acids are significantly increased in the tissues of fish to which vegetable oils are added to their diets, while the percentage of PUFAs (n-3) decreases. (Şener and Yıldız, 2003; Yıldız and Şener, 2004).

2.3. EFFECT OF LIPIDS NUTRITION ON FISH REPRODUCTION PERFORMANCE

Nutrition is a key factor controlling gonadal development and fecundity. Spawning success and offspring survival rate depends on quantity and quality of lipids and fatty acids profile in fish broodstock feed (Izquierdo *et al*., 2001). The sperm membrane flexibility and other characteristics composed from phospholipids and cholesterol compounds, and types of fatty acids, for that its effect on sperm performance and efficiency for reach the highest rate of egg fertilization (Muller *et al*., 2008; Wassall and Stillwell, 2009; Beirão *et al*., 2012; Bell *et al*., 1997b; Henrotte *et al*., 2010; Labbé *et al*., 1995; Lahnsteiner *et al*., 2009; Muller *et al*., 2008). Sperm cells contain a high percentage of PUFAs, unlike the somatic cells because it This reflects the importance of the presence of these fatty acids in the diet of fish broodstock than commercial stock, because raised plasma membrane fluidity is flexible with water osmotic

influences to enhance sperm motility in external fertilization media. And furthermore, some researchers noticed; the composition of sperm cells is greatly affected by the content of the feed formulation from phospholipids, cholesterol, and an assortment of fatty acids(Bell *et al*., 1997b; Labbé *et al*., 1995; Pustowka *et al*., 2000; Nandi *et al*., 2007; Asturiano *et al*., 2001). Beir~ao *et al*., (2015) mentioned that the presence of docosahexaenoic acid (DHA) with suitable levels in Senegalese sole (*Solea senegalensis*) diet raises spermatozoa progressive percentage (%) and velocity (VCL) speed (μm/s), and general sperm quality. Increased levels of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) fatty acid in European eel diets lead to more sperm volumes and high sperm motility (Butts *et al*., 2015). The deficiency of essential fatty acids (n-3) during spawning season in freshwater fish diet as rainbow trout (*Oncorhynchus mykiss*) appeared in decline sperm motility parameters, however suitably ratio of HUFAs/PUFAs reflected the highest percentage of sperm motility and duration motility time (Vassallo-Agius *et al*., 2001; Hajiahmadian *et al*., 2016). The dietary lipids and adipose tissues considered a mainly sources to provide vitellogenesis with fatty acids to synthesize egg yolk until maturation egg stage (Wiegand, 1996). The spawning processes steps, yolk sac development, and stages of embryonic evulation effects with essential fatty acids type of dietary lipids and storage lipids as building units and sources of energy to push the Metabolic synthesis in gonads organs (Ohs *et al*., 2013). The needs for long-chain PUFAs (n-3) increases through the early developmental stages of the zygote and larvae, in relation to the synthesis of the nervous system and optical tissues (Bruce *et al*., 1999; Sargent *et al*., 1999).

2.4. EMULSIFICATION

2.4.1.Description of Emulsifiers and Emulsifier Components

Emulsification it is a process to facilitate the mixing of phase that differ in terms of polarity and non-polarity or hydrophilic and hydrophobic, in order to form a homogenous medium composed from droplets size (0,1 to 100 microns), thus reducing the surface tension between liquids of a watery and oily nature to create miscibility phase (Muñoz *et al*., 2007). At the homogenization phase, the surfaces of the emulsifying materials are characterized by the ability to create a state of static kinetic emulsification by attracting the surfaces of the newly formed droplets. For more physical stability of the emulsion phase, the emulsifiers create barriers across the formed interfacial membranes; this also improves chemical stability and

significantly slows fatty acid oxidation and other reactions (Faraji *et al*., 2004; Lee *et al*., 2019; Qian *et al*., 2012).

2.4.2. Emulsifier Used in Experiments Animals Farm and Fish Feeding

The dietary lipids transformed to large droplet sizes inside the fish intestinal canal which negatively affects lipid degradation by lipase enzymes (Gargouri *et al*., 1983). Emulsifiers can be converting nonpolar particle surfaces to polar particle surfaces thus, the admixing of fatty substances with the aqueous media and diffusion within, which helps the work of lipase enzymes (Al-Marzooqi and Leeson, 1999). In normal situation, bile salts are secreted in the intestine lumen to emulsify dietary fats (Stamp and Jenkins, 2008). And also proposed that the activation of the lipase enzymes group is influenced mainly by bile salts. Bile acids are primarily composed of cholic acid and chenodeoxycholic acid which are derived from cholesterol compounds and can used its level in the blood as sign of bile status (Begley *et al*., 2005; Crespo and Esteve-Garcia, 2003). Recently introduced synthetic emulsifiers in diet with a high level of fat improve lipid digestive, utilization, and metabolism (de Oliveira *et al*., 2019; Siyal *et al*., 2017). The addition of bile acids (including cholic acid and chenodeoxycholic acid) and bile salts (taurocholate) or synthetic emulsifiers in the feed of the broiler chick's herd raises the utilization of food fats, which reflects positively on the growth rate (Lai, 2018; San Tan *et al*., 2016). Bontempo *et al*., (2018) explained that; the vegetal bidistilled oleic acid and glycerol polyethylene glycol ricinoleate as emulsifier supplements in broiler chicken feed play a role to enhance feed utilization, the percentage of net edible tissue, fatty acids metabolism, and growth rate. Furthermore, Siyal *et al*., (2017) mentiond that the supplementation of emulsifiers had direct effect on dietary fatty acids digestibility in broiler chickens digestion system thus, increasing the utilization of diet lipids, which results in improving the growth of chickens. Lysolecithin or Lysophosphatidylcholine (LPC) It is a metabolized lecithin derivative known for its emulsifying properties due to strong surface reactions and emulsifying ability 5 time more than lecithin which associates lipids hydrolysis and compose micelles for absorption and transfer the fatty acids (Robinson, 1961; Casals *et al*., 1984; Dobiášová *et al*., 1975; Zhang *et al*., 2011). LPC has uses in various fields such as the pharmaceutical industry and in feeding various farm animals, it also introduced into the feed of different species of fish(crucian carp (*Carassais auratus gibelio*), hybrid tilapia (*Oreochromis aureus* ♂×*Oreochromis niloticus*♀), and turbot (*Scophthalmus maximus L*.) to

study their response to feed fat utilization and growth performance (Li *et al*., 2010a; Li *et al*., 2010b; Li *et al*., 2019).

2.5. ZEBRAFISH

2.5.1. Scientific Classification and Original Native

Zebrafish, Danio rerio, pertaining to the Danionidae family, is a tropical species Danio rerio, Zebra danio : aquarium (fishbase.se). The outer skin of the fish has distinct stripes and is covered with dark blue, silvery-white and golden-yellow stripes depending on the light in the environment (Mills, 1994).

South Asia region is the original home of the Zebrafish, and from there it spread widely to the rest of the Asian countries; (Nepal, India, Bangladesh, Pakistan, and Myanmar,), Then to other countries of the world (Spence *et al*.,2008). The nature of the tropical and semi-tropical climate of the geography area of the zebrafish had a great impact in determining its requirements of environmental factors. The nature of the habitat of zebrafish varies in it natural places in terms of the sizes and shapes of the different freshwater bodies; small pond , irrigation streams and channels, drains of rice farms, slow or speed rivers. (Bhat, 2003; Daniels, 2002; Menon, 1999).

2.5.2. The Life Cycle of Zebrafish

Zebrafish females lay eggs, which are fertilized directly by the males, and then left without parental care. Embryonic development begins shortly after the fertilization of the eggs, with the divisions of the zygote, It is surrounded by a chorion, membrane which protects it relatively for the first 48 hours of life. In the late stages of embryonic development, the hatching point is determined by the strength of the chorion membrane and the muscular movement of the larva inside. (Laale, 1977). Shortly after fertilization, the egg cytoplasm migrates from the vegetal pole to the animal pole and surrounds the yolk-free nucleus, the part where cell divisions take place (segmentation) is called the blastodisc. The first division begins 35 minutes after fertilization and is completed in 45 minutes. The cleavage period, which begins with the first cell division, refers to the process that includes 64 cell stages with regular divisions. The blastula period, which begins with 128 cell divisions, continues until the gastrula period, which begins with 50% epiboly stage. The gastrula period continues until

the end of the "bud" phase, which starts at the 10th hour. At this stage, germ ring, embryonic shield, epiblast, hypoblast, evacuation zone are seen. In the segmentation period, which is characterized by the formation of somites, the tail bud is formed and body movements are observed for the first time. Pigmentation, blood circulation, fin formations and foregut development are observed in the pharyngula stage (24th hour) following the end of the 26-somite stage in which otoliths appear. In the hatching stage (48th hour), cartilage formation in the pectoral fin and head region, hatching at irregular intervals, rapid morphogenesis of the primary organ systems are observed. From the 72nd hour after fertilization, the larval stage begins. At this stage, the formation of air sacs and short-term movements other than normal swimming behavior are observed in the 3.5 mm long larva. In the first few days, it starts to take feed without completely finishing the yolk sac (Kimmel *et al*.,1995; Ekici, 2007). The digestive system begins to form inside the zebrafish embryo, with composed the intestine from the layer of endodermal rod cells at 42 hours post-fertilization. The esophagus, liver, pancreas, and pharynx formed at 48 hours post-fertilization, then the mouth and anus appear at the completion of 72 hours post-fertilization immediately at hatching time. (Holmberg *et al*., 2004; Wallace *et al*., 2005). The small secretory cell formed the adhesion gland after hatching on the ventral larvae head to be easily attached to hard edges surrounding the water to fill the swim bladder with sac atmospheric air (Laale, 1977). Until the completed of 5 DPF, the larvae depend on the nutrients stored in the yolk sac, because gastrointestinal tract open and digestive glands secreted enzymes at 6 DPF after that larvae can start intake external feed from water medium, and at 7 DPF the larvae completely change to outer feeding and yolk sac total decay (Jardine and Litvak 2003; Wallace *et al*., 2005; Wilson, 2012). Metamorphosis happened about 14-29 DPF, It is the intermediate phase between the larvae stage and juvenile stage, it is similar to adult fish in all morphological and color characteristics, but differs in size and sexual maturity (Parichy, 2003). On the 30th day after fertilization, juvenile fish reaching a length of about 1 cm show fin formation and pigmentation of the adult individual. The 90-day-old zebrafish is about 2 cm long and has reached reproductive maturity. The 1000-day zebrafish, 4-5 cm long, is at the end of its life cycle. Zebrafish reach puberty at an early age and complete their life cycle within 10 weeks, It is characterized by high fertility and its females can lay 200 eggs per week when provided all optimal environmental conditions. (Brand *et al*., 2002; Carpio and Estrada, 2006).

2.5.3. Environmental Requirements for Zebrafish

Zebrafish can be classified as eurythermal because they show tolerance to large temperature ranges (6.7- 41.7°C) (Cortemeglia and Beeringer, 2005; Schaefer and Ryan, 2006). Rapid growth in natural environments was detected in months when the water temperature rose to 34 ° C (Spence *et al*., 2007). The high tolerance of temperature shows that it can adapt to various conditions in natural habitats (Spence *et al*., 2008). Despite this wide temperature tolerance range in natural environments, it is cultivated at a temperature at 28°C under laboratory conditions (Westerfield, 1995; Westerfield, 2000); However, 28.5°C is the temperature value used in embryonic development (Kimmel *et al*., 1995). Zebrafish usually prefer alkaline waters in natural environments, as well as pH values lower. In laboratory conditions, it is preferred that the pH value is between 7-8 (Harper and Lawrence, 2011). It is recommended that alkalinity is worth 50-150 mg/l $CaCO_3$ in cultivation. In zebra fish systems, it is necessary to provide low alkalinity to keep pH in balance (Harper and Lawrence, 2011). Zebrafish prefers the hardness values over 100 mg/l $CaCO_3$ (Brand *et al*., 2002). In general, they consume more oxygen than large fish because of high metabolic speeds of small -bodied tropical fish such as zebrafish (Helfman *et al*., 1997). For this reason, it requires ~ 7.8 mg/L of the dissolved oxygen level at a water temperature of 28.0°C (Popma and Masser, 1999).

2.5.4. Nutritional Requirements of Zebrafish

Zebrafish have ability to elongate essential fatty acid chains and change the sites of bonds on it for transfer to very essential highly unsaturated fatty acids (HUFAs), such as eicosapentaenoic acid (20:5n-3; EPA), docsahexaenoic acid (22:6n-3; DHA), and arachidonic acid (20:4n-6; AA) as similar to other freshwater fish in their fatty acid metabolism pathways (Tocher *et al*., 2001). The protein requirement of zebrafish is considered at intermediate level between the carnivorous and the omnivorous fish, Perhaps this high requirement for protein, unlike the other of omnivorous fish, is due to the small size of the zebra fish, and there is opposite relationship between fish protein requirement and size (National Research Council,1993; Lochmann and Phillips 1994; and Chong *et al*., 2000).

2.5.5. Using Zebrafish as a Model in Research Fish Feeding Experiments

In addition to human diseases, zebrafish have advantages such as being able to be produced in small tanks with a maximum length of 4-5 cm in aquaculture studies, having a short breeding

cycle as a model organism, producing a large number of eggs and being easily manipulated (Clark, 2003). By the virtue of these advantages, many studies on different subjects have allowed molecular analyzes to be made compared to other fish species. The zebrafish genome is similar in a large number of genetic locations to a number of different fish species, example; green spotted pufferfish (*Tetraodon nigriviridis*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), catfish (*Ictalurus punctatus*), medaka (*Oryzias latipes*), and fugu (*Takifugu rubripes*), and also even similar with human genomes (Barbazuk *et al*., 2000; Woods *et al*., 2005; Rexroad *et al*., 2005; Quinn *et al*., 2008; Liu *et al*., 2009). Zebrafish are evolutionarily closer to commonly aquacultured species than to mammals used as model organisms. The fact that zebrafish have omnivorous feeding habits indicates that they can be used as a model in feeding studies of fish species with carnivorous or herbivorous feeding characteristics in aquaculture. However, a large number of researches are needed on zebrafish nutrition and protein, carbohydrates and lipid requirements to support the view that this species can be used as a model in feeding studies (Ulloa *et al*., 2011). Many applied studies in the field of aquaculture nutrition research have used zebrafish (*Danio rerio*) as a model organism to examine the effect of nutrients and feed additives on fish growth, reproduction (Araújo *et al*., 2017) and used zebrafish (*Danio rerio*) to examine the effect of fatty acids of vegetable oils (olive, linseed, and corn oil) on broodstock nutrition on oogenesis, embryogenesis and flesh structure. In this study the fatty acids had been measured in experimental diets and zebrafish tissue, and fatty acids effects on level of oestradiol which reflects the developing of ovaries and eggs quality before fertilization and during embryo developments. In this study the fatty acids had been measured in formulated nourishments and zebrafish tissue, and fatty acids effects on level of oestradiol which projects the oogenesis and grade of egg before fertilization and during embryogenesis.

Zebrafish were used in many experiments related to dietary fats and fatty acids; according to Adam *et al*., (2017) study, zebrafish used as the model organism to compare between high and low level of arachidonic acid (ARA) and their effects on the general metabolism. Jaya *et al*., (2008) introduced dietary HUFAs in zebrafish diet to understood the change of fatty acids metabolism during spawning period and effects on activity of desaturase and elongase genes in liver, And its consequences from the changes that occur in the fatty acids of the tissues. Also, even anti-nutritional effects have been studied in experiments with zebrafish as

experimental units; according to Hedrera *et al*., (2013) study soy saponin inflammation effects in intestinal of zebrafish larvae.

3. MATERIAL AND METHODS

3.1.MATERIALS

3.1.1.The Experiment Site

This research study was conducted from March/2021 until June/2021 on zebrafish experiment studies in the Aquatic Vertebrate Living Experiment Unit (İUSUCAN), Inland Fisheries Production Research and Application Station affiliated with Faculty of Aquatic Sciences Istanbul University at Sakarya Province in Sapanca.

Figure 3.1: Aquatic Vertebrate Living Experiment Unit (İUSUCAN).

3.1.2. Zebrafish Housing

In this experiment, we used a eighteen plastic tanks (3.5L) (three tanks for each group diet) in recirculation zebrafish system (Tecniplast Company – Italy). Each tank had baffles on the outlet side (300, 500 and 800 microns according to the fish size) to discharge the water. The effluent water from experimental tanks was treated via a fine mechanical filter, and carbon and UV filters. Water temperature (28° C), pH(6-7) , dissolved oxygen (7.8 mg/L), conductivity and salinity(450-490ppm) were automatically monitored and controlled with a computerized system.

Figure 3.2 : Zebrafish housing system device.

3.1.3.Zebrafish Population

180 zebrafish larvae (*Danio rerio* Hamilton, 1822) AB line as experimental units, were obtained from İUSUCAN.

3.1.4. Emulsifier Substance

The type of emulsifier substance (Polyglycerol polyricinoleate) that used in this experiment with five diets by 0.06%, obtained from ASPEK KIMYA GIDA company (Turkey) (1kg: 360,84TL).

Figure 3.3: Polyglycerol polyricinoleate bottles.

In figure(3.4) show the chemical structure of polyglycerol polyricinoleate

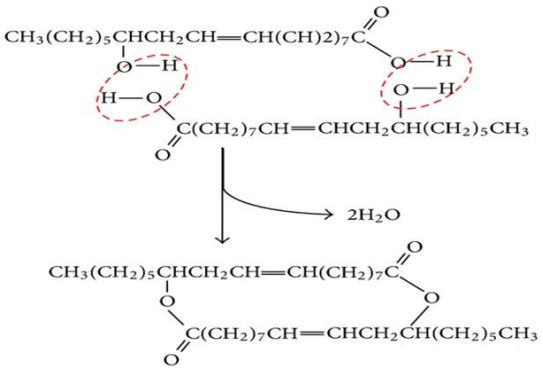

Figure 3.4: Chemical composition of polyglycerol polyricinoleate.

3.1.5. Experimental Feed Ingredients

All ingredients were obtained from National Turkish Companies and local markets to composed the control and five experimental diets; Fish meal 650g/kg, Fish oil 30-90g/kg, Sunflower oil 60g/kg, linseed oil 60g/kg, Gelatin 60g/kg, Corn starch 103g/kg, Di-calcium phosphate 15g/kg, Vitamin and mineral premix 3g/kg, Lysine 10g/kg, Methionine 5g/kg, Cellulose 60g/kg, and Polyglycerol polyricinoleate (PGPR) 6g/kg (Table3.1)

Figure 3.5: Zebrafish feeds texure

Table3.1: Composition of control and experimental diet groups

Ingredients of control and experimental diets (g/kg)	Control group	Control+ PGPR group	S6%L0% group	S6%L0%+ PGPR group	S0%L6%+ group	S0%L6%+ PGPR group
Fish meal	650	650	650	650	650	650
Fish oil	90	90	30	30	30	30
Sunflower oil	0.0	0.0	60	60	0.0	0.0
Linseed oil	0.0	0.0	0.0	0.0	60	60
Gelatin	60	60	60	60	60	60
Corn starch	107	101	107	101	107	101
Dicalcium phosphate	15	15	15	15	15	15
Vitamin and mineral premix	3	3	3	3	3	3
Lysine	10	10	10	10	10	10
Methionine	5	5	5	5	5	5
Cellulose	60	60	60	60	60	60
PGPR[1]	0.0	6	0.0	6	0.0	6
Total	1000	1000	1000	1000	1000	1000

PGPR[1]: Polyglycerol polyricinoleate, PGPR (E-476)

Table3.2: A proximate analysis of control and experimental diet groups

Proximate composition	Control group	Control+ PGPR group	S6%L0% group	S6%L0%+ PGPR group	S0%L6%+ group	S0%L6%+ PGPR group
Crude protein (%)	45.0416	44.9543	44.9411	45.0085	45.0458	44.9888
Crude lipid (%)	14.227	15.6613	16.5180	14.0933	15.7980	15.6177
NFE (%) [1]	19.7573	18.8222	17.7189	20.006	18.3741	18.5877
Cellulose (%)	5.9996	6.0099	6.0030	5.9873	6.0003	5.9883
Ash (%)	8.8763	8.5784	9.0695	8.8282	8.8570	8.8927
Gross energy (MJ/kg DM)[2]	19.618	19.9878	20.13342	19.5848	20.0005	19.9413

[1]NFE(%): Nitrogen-free extract (soluble carbohydrate) calculated as 100 - (Crude protein % + Crude lipid + Cellulose %+ Ash % + moisture %).

[2]Gross energy (MJ/kg DM): Calculated as total carbohydrate .17.2 kJ/g; fat .39.5 kJ/g; and protein .23.5 kJ/g (Perera, 2016).

3.1.6. Observation of Sperm Motility

Sperm motility observations was carried out using Makler counting chamber under CX41 microscope (Olympus, Japan) to show with CEROS II (Hamilton-Thorne, Beverly, MA, USA) program (CASA System).

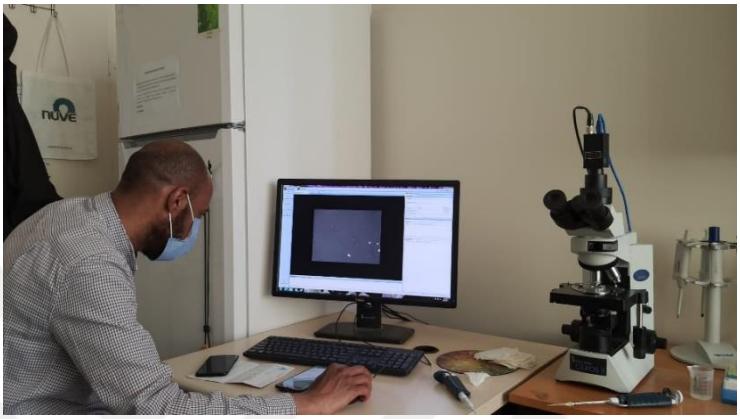

Figure 3.6: Observation of zebrafish sperm motility with CASA system.

3.1.7. Screening of Embriyonic Development

Embryogenesis stages, fertilized egg and yolk diameters, larval length measurements were performed under a stereo microscope with Leica Application Suite (LAS) integrates program.

3.1.8. Zebrafish Mating Tanks

Tanks with a volume of 3.3L were used for mating zebrafish brootstocks. By placing two rows of marbles on the bottom of these tanks, the broodstock fish were prevented from eating the eggs.

Figure 3.7: Zebrafish mating tanks.

3.2. METHODS

3.2.1. Preparation of Zebrafish Diets

The feed treartments were prepared in the Nutrition Laboratory Faculty of Aquatic Sciences Istanbul University, with sensitive balance and an electric blender was used to take the required amount from each ingredient and reach a high degree of homogeneous between it in diets, for obtain a balanced diet according to fish nutrition requirements. The control feed group just comprehend fish oil as provenance of without vegetable oils and PGPR as an emulsifier material ; the S6%L0% diet group included fish oil 3% and sunflower oil 6% as a ratio (1:2) without Linseed oil and PGPR, S0%L6% diet group involved fish oil 3% and linseed oil 6% as a ratio (1:2) without sunflower oil and PGPR, control+PGPR, S6%L0%+PGPR and S0%L6%+PGPR the same as control, second and third diet group just included PGPR with 0.06% (Table3.1). All control and experimental diet groups had equal amounts of other ingredients (Table3.1). After preparation; diets were divided into three types according to size (0.07± 0.01mm for larvae, 0.150±0.015mm for fingerlength,and 0.250±0.035mm for the adult stage).

3.2.2. Feeding and Daily Tasks During Experiment Period

In the first three days of the trail; groups were fed a control diet (adaptation days), then started body weight was taken for every group diet with electric sensitive balance. Zebrafish were nutrified twice times a day with trail diets and one time a day with artemia during the experiment (at 09: 00, 13: 00, and 17: 00 h). The faeces had collected very carefully for the sensitive fourth time the day before and after feeding from the bottom of the tank with a glass pipette and put in plastic bottles and then kept in the refrigerator (20°- C). The digital screen of the zebrafish system was been constantly monitored to ensure that all environmental requirements (pH 6.5-7.5, 26-28°C, and 450 – 490 ppm) by the zebrafish are within the optimum limits.

3.2.3. Weighting to Calculate Growth Performance Measurements

On the weighing day, the fish were fasted and before weighting done anesthetization with 164 mg/L of MS-222 for 1-2 minutes to avoid stress and ensure the welfare of the fish (Matthews et al., 2018), then weighted with eletric digital balance after dried on toilet papers. The

outgrowth and FCR performance of experimental zebrafish were accounted using the following formulas (Ricker, 1979, Silva *et al*., 1995; Agbo, 2008; Bob-Manuel, 2013):

$$WG = ((FBW-IBW)/FBW)\times 100 \quad (3.1)$$

$$SGR = ((\ln FBW - \ln IBW)/D)\times 100 \quad (3.2)$$

$$FCR = FI\ (g)/WG\ (g) \quad (3.3)$$

$$PER = WG(g)/CPFI(g) \quad (3.4)$$

$$AD = ((NI-NF)/NI)\times 100 \quad (3.5)$$

3.2.4. Approximate analysis to determine chemical composition of diet samples

The control and experimental diet groups samples were submitted for approximate analysis (AOAC, 1990) in Nutrition laboratory, Faculty of Aquatic Sciences Istanbul University, to determined; DM (%), Ash(%) , CP(%) , EE(%) and CC(%).

3.2.4.1. Dry matter (%)

5 gram (weight before drying) of sample put inside oven at 105 C° for 18 hour, after that transferred to desiccator until reach to room temperature then weighted (weight after drying)

$$Moisture\ (\%) = ((WBD- WAD)/WBD)\times 100 \quad (3.6)$$

$$DM(\%) = 100- Moisture\ (\%) \quad (3.7)$$

3.2.4.2. Ash (%)

1 gram (weight before burning) of sample weighted in crucible and put inside Gallankamp muffle furnace at (550 – 600 C°) for 4-6 hour, after that transferred to desiccator until reach to room temperature then weighted (weight after burning).

$$Ash\ (\%) = ((WBB-WAB)/WBB)\times 100 \quad (3.8)$$

3.2.4.3. Crude Protein(%)

1 gram of sample added in digestion Kjeldahl tube (250 ml) with 2 catalyst and 20 ml of concentrated sulfuric acid (96 – 98 % conc.), then transfer the tube in isolation casket calefactor with sucking gases system. When temperature in heater raised to 450 C° the digestion Kjeldahl tubes was kept until liquid becomes transparent. The transparent liquid was transferred in volume flask (100ml) and completed to 100 ml with distilled water. Samples was distillated with sodium hydroxide (40% conc.), final distillate was received in boric acid (2% conc.), after that titration against 0.1142 N sulfuric acid.

$$\text{Nitrogen (\%)} = ((\ 1.4007 \times (V_a - V_b\ ) \times N\ ) / SW) \times 100 \quad (3.9)$$

$$\text{CP (\%)} = \text{Nitrogen (\%)} \times 6.25 \quad (3.10)$$

3.2.4.4. Ether Extract (%)

Soxhlet extraction apparatus was used to determined crude lipids, empty Soxhlet beaker was weighted, and 5g of feed was put inside, then 100 – 150 ml of petroleum-ether put to beakers before fixed in apparatus. The oil is separated during the heating process and the organic solvent permeates into the sample to dissolve with it. After the completion of the time, the beakers are kept inside the oven (105°C) at the drying temperature to evaporate the solvent particles away from the oil. Then weighting after arrived temperature room.

$$\text{E E (\%)} = W_a - W_b \times 100 / SW \quad (3.11)$$

3.2.4.5. Crude Cellulose (%)

1 gram of sample was weighted and put in glass beaker (250ml), then 100ml of sulfuric acid (1.25% conc.) was added before but inside the in refluxing heater apparatus and kept for 30 minute after boiling. After that 10ml of potassium hydroxide (28% conc.) was add and heating just half hour. Sintered glass filled by quartz sand at a height of 8-10 mm was weighted before filter process. Sand was washed inside the sintered glass crucible well with hot distilled water. Sample was transferred from refluxing heater apparatus to filter in sintered glass crucible and washed twice time with distilled water and 10ml sulfuric acid (1.25% conc.) and then again twice time with distilled water. And washed finally by acetone before transferred to oven at 130 C° for one hour, then put in desiccator and weighted (Sintered glass

crucible, cellulose and ash weight). After that samples put inside Gallankamp muffle furnace at (550 – 600 C°) for 30 minute, then transferred to desiccator until arrived temperature room and weighted (Sintered glass crucible and ash weight).

Crude cellulose (%) = ($(W_A - W_0) - (W_B - W_0)/SW) \times 100$ (3.12)

3.2.5. Fatty Acids Analysis of Diets, Tissues, and Faeces

Diets, tissues and feces samples of control and experimental groups were submitted to fatty acids analysis in Central Research and Application Laboratory, Kastamonu University. The method of Gas Chromatograph Mass Spectrometer was used to determine fatty acids in samples. Before analysis, 10 mL of high purity C_6H_{14} and 0.5 milltre of 2 N CH_5KO_2 solutions were added to 0.1 g oil, and the solution was left far away from light for 2 hours. After pretreatment, oil content analysis was performed with GCMS device. During the analysis, He_2 inside brand canicular column work as tranfer. Column furnace hot adjusted at 40°C, surface at 250°C, ionic thermocouple at 200°C and insertion temperature at 250°C. The injection volume is 1µL, and the split (1/5) method was used for injection. During the analysis, three min at 40°C, from 40°C to 240°C with 4°C/min increment, 240°C for ten mintue, from 240-260°C with 4°C min^{-1} increment, 260 °C at 10 min, the oven program was applied, which lasted for a total of 78 minutes (Meier, 2006; and Kesbiç, 2019).

3.2.6. Sperm Motility Test

When the fish arrived at adult age, three males were selected randomly from each group to submit for sperm motility test with a Computer-Aided Sperm Analysis (CASA) system. After anesthetizing with 164 mg $L^{-1}$of MS-222 and rinsed in fresh system water, sperm collected in the capillary glass tube (10µl) (Sigma –Aldrich P0674) by holding the fish steady with a sponge and applying a slight pressure to the abdominal wall with a forceps. Sperm quality was determined using a CASA system (Olympus CX41, AgroLegato, Budapest, Hungary) and a CASA system with the phase-contrast objective. 10µl of sperm sample mixed with 100 µl of zebrafish system water (as sperm activation medium) on Makler counting chamber under CX41 microscope (Olympus, Japan) to show with CEROS II program (CASA System), the sperm motility and progressive motility SKPs parameters values.

3.2.7. Estimation of Fertility and Hatching Rate of Eggs

Daily light and dark duration were adjusted by controlled hour numbers of artificial lights to 10: 14 hours a day. When zebrafish get a maturation size photoperiod is important for stimulated mating behavior and the genital endocrine glands and sexual behavior. Before mating; mating tanks were prepared to add small glass balls on their bottoms. Every morning at first minutes of light duration, males that exhibited sexual behavior were isolated with female bloated bellies (3:1) from the same group and put together in mating tanks (Ruhl et al., 2009). Mated individuals monitor regularly until eggs were released from the females, collected 5 minutes later, and then counted themin numbers and them put on the lab. plates. Eggs were incubated at 28°C.

3.2.7.1. Fertility Rate (%)

The total eggs number were been calculated that released by the female zebrafish, then the unfertilized eggs (white color) were been removed and calculated again before put in incubator.

$$\text{Fertility rate (\%)} = ((\text{TEN} - \text{UEN})/\text{TEN}) \times 100 \quad (3.13)$$

TEN: Total eggs number

UEN: Unfertilized eggs number

3.2.7.2. Hatching Rate (%)

After the incubation period, hatched larvae were been calculated from the total number of fertilized eggs which put from begining in incubator.

$$\text{hatching rate (\%)} = (\text{HLN}/\text{TFEN}) \times 100 \quad (3.14)$$

HLN: hatched larvae number

TFEN: Total fertilized eggs number

3.2.8. Measuring the Egg and Yolk Diameter, and Larvae Length and Evaluation of Embryonic Development

The egg and yolk egg diameter of fertilized egg samples were determined from the first incubation hour under a microscope with Leica Application Suite (LAS) integrates program, and embryonic development stages(Kimmel *et al*., 1995; Araújo *et al*., 2017) was been following up until hatching point after that the length of hatched larvae was taken.

Table3.3: Score system used to characterize the developmental stage of zebrafish embryo (Kimmel *et al*., 1995; Araújo *et al*., 2017).

Embryo stage name	Time (hours)	Score
Dome	4.3	0.6
50% Epiboly	5.3	0.7
Germ-ring	5.7	0.8
60% Epiboly	6.0	0.8
70% Epiboly	7.0	1.0
80% Epiboly	8.0	1.1
90% Epiboly	9.0	1.3
95% Epiboly	9.5	1.3
Bud	10.0	1.4
3 Somite	11.0	1.5
6 Somite	12.0	1.6
14 Somite	16.0	2.2
18 Somite	18.0	2.6
21 Somite	19.5	2.6
26 Somite	22.0	3.1
Prim 6	25.0	3.3
Prim 12	30	4.2
Prim 20	34.0	4.9
Prim 25	36.0	5.0
Prim 26	38.0	5.1
High pec	42.0	5.8
Long pec stage	48.0	6.7
Pec Fin stage	60.0	8.3

3.2.9. Statistical Analysis

The parameters of growth and reproductive and fatty acids submitted for statistical analysis using SPSS program version 21.0 with one-way analysis (ANOVA). Comparative evaluation of averages with Duncan's to determine the significant differences between it within ($p < 0.05$) level.

4. RESULTS

4.1. Fatty Acid Analysis Results and Digestibility (%)

4.1.1. The Quantity and Quality of Fatty Acids Present in Feeds

In Table(4.1) control and control+PGPR diet content, only fish oil (90g/Kg) was the high percentage in C12:0, C13:0, C17:0, C18:0, C20:0, C21:0, C20:1, C20:3(n-3), C20:4(n-6), C23:0, C24:1, C21:5(n-3), and C22:6(n-3) fatty acid with ($p< 0.05$). Feeds involved (30g/Kg) fish oil and (60g/Kg) of sunflower oil (S6%L0% and S6%L0%+PGPR) were high percentages in C8:0, C18:1(n-9), C18:2(n-6), C22:0, and C24:0 fatty acid with ($p< 0.05$). Diets containing (30g/Kg) fish oil and (60g/Kg) linseed oil (S0%L6% and S0%L6%+PGPR) were high percentages in C18:3(n-3) ($p< 0.05$).

Table4.1: Fatty acids (%) from total lipids in control and experimental diet groups.

Types of fatty acids (%)	Control group	Contro+ PGPR group	S6%L0% group	S6%L0+ PGPR group	S0%L6% group	S0%L6+ PGPR group
C8:0	0.003± 0.0003^{b}	0.003± 0.0001^{b}	0.004±0.001^{a}	0.003±0.0003^{b}	0.003± 0.0001^{b}	0.003± 0.0002^{b}
C10:0	0.006± 0.0004^{abc}	0.006± 0.0001^{a}	0.006±0.001^{ab}	0.005± 0.0004^{d}	0.005± 0.0001^{bc}	0.005±0.0002^{cd}
C12:0	0.016± 0.001 ^{a}	0.018± 0.0002^{a}	0.012± 0.002^{b}	0.009± 0.001^{c}	0.012± 0.0002^{b}	0.011±0.0004 ^{b}
C13:0	0.016± 0.001^{a}	0.017± 0.0001^{a}	0.012± 0.002^{b}	0.009± 0.001^{d}	0.011± 0.0002^{bc}	0.01± 0.0004^{c}
C14:0	0.843± 0.019^{b}	0.918± 0.003^{a}	0.451±0.059^{c}	0.373± 0.031^{d}	0.4561± 0.009^{c}	0.429±0.001^{b}
C16:0	3.735± 0.0806^{b}	4.092± 0.008^{a}	2.944±0.315^{c}	2.509± 0.199^{d}	2.943± 0.038^{c}	2.85± 0.107^{c}
C16:1(n-7)	0.939± 0.013^{b}	1.016± 0.006^{a}	0.503±0.064^{c}	0.413± 0.034^{d}	0.515±0.01^{c}	0.473± 0.02^{d}
C17:0	0.179± 0.004^{a}	0.192± 0.002^{a}	0.123± 0.017^{b}	0.1031± 0.0088^{c}	0.128± 0.0016^{b}	0.12± 0.005^{b}
C18:0	0.861± 0.017^{ab}	0.935± 0.01^{a}	0.844± 0.075^{b}	0.7241± 0.0600^{c}	0.921± 0.0129^{ab}	0.911± 0.045^{ab}
C18:1(n-9)	3.49± 0.054^{c}	3.929± 0.051^{b}	4.551± 0.238^{a}	3.8873± 0.2960^{b}	3.7984± 0.0473^{b}	3.745± 0.166^{c}
C18:2(n-6)	0.313± 0.007^{d}	0.42± 0.014^{d}	5.169± 0.111^{a}	4.547± 0.345^{b}	1.457± 0.0212^{c}	1.503± 0.064^{c}
C20:0	0.112± 0.003^{a}	0.119± 0.003^{a}	0.080± 0.008^{b}	0.065± 0.006^{c}	0.072± 0.003^{bc}	0.068± 0.005^{c}

Table4.1: Fatty acids analysis in control and experimental diet groups (%) (continued).

Types of fatty acids (%)	Control group	Control+ PGPR group	S6%L0% group	S6%L0+ PGPR group	S0%L6% group	S0%L6+ PGPR group
C18:3(n-3)	0.116± 0.001^{cd}	0.195± 0.017^{c}	0.058± 0.012^{c}	0.054± 0.004^{d}	3.709± 0.047^{b}	3.927± 0.171^{a}
C20:1	0.246± 0.006^{a}	0.257± 0.005^{a}	0.182± 0.021^{bc}	0.1611± 0.0138^{c}	0.198± 0.005^{b}	0.186± 0.012^{b}
C21:0	0.026± 0.001^{a}	0.027± 0.001^{a}	0.019± 0.003^{b}	0.0139± 0.0012^{c}	0.018± 0.0003^{b}	0.017± 0.001^{b}
C22:0	0.046± 0.001^{c}	0.049± 0.001^{c}	0.084± 0.003^{a}	0.0705± 0.0057^{b}	0.041± 0.001^{d}	0.039± 0.002^{d}
C20:3(n-3)	0.028± 0.001^{a}	0.031± 0.001^{a}	0.019± 0.003^{c}	0.0145± 0.001^{d}	0.022± 0.001^{b}	0.02± 0.001^{bc}
C20:4(n-6)	0.11± 0.004^{a}	0.112± 0.003^{a}	0.061± 0.008^{b}	0.0474± 0.0054^{c}	0.062± 0.001^{b}	0.054± 0.003^{bc}
C23:0	0.0205± 0.0003^{a}	0.0213± 0.001^{a}	0.018± 0.002^{b}	0.014± 0.002^{d}	0.017± 0.0004^{bc}	0.016± 0.001^{d}
C20:5(n-3)	0.9937± 0.0092^{b}	1.057± 0.042^{a}	0.408± 0.055^{c}	0.313± 0.027^{d}	0.419± 0.011^{c}	0.371± 0.021^{c}
C24:0	0.0362± 0.0013^{b}	0.037± 0.002^{b}	0.046± 0.003^{a}	0.037± 0.0003^{b}	0.034± 0.001^{bc}	0.032± 0.002^{c}
C24:1 (n-9)	0.1234± 0.0040^{a}	0.126± 0.004^{a}	0.084± 0.011^{b}	0.07± 0.008^{c}	0.087± 0.001^{b}	0.079± 0.005^{bc}
C22:5(n-3)	0.0989± 0.0006^{a}	0.104± 0.004^{a}	0.0443± 0.0062^{b}	0.0346± 0.0046^{c}	0.045± 0.002^{b}	0.04± 0.002^{bc}
C22:6(n-3)	1.87± 0.014^{a}	1.982± 0.086^{a}	0.794± 0.098^{b}	0.615± 0.076^{c}	0.826± 0.014^{b}	0.709± 0.046^{bc}

4.1.2. Fatty Acid Analysis of Whole Zebrafish Body.

In the (Table4.2) show the fatty acids profile percentage of zebrafish tissues in control and experimental groups. The fatty acids C8:0 and C10:0 were high percentages in fish tissue of control+PGPR, S6%L0%+PGPR, and S0%L6%+PGPR groups ($p < 0.05$). The fatty acids C12:0, C13:0, and C14:0 were high percentages in fish tissue of the control+PGPR group ($p < 0.05$). The fatty acid C16:0 was a high percentage in fish tissue of control+PGPR and S0%L6%+PGPR groups ($p < 0.05$). The fatty acids C16:1(n-7), C17:0, and C18:0 were high percentages in fish tissue of the control+PGPR group ($p < 0.05$). The fatty acid C18:1(n-9) was a high percentage in fish tissue of the S0%L6%+PGPR group ($p < 0.05$). The fatty acid C18:2(n-6) was a high percentage in fish tissue of S6%L0% and S6%L0%+PGPR groups ($p < 0.05$). The fatty acid C20:0 was a high percentage in fish tissue of the control+PGPR group ($p < 0.05$). The fatty acid C18:3(n-3) was a high percentage in fish tissue of the S0%L6% (PGPR) group ($p < 0.05$). The fatty acid C20:1 was a high percentage in fish tissue of control+PGPR and S0%L6%+PGPR groups ($p < 0.05$). The C21:0 and C22:0 were high percentages in the fish tissue of the control+PGPR group ($p < 0.05$). The fatty acid C20:3(n-3) was a high percentage in fish tissue of the S0%L6%+PGPR group ($p < 0.05$). The fatty acid C20:4(n-6) was a high percentage in fish tissue of the control+PGPR group ($p < 0.05$). The fatty acid C23:0 was a high percentage in fish tissue of control+PGPR, S6%L0%+PGPR, and S0%L6%+PGPR groups ($p < 0.05$). The fatty acid C20:5(n-3) was the high percentage in fish tissue of control, S0%L6%, control+PGPR, and S0%L6%+PGPR groups with significantly different ($p < 0.05$). The C24:0 and C24:1 were high percentages in fish tissue of the control+PGPR group ($p < 0.05$). C21:5(n-3a) was a high percentage in fish tissue of S0%L6% and S0%L6%+PGPR groups ($p < 0.05$). The C22:6(n-3) was a high percentage in fish tissue of the S0%L6% group ($p < 0.05$).

Table4.2: Comparison of fatty acids analysis results in whole zebrafish body between all groups.

Types of fatty acids (%)	**Control group**	**Control+ PGPR group**	**S6%L0% group**	**S6%L0% + PGPR group**	**S0%L6% group**	**S0%L6%+ PGPR group**
C8:0	0.005± 0.001^{c}	0.014± 0.003^{a}	0.004± 0.0002^{c}	0.013± 0.003^{a}	0.005± 0.001^{c}	0.009± 0.002^{b}
C10:0	0.009± 0.002^{b}	0.016± 0.002^{a}	0.008± 0.0002^{b}	0.014± 0.001^{a}	0.01± 0.001^{b}	0.015± 0.002^{a}
C12:0	0.019± 0.005^{d}	0.033± 0.003^{a}	0.016± 0.001^{d}	0.025± 0.002^{bc}	0.021± 0.001^{cd}	0.029± 0.003^{ab}
C13:0	0.018± 0.004^{cd}	0.03± 0.003^{a}	0.014± 0.001^{c}	0.022± 0.002^{bc}	0.018± 0.0004^{cd}	0.026± 0.003^{ab}
C14:0	0.534± 0.011^{b}	0.831± 0.044^{a}	0.341± 0.007^{c}	0.464± 0.022^{b}	0.467± 0.009^{b}	0.505± 0.009^{b}
C16:0	7.577± 1.551^{cd}	11.34± 0.447^{a}	6.408± 0.156^{d}	9.016± 0.266^{b}	8.672± 0.098^{bc}	10.683± 0.352^{a}
C16:1(n-7)	1.216± 0.247^{bc}	1.656± 0.111^{a}	0.839± 0.02^{d}	1.131± 0.021^{c}	1.177± 0.014^{bc}	1.377± 0.034^{b}
C17:0	0.226± 0.042^{b}	0.34± 0.018^{a}	0.182± 0.007^{c}	0.245± 0.009^{b}	0.23± 0.017^{b}	0.264± 0.007^{b}
C18:0	1.862± 0.383^{c}	3.753± 0.136^{a}	2.014± 0.053^{c}	2.666± 0.085^{b}	2.164± 0.037^{c}	2.876± 0.196^{b}
C18:1(n-9)	3.49± 0.054^{c}	3.929± 0.051^{b}	4.551± 0.238^{a}	3.887± 0.296^{b}	3.798± 0.047^{b}	3.745± 0.166^{c}
C18:2(n-6)	0.313± 0.007^{d}	0.42± 0.014^{d}	5.169± 0.111^{a}	4.547± 0.345^{b}	1.457± 0.021^{c}	1.503± 0.064^{c}
C20:0	0.055± 0.01^{c}	0.109± 0.004^{a}	0.055± 0.002^{c}	0.08± 0.002^{b}	0.063± 0.001^{c}	0.082± 0.007^{b}

Table4.2: Comparison of fatty acids analysis results in whole zebrafish body between all groups (continued).

Types of fatty acids (%)	Control group	Control+ PGPR group	S6%L0% group	S6%L0%+ PGPR group	S0%L6% group	S0%L6%+ PGPR group
C18:3(n-3)	4.852±1.093^{c}	5.941±0.626^{b}	4.75±0.192^{c}	4.416±0.376^{c}	6.599±0.354^{b}	9.163±0.328^{a}
C20:1	0.12± 0.024^{bc}	0.15± 0.005^{a}	0.114±0.002^{c}	0.120±0.005^{bc}	0.137±0.006^{ab}	0.1508±0.009^{a}
C21:0	0.014±0.004^{c}	0.075±0.007^{a}	0.012±0.001 ^{c}	0.056±0.002^{ab}	0.02±0.001^{c}	0.047±0.033^{b}
C22:0	0.052±0.009^{c}	0.092±0.006^{a}	0.058± 0.001^{c}	0.081±0.001^{b}	0.06±0.002^{c}	0.080±0.006^{b}
C20:3(n-3)	0.329±0.072^{c}	0.395±0.043^{b}	0.292±0.013^{c}	0.291±0.025^{c}	0.393±0.022 ^{b}	0.529±0.019^{a}
C20:4(n-6)	0.085±0.016^{b}	0.103±0.008^{a}	0.07±0.003^{b}	0.079±0.003^{b}	0.085± 0.0004^{b}	0.082±0.007^{b}
C23:0	0.013±0.003^{b}	0.025±0.002^{a}	0.012±0.002^{b}	0.021±0.002^{a}	0.015±0.001^{b}	0.024±0.004^{a}
C20:5(n-3)	0.518±0.108^{a}	0.591±0.066^{a}	0.324± 0.015^{b}	0.319±0.033^{b}	0.55±0.013^{a}	0.59±0.024^{a}
C24:0	0.023±0.012^{b}	0.042±0.004^{a}	0.022±0.010^{b}	0.03± 0.012^{ab}	0.03± 0.001^{ab}	0.032±0.012^{ab}
C24:1	0.013±0.002^{b}	0.032±0.022^{a}	0.012± 0.002^{b}	0.015±0.001^{ab}	0.015± 0.002^{ab}	0.02±0.001^{ab}
C22:5(n-3)	0.236±0.051 ^{b}	0.222± 0.028^{b}	0.197±0.008^{bc}	0.17± 0.023^{c}	0.294±0.007^{a}	0.288±0.013^{a}
C22:6(n-3)	1.317±0.303^{ab}	1.222± 0.151^{ab}	0.887±0.049^{cd}	0.669±0.071^{d}	1.483±0.023^{a}	1.087±0.04^{bc}

4.1.3. Fatty Acid Analysis of Faeces

In Table(4.3) there is similarity percentage the C8:0 ($p > 0.05$) between the control and experimental groups' faeces. The fish faeces of the control group were a high percentage of C23:0 ($p < 0.05$). The fish faeces of S6%L0% group were high percentage of C18:2(n-6), C20:1, C22:0, C20:4(n-6), C24:0, and C24:1 ($p < 0.05$). The C10:0, C12:0, C13:0, C14:0, C16:0, C16:1(n-7), C17:0, C18:0, C20:0, C20:1, C21:0, C22:0, C20:3(n-3), C20:4(n-6), C23:0, C20:5(n-3), C24:0, C24:1, C22:5(n-3), and C22:6(n-3)fatty acids were high percentage in fish faeces of control+PGPR group ($p < 0.05$). The fish faeces S6%L0%+PGPR group was a high percentage of C18:1(n-9) ($p < 0.05$). The fish faeces S0%L6%+PGPR group was high percentage of C18:1(n-9) and C18:3(n-3) ($p < 0.05$).

Table4.3: Fatty acids analysis results in the fish faeces of control and experimental groups.

Types of fatty acids (%)	Control group	Control+ PGPR group	S6%L0% group	S6%L0%+ PGPR group	S0%L6% group	S0%L6%+ PGPR group
C8:0	0.008±0.002	0.012±0.003	0.012±0.004	0.010±0.001	0.008±0.003	0.008±0.002
C10:0	0.012±0.005 ^{ab}	0.02±0.005^{a}	0.015±0.006^{ab}	0.01±0.001^{b}	0.01±0.005^{b}	0.012±0.003^{ab}
C12:0	0.019±0.008 ^{b}	0.031±0.007^{a}	0.024±0.009^{ab}	0.017±0.001^{b}	0.016±0.007^{b}	0.019±0.005^{b}
C13:0	0.014±0.009 ^{b}	0.026 0.007^{a}	0.020±0.009^{ab}	0.012±0.001^{b}	0.013±0.007^{b}	0.016±0.005^{ab}
C14:0	0.160± 0.04^{c}	0.278±0.029^{a}	0.242±0.066^{ab}	0.146±0.01^{c}	0.146±0.031^{c}	0.181±0.036^{bc}
C16:0	1.455± 0.431^{c}	2.54±0.342^{a}	2.34919101± 0.564854^{ab}	1.414±0.083^{c}	1.439±0.335^{c}	1.694±0.366^{bc}
C16:1(n-7)	0.273±0.054 ^{b}	0.491±0.044^{a}	0.329±0.06^{b}	0.242±0.007^{b}	0.259±0.046^{b}	0.296±0.049^{b}
C17:0	0.073±0.023 ^{c}	0.125±0.014^{a}	0.07±0.031^{c}	0.065±0.005^{c}	0.115±0.016^{ab}	0.084±0.017^{bc}
C18:0	0.552±0.165 ^{c}	0.929±0.017^{a}	0.866±0.195^{ab}	0.53±0.068^{c}	0.539±0.141 ^{c}	0.624±0.134^{bc}
C18:1(n-9)	0.21±0.044^{b}	0.38±0.027^{b}	0.305±0.062^{b}	0.954±0.114^{a}	0.21±0.04^{b}	1.056±0.236^{a}
C18:2(n-6)	0.067±0.017 ^{c}	0.135±0.113^{bc}	0.317±0.097^{a}	0.213±0.035^{b}	0.085±0.021^{c}	0.15± 0.06^{bc}
C20:0	0.039±0.017 ^{bc}	0.072±0.001^{a}	0.062±0.019^{ab}	0.036±0.002^{c}	0.037±0.012^{bc}	0.045±0.011^{bc}

Table4.3: Fatty acids analysis results in the fish faeces of control and experimental groups (continued).

Types of fatty acids (%)	Control group	Control+ PGPR group	S6%L0% group	S6%L0%+ PGPR group	S0%L6% group	S0%L6%+ PGPR group
C18:3(n-3)	0.046± 0.006^{c}	0.114± 0.014^{ab}	0.113± 0.060^{ab}	0.054± 0.013^{bc}	0.079± 0.019^{bc}	0.145± 0.043^{a}
C20:1	0.093± 0.029^{b}	0.159± 0.016^{a}	0.162± 0.041^{a}	0.087± 0.008^{b}	0.089± 0.017^{b}	0.111± 0.021^{b}
C21:0	0.018± 0.011^{b}	0.035± 0.008^{a}	0.028± 0.011^{ab}	0.016± 0.001^{b}	0.017± 0.008^{b}	0.022± 0.006^{ab}
C22:0	0.03± 0.014^{b}	0.054± 0.008^{a}	0.055± 0.017^{a}	0.032± 0.003^{b}	0.03± 0.01^{b}	0.035± 0.008^{ab}
C20:3(n-3)	0.015± 0.008^{b}	0.029± 0.006^{a}	0.026± 0.01^{ab}	0.014± 0.002^{b}	0.014± 0.007^{b}	0.020± 0.006^{ab}
C20:4(n-6)	0.031± 0.014^{b}	0.061± 0.008^{a}	0.056± 0.017^{a}	0.031± 0.004^{b}	0.03± 0.01^{b}	0.047± 0.01^{ab}
C23:0	0.019± 0.011^{a}	0.035± 0.008^{a}	0.029± 0.011^{ab}	0.016± 0.001^{b}	0.018± 0.008^{b}	0.021± 0.005^{ab}
C20:5(n-3)	0.033± 0.013^{c}	0.093± 0.009^{a}	0.056± 0.016^{b}	0.032± 0.003^{c}	0.029± 0.009^{c}	0.069± 0.016^{b}
C24:0	0.029± 0.012^{b}	0.052± 0.008^{a}	0.048± 0.014^{a}	0.029± 0.003^{b}	0.03± 0.01^{b}	0.034± 0.007^{ab}
C24:1	0.069± 0.018^{b}	0.119± 0.009^{a}	0.111± 0.023^{a}	0.058± 0.006^{b}	0.065± 0.011^{b}	0.076± 0.008^{b}
C22:5(n-3)	0.01± 0.006^{b}	0.023± 0.003^{a}	0.017± 0.006^{ab}	0.009± 0.001^{b}	0.009± 0.004^{b}	0.014± 0.005^{b}
C22:6(n-3)	0.055± 0.019^{d}	0.163± 0.015^{a}	0.110± 0.028^{b}	0.058± 0.011^{cd}	0.053± 0.012^{d}	0.092± 0.025^{bc}

4.1.4. Fatty Acids Digestibility (%)

In Table(4.4) there were propinquity between control and experimental groups ($p > 0.05$) in C8:0, C10:0, C12:0, and C13:0, C21:0, C24:0 digestibility(%). S6%L0% group was low digestibility (%) in C14:0 and C16:0 significantly ($p < 0.05$) among other groups. The C16:1(n-7) in control was high digestibility (%) ($p < 0.05$) thereamong anthor groups. S6%L0% group was low digestibility (%) in C17:0, C18:0, C20:1, C20:3(n-3), C20:4(n-6), C20:5(n-3), C22:5(n-3), and C22:5(n-3) ($p < 0.05$) among other groups. S6%L0%+PGPR was low digestibility (%) in C18:1(n-9) significantly ($p < 0.05$) among other groups. Group diets content fish oil was low digestibility (%) in C18:2(n-6) ($p < 0.05$) compare with other groups. S0%L6%+PGPR group was high digestibility (%) in C22:0 ($p < 0.05$) compare with other groups.

Table4.4: Fatty acids digestibility (%) results in the control and experimental groups.

Types of fatty acids (%)	Control group	Control+ PGPR group	S6%L0% group	S6%L0%+ PGPR group	S0%L6% group	S0%L6%+ PGPR group
C8:0	17.971± 4.683	15.220± 17.681	16.631± 18.676	28.525± 8.327	26.139± 24.721	35.286± 22.162
C10:0	35.612± 7.627	28.394± 16.635	26.821± 29.340	49.177± 7.005	46.747± 22.665	47.726± 20.049
C12:0	65.071± 19.014	58.717± 8.693	41.749± 21.077	57.461± 5.833	58.304± 14.031	59.536± 14.337
C13:0	72.185± 21.311	62.895± 9.102	49.629± 20.536	66.862± 4.682	66.197± 14.321	63.544± 14.051
C14:0	94.195± 2.092 [a]	92.878± 0.925 [a]	84.227± 4.331 [b]	90.620± 1.417 [a]	90.425± 1.917 [a]	90.192± 2.840 [a]
C16:0	88.059± 4.852 [a]	85.443± 1.941 [a]	76.625± 5.734 [b]	86.542± 1.205 [a]	85.367± 3.465 [a]	86.174± 4.180 [a]
C16:1(n-7)	91.201± 2.500 [a]	88.664± 1.139 [ab]	80.731± 4.133 [c]	85.910± 2.197 [abc]	84.855± 3.167 [bc]	85.499± 3.624 [bc]
C17:0	87.530± 5.417 [a]	84.708± 1.824 [a]	72.437± 7.484 [b]	84.815± 2.529 [a]	83.807± 3.282 [a]	83.655± 4.592 [a]
C18:0	80.320± 8.133 [a]	76.756± 3.691 [ab]	69.955± 7.246 [b]	82.619± 1.35 [a]	82.470± 4.884 [a]	84.109± 4.614 [a]
C18:1(n-9)	98.177± 0.552 [a]	97.729± 0.183 [a]	98.053± 0.355 [a]	94.109± 1.054 [b]	98.346± 0.333 [a]	93.447± 1.967 [b]
C18:2(n-6)	93.476± 2.442 [b]	92.470± 0.455 [b]	98.218± 0.515 [a]	98.875± 0.241 [a]	98.271± 0.336 [a]	97.663± 1.101 [a]
C20:0	89.154± 6.135[a]	85.792± 1.991 [ab]	77.347± 6.965 [b]	86.758± 2.056 [ab]	84.888± 4.027 [ab]	84.742± 4.554 [ab]

Table 4.4: Fatty acids digestibility (%) results in the control and experimental groups (continued).

Types of fatty acids (%)	Control group	Control+ PGPR group	S6%L0% group	S6%L0%+ PGPR group	S0%L6% group	S0%L6%+ PGPR group
C18:3(n-3)	88.035± 2.988 ^{ab}	86.239± 2.246 ^{ab}	43.461± 25.7 ^{b}	76.710± 4.230 ^{b}	99.361± 0.12^{a}	99.140± 0.317 ^{a}
C20:1	88.338± 5.131 ^{a}	85.424± 1.526 ^{a}	74.150± 5.96 ^{b}	86.982± 2.491 ^{a}	86.565± 2.65^{a}	86.106± 4.157 ^{a}
C21:0	77.345± 16.576	70.205± 5.719	55.535± 16.973	72.796± 4.551	71.940± 11.29	69.437± 10.981
C22:0	79.962± 11.936^{ab}	73.756± 3.422 ^{b}	80.976± 5.68^{ab}	89.087± 2.210 ^{a}	78.799± 5.68^{ab}	79.165± 6.434 ^{ab}
C20:3(n-3)	83.793± 10.832 ^{a}	77.992± 3.296 ^{a}	60.708± 14.2^{b}	77.406± 3.927 ^{a}	80.777± 7.22^{a}	76.358± 8.763 ^{ab}
C20:4(n-6)	91.245± 4.666 ^{a}	87.215± 1.500 ^{ab}	72.944± 9.09^{c}	84.461± 3.251 ^{ab}	85.654± 3.53^{ab}	79.622± 6.585 ^{bc}
C23:0	71.493± 19.962	61.757± 7.244	54.018± 16.47	72.152± 4.921	69.957± 11.49	68.195± 10.785
C20:5(n-3)	98.987± 0.522 ^{a}	97.943± 0.152 ^{a}	95.948± 1.17^{b}	97.559± 0.457 ^{a}	97.984± 0.37^{a}	95.699± 1.252 ^{b}
C24:0	74.936± 14.038	66.900± 3.672	69.344± 8.648	81.358± 3.994	74.649± 6.451	75.131± 6.872
C24:1	82.672± 7.089 ^{a}	77.789± 2.179 ^{a}	61.056± 9.30^{b}	79.976± 4.546 a	77.819± 3.08^{a}	77.605± 3.960 ^{a}
C22:5(n-3)	96.839± 2.154 ^{a}	94.861± 0.514 ^{ab}	88.504± 4.29^{c}	93.446± 1.128 ^{ab}	93.895± 2.08^{ab}	91.837± 3.239 ^{bc}
C22:6(n-3)	99.103± 0.418 ^{a}	98.072± 0.210 ^{ab}	95.926± 1.04^{c}	97.706± 0.632 ^{b}	98.118± 0.21^{ab}	96.987± 1.015 ^{bc}

4.2. Lipids in Whole Fish Body and ∑SFAs, ∑MUFAs, ∑HUFAs and ∑PUFAs

In Figure(4.1) The fish body in control+PGPR and S0%L6%+PGPR (Figure8) content high lipids percentage with significant different ($p<0.05$) thereamong the other groups.

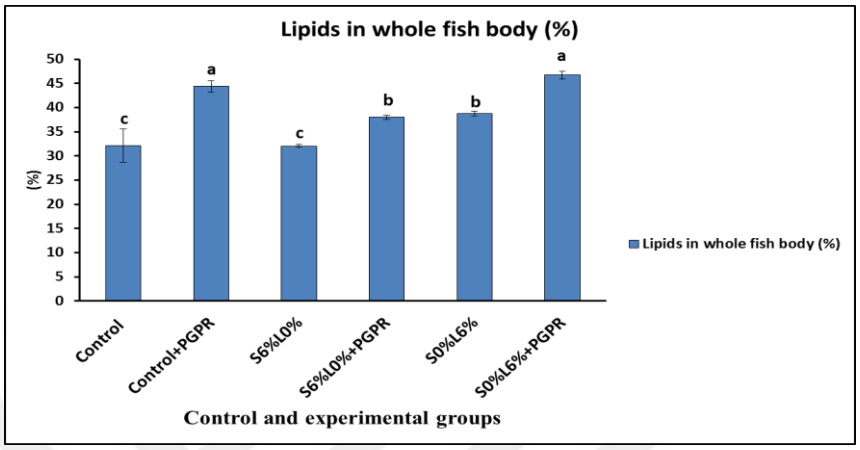

Figure4.1: Illustrate total lipids in whole fish body (%) in control and experimental groups.

In (Figure4.2) The control+PGPR diet and fish body group had high ∑SFAs with significant different ($p<0.05$) than other groups.

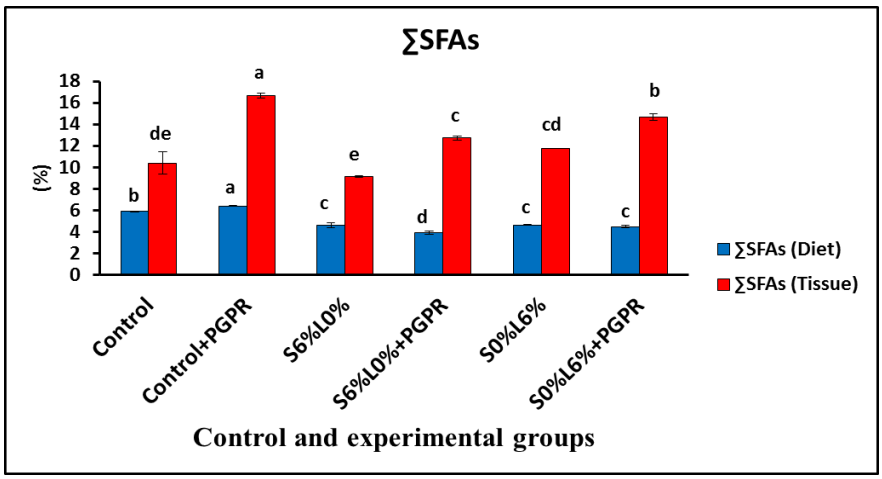

Figure4.2: Illustrate ∑SFAs(%) in diets, and fish body.

In (Figure4.3)The control+PGPR and S6%L0% group had high in total mono-unsaturated fatty acids (∑MFAs) percentage in diet with significant different ($p<0.05$), while S0%L6% (PGPR) group had high in total mono-unsaturated fatty acids (∑MFAs) percentage in fish tissue with significantly ($p<0.05$).

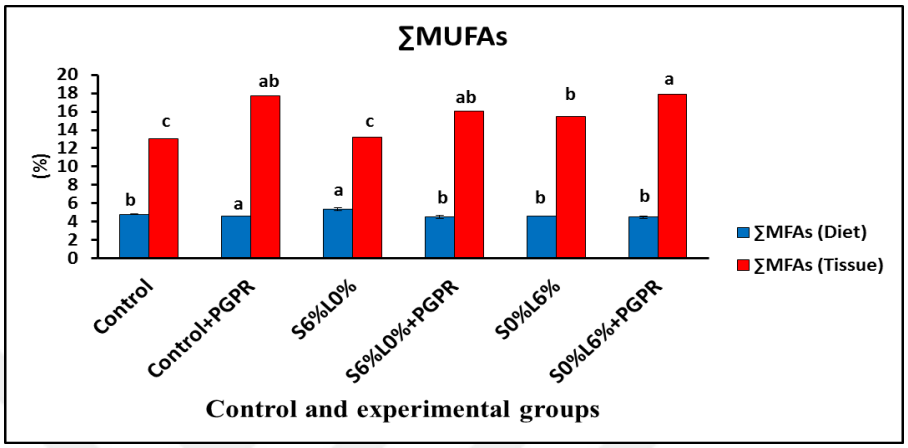

Figure4.3: Illustrate ∑MFAs (%) in diets, and fish body.

In Figure(4.4) The total high unsaturated fatty acids(∑HUFAs) and poly unsaturated fatty acids (∑PUFAs) percentage had high percentage in diet of S6%L0%, S0%L6% and S0%L6%+PGPR group with significant different ($p<0.05$), however S0%L6%+PGPR group had high total poly mono-unsaturated fatty acids (∑PUFAs) percentage in fish body with significant different ($p<0.05$) among other groups.

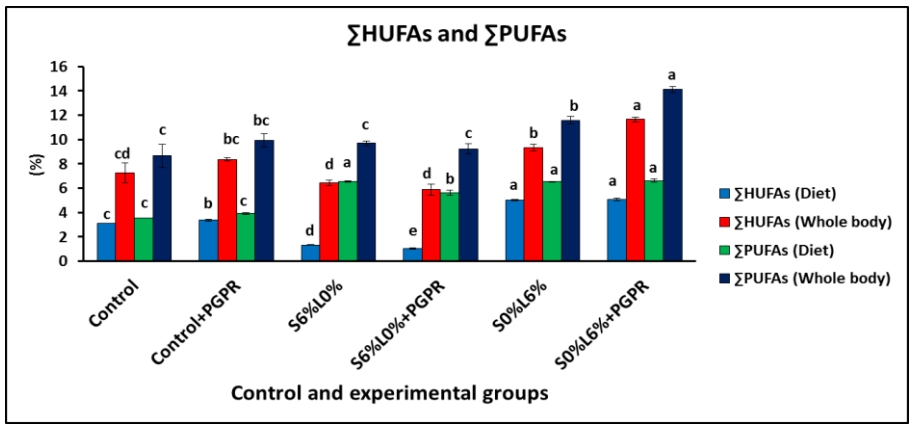

Figure4.4: Illustrate∑HUFAs and ∑PUFAs(%) in diets, and fish body.

4.3. Survival Rate , Growth Measurements , and Diets Exploitation Efficiency

In Figure(4.5) There were no more differentiation in fish death conditions between groups ($p>0.05$).

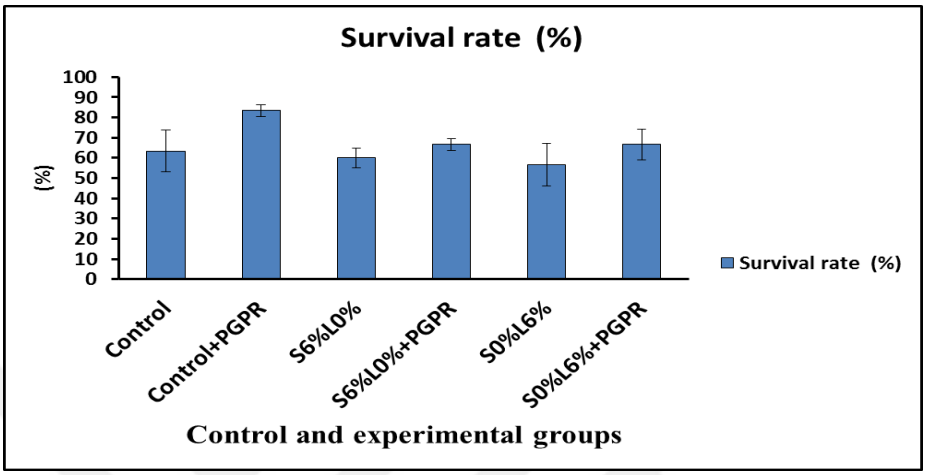

Figure4.5: Show fish survival rate in control and experimental group until the end of the experiment.

In Figure(4.6) and Figure(4.7) From the first feed day until completed the experiment; changing of individual mean live body weight and weight gain show the effects of control and experimental feeds on weight changes of zebrafish. The general, male and female final weight and weight gain had high values in S0% L6%+PGPR group ($p< 0.05$) among other groups.

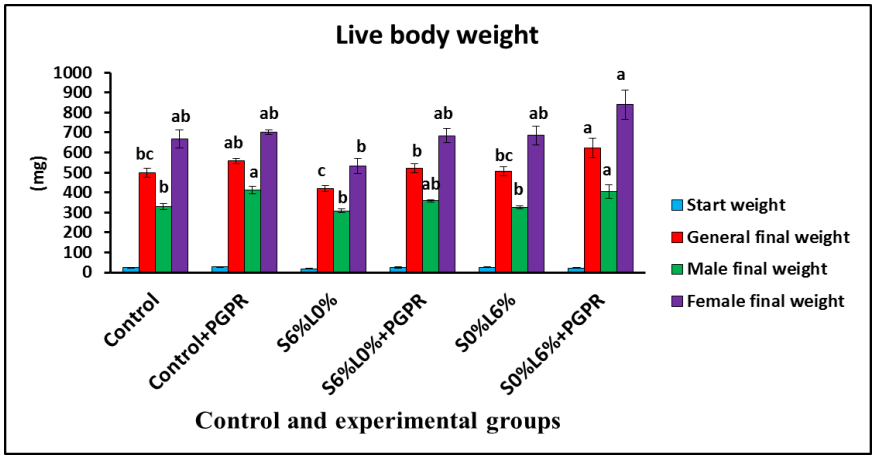

Figure4.6: Illustrate the fish live body weight (mg) at the beginning until final of the experiment period.

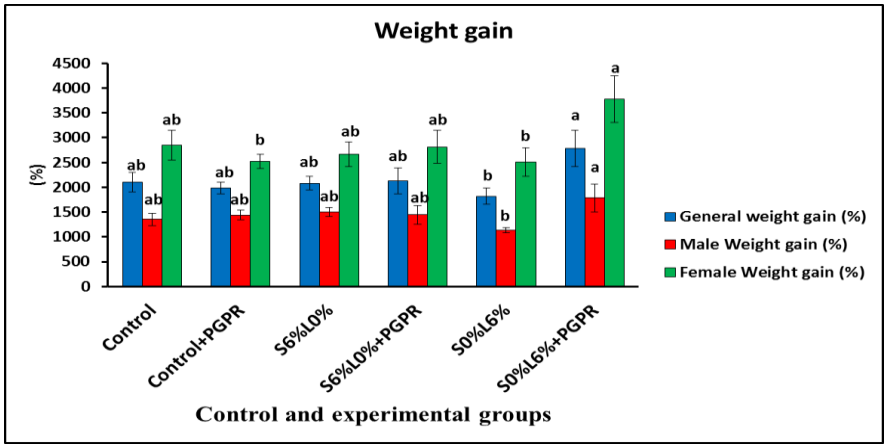

Figure4.7: Illustrate the fish live body weight gain (%) at the eventually of the experiment.

In Figure(4.8) the individual mean of daily obtained weight increasing SGR values for male, female, and together was high in S0% L6%+PGPR with significant different ($p < 0.05$) between other groups.

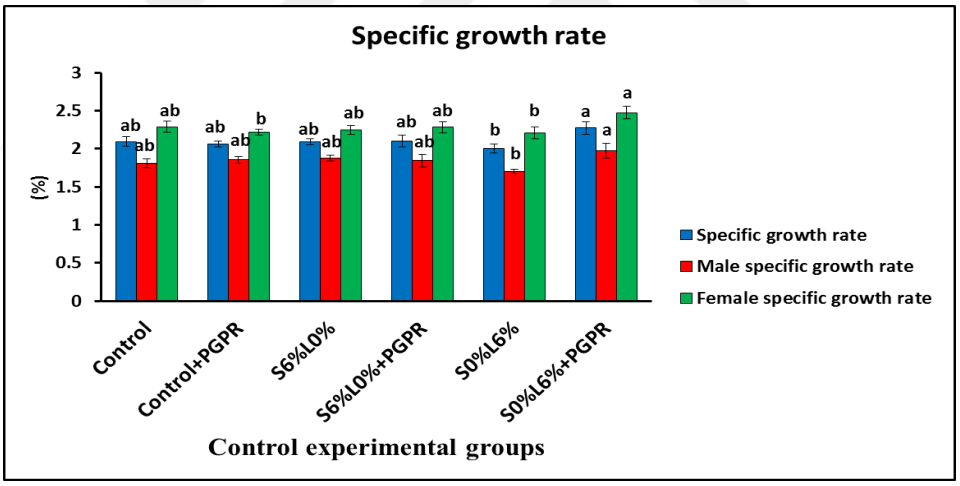

Figure4.8: Illustrate SGR at last of the experimental period.

In Figure(4.9) the S0%L6% and S0%L6%+PGPR group had high daily dry matter feed intake ($p>0.05$), but high with significantly ($p< 0.05$), in daily lipids feed intake and digested lipids values.

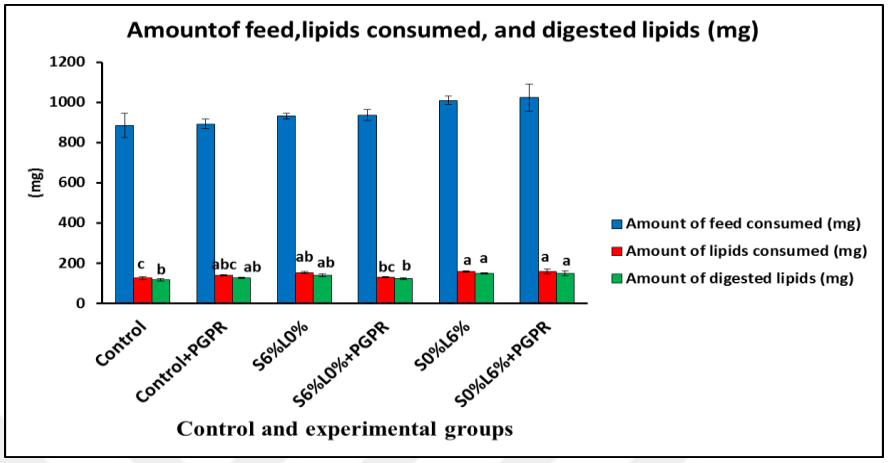

Figure4.9: Illustrate amount of diet and dietary lipid (mg) that consumed by fish and digested lipids amount (mg) during experiment period.

In Figure(4.10) no significant different ($p>0.05$) thereamong all groups in feed digestibility percentage, but lipids digestibility percentage was high with significant different ($p<0.05$) in control+PGPR and S0%L6%+PGPR group.

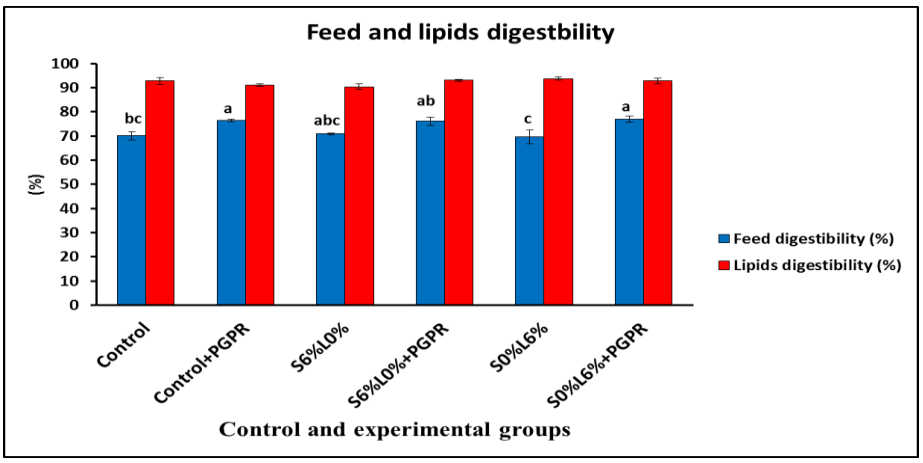

Figure4.10: Illustrate feed and lipids digestibility (%) of fish during experiment period.

In Figure(4.11) the S0%L0% (PGPR) and S0%L6% (PGPR) group both had significant different ($p<0.05$) in FCR and PER.

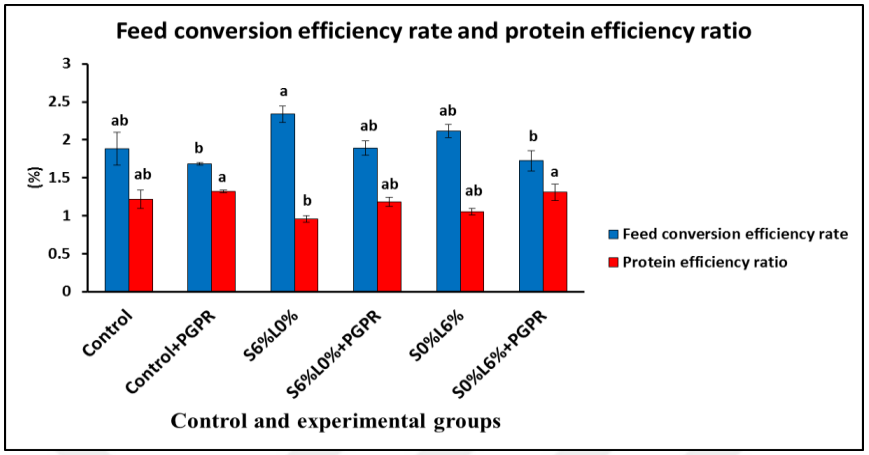

Figure4.11: Illustrate FCR and PER of zebrafish during experiment period.

4.4. Reproductive Performance

In Figure(4.12); SKPs (μm/s) values in motility was no significant difference ($p>0.05$) between groups at the sixth second from the start of the sperm motility, but the S6%L0% group had high values significantly ($p<0.05$) at the ninth and twelfth second from starting motility point.

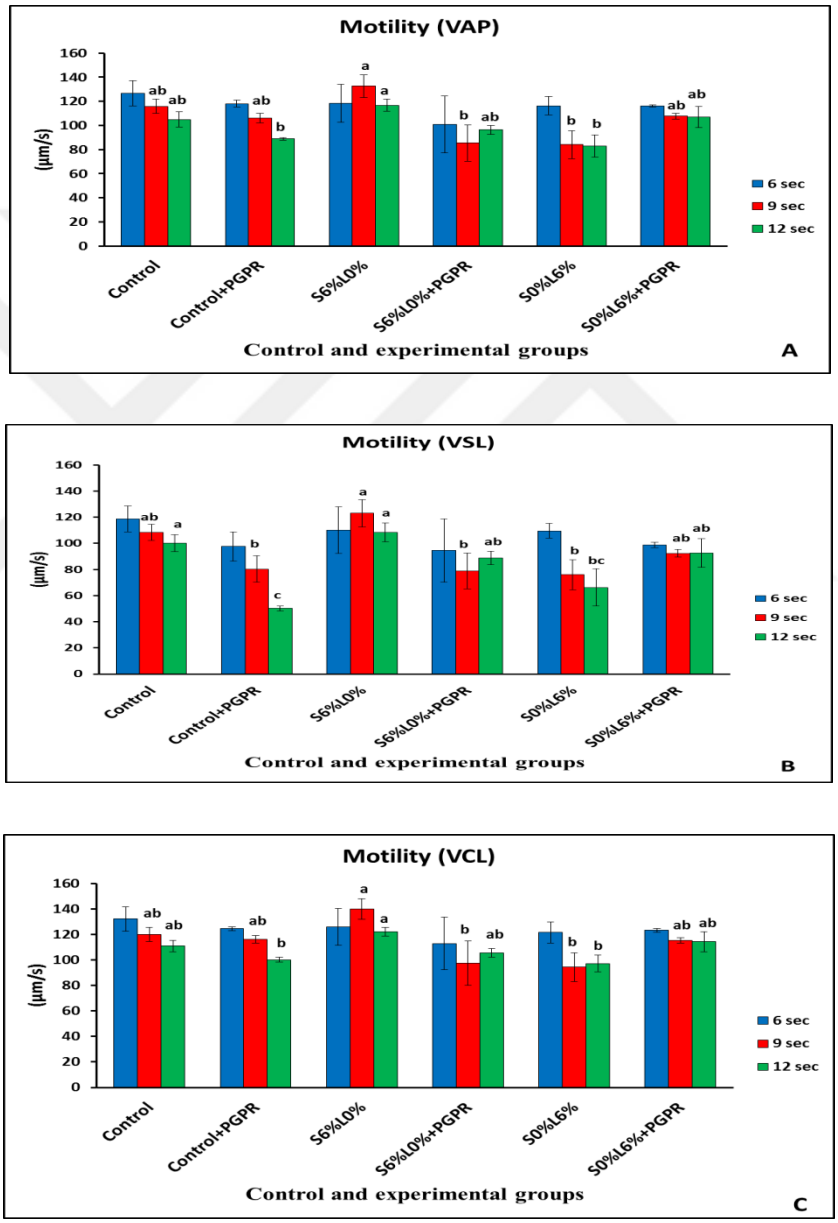

Figure4.12: Illustrate the effect of emulsifier material with sunflower and linseed oil on SKPs of mobility changes (μm/s) for zebrafish males in all groups at three time points (6th , 9th , and 12th sec.).

In Figure(4.13); the S6%L0%+PGPR group had high values with ($p< 0.05$) in progressive motility SKPs (μm/s), with S6%L0% group in progressive motility VSL (μm/s) at the 6th sec. from the start of the sperm motility. S6%L0% and S0%L6%+PGPR groups had ($p< 0.05$) in progressive VAP, VCL, and VSL (μm/s) at the 9th sec., while no significant difference ($p> 0.05$) between groups in progressive VAP (μm/s)) at 12thsec.from the start of sperm motility. S6%L0% and S6%L0%+PGPR group progressive motility in VCL (μm/s) value at the 12th sec. was raise ($p< 0.05$) and S6%L0% group in VSL (μm/s).

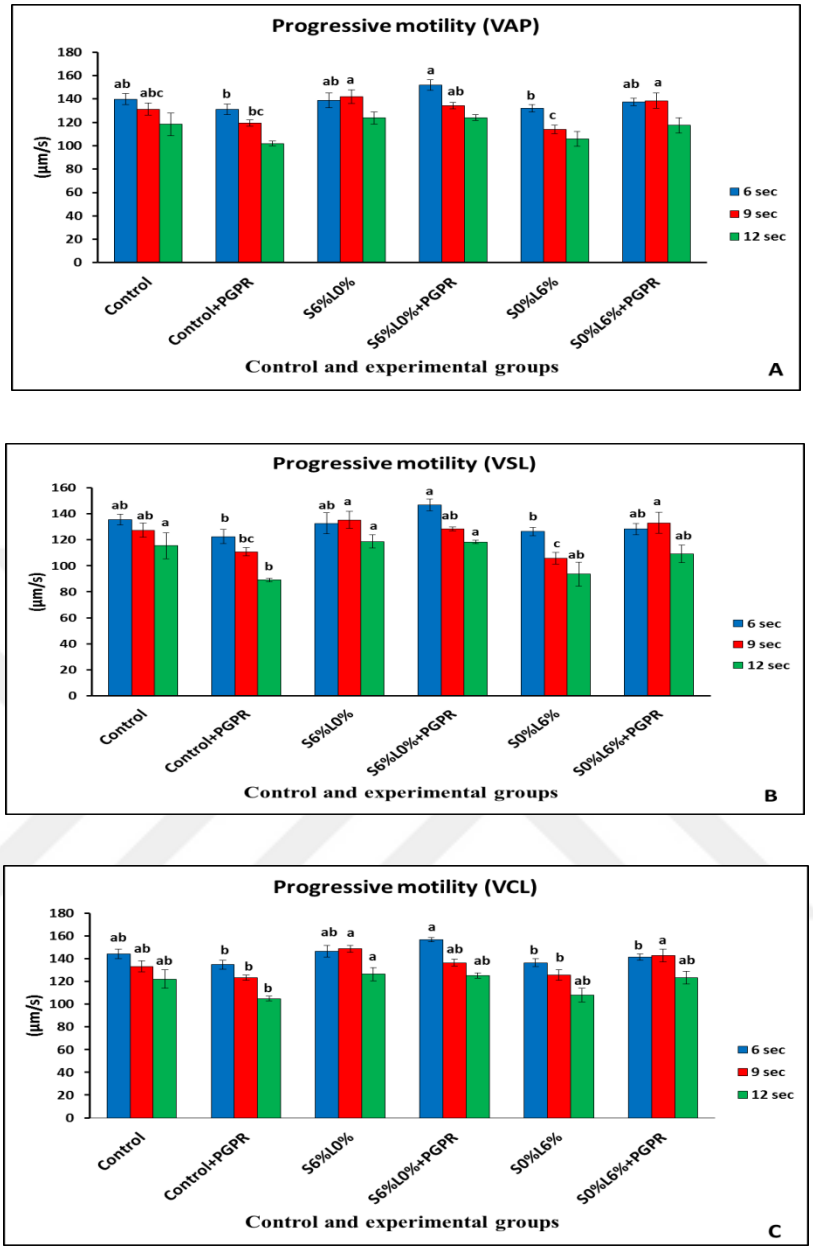

Figure4.13: Illustrate the effect of emulsifier material with sunflower and linseed oil on progressive motility SKPs (µm/s) of zebrafish males in all groups at three time points (6th , 9th , and 12th sec.).

In Figure (4.14) motility time from the zero second until vibration point time semi equal ($p > 0.05$) among the groups, but S0%L6%, control+PGPR, and S0%L6%+PGPR sperm had longer total motility time from the zero second to complete stop ($p < 0.05$).

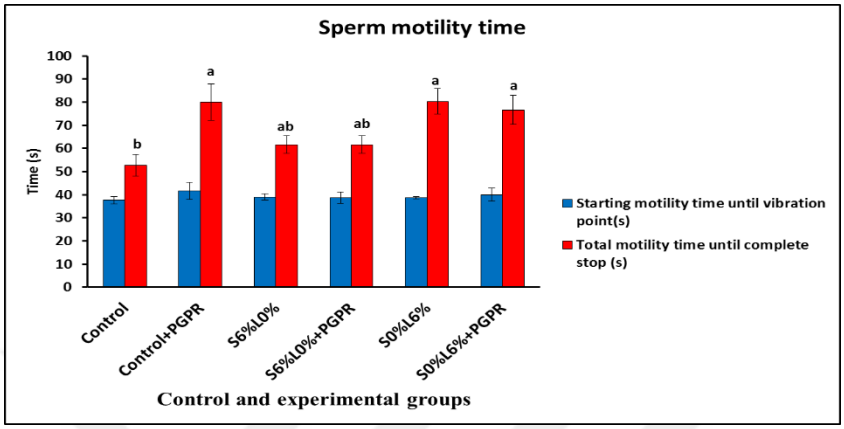

Figure4.14: Illustrate the effect of emulsifier material with sunflower and linseed oil on sperm motility time (s) from starting motility time until vibration point and total from beginning until a complete stop.

In Figure(4.15) the control, control+PGPR, and S0%L6% groups had high fertilized egg percentage and hatching rates ($p<0.05$) among other groups.

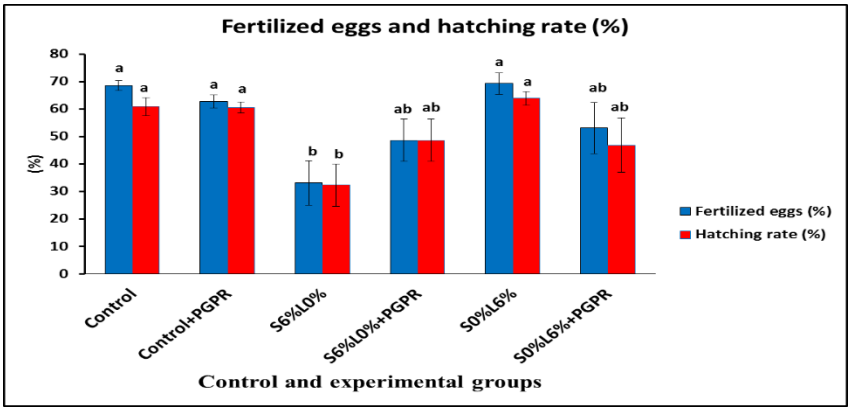

Figure 4.15: Illustrate the effect of emulsifier material with sunflower and linseed oil on fertilized and hatching rate (%) of control and experimental groups eggs.

In Figure(4.16) the groups; control+PGPR, S6%L0%+PGPR, and S0%L6%+PGPR had the biggest egg diameter (µm) ($p<0.05$), while only S0%L6%+PGPR group had the biggest yolk diameter (µm) ($p<0.05$). control+PGPR and S6%L0%+PGPR had the longest larvae length (µm) ($p<0.05$).

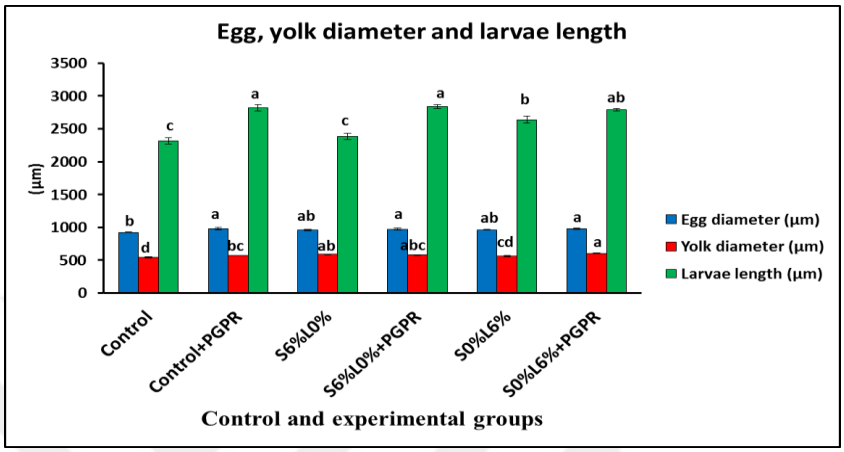

Figure4.16: Illustrate the effect of emulsifier material with sunflower and linseed oil on the egg, yolk diameter, and larvae length (µm).

In Figure (4.17) the S6%L0%+PGPR and S0%L6%+PGPR groups had high score degree ($p< 0.05$) of embryo development at 9 hpf ((90%-epiboly). The embryo development at 25 and 48 hpf (prim-6 and long pec) in all groups had close score degrees ($p> 0.05$). The control group was less score degree ($p< 0.05$) at 35 hpf of embryo development (prim-25).

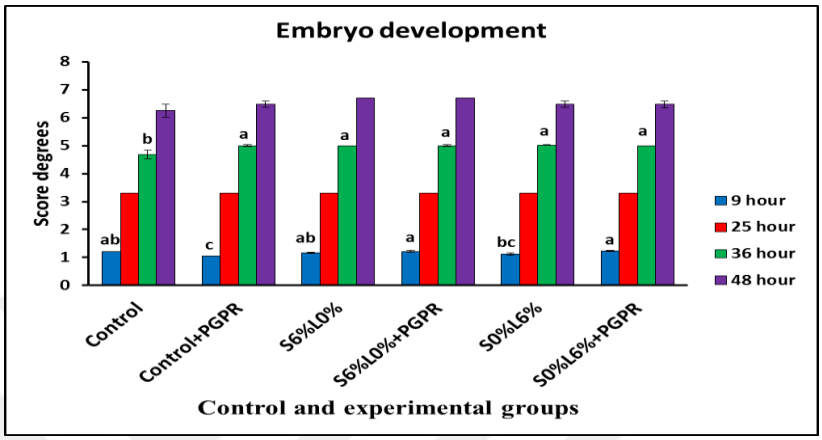

Figure4.17: Illustrate the effect of emulsifier material with sunflower and linseed oil on embryo development in four embryo stages; (90%-epiboly, prim-6, prim-25, and long pec) at four times (9, 25, 36, and 48 hour of post-fertilization) with embryo developing scores (Araújo et al., 2017); (1.3, 3.3, 4.9, and 6.7 respectively) at times; (9, 25, 35, and 48-hour post fertilization) from total incubation period.

5. DISCUSSION AND CONCLUSION

In this study, when the result of essential fatty acids analysis of fish oil, sunflower oil, and linseed oil (Wasseff et al., 2015) was compared with the fatty acid results of the oils used in zebrafish feed in the study (Table 4.1), the results showed parallelism. In (Table4.1) the C20:4(n-6), C20:5(n-3), 22:5(n-3), and C22:6 (n-3) were high percentages with significant different ($p<0.05$) in the diets content fish. While the sunflower oil diets were a high percentage of C18:2(n-6) with a significant difference ($p<0.05$) and linseed oil diets were a high percentage of essential acids; 18:3(n-3) with significantly different ($p<0.05$). The fact that there was no difference between the fatty acid data of the feed created using the oils used in the zebrafish feed formulated in our study and the fatty acid results of the forms of the oils before they were added to the feeds shows that the feed formulation used in zebrafish nutrition was formed correctly in the study. The more effects of emulsifier on saturated fatty acids digestibility than other long-chain fatty acids principally on the C16:0 and C18:0 fatty acids (Siyal *et al*., 2017).

There is no difference in saturated fatty acids of zebrafish tissues profile when using vegetable oils as a complete replacement to fish oil in the diet while linoleic acid and linolenic acid were high percentages in fish fed vegetable oils diet and other essential fatty acids were high in fed fish oil diet (Tocher *et al*., 2001). There is no more differentiation between saturated fatty acids percentages in the tissue of herbivorous, omnivorous, and carnivorous freshwater or marine fish, and C16:0 was a high percentage in the tissue (ranges between19.4 ± 4.4 to 28.5 ± 6.6% from total fatty acids) of both marine and freshwater fish among other saturated fatty acids profiles (Parzanini *et al*., 2020). In (Table4.2) the accumulation of saturated fatty acids was a high percentage in fish tissues that fed diets content PGPR with significantly different ($p<0.05$), then diets without PGPR, and this corresponds positively with the digestibility (%) results in (Table4.4). The percentage of C16:0 was high (Table4.2) in the tissue of every fish group than in other saturated fatty acids in the same group, especially in fish tissue that fed diet content PGPR. The C18 PUFAs are slow convert to other types of fatty acids in marine fish chiefly carnivorous species opposite than freshwater species that can transfer easy 18: 2n-6 and 18: 3n-3 to HUFAs in body lipids (Sargent et al., 2002; Tocher, 2003). In (Table4.1) the sunflower and linseed oil diet groups content high level of C18

PUFAs than fish oil diet groups, but zebrafish as freshwater fish have a good ability to transfer to HUFAs in whole body lipids (Figure 4.4).

In (Table4.3) control and control+PGPR groups faeces include less percentage of C18:2(n-6) fatty acids than other fish groups maybe it is normal because the fatty acid analysis of their diets (Table4.1) content less percentage of C18:2(n-6), and also the digestibility (%)(Table4.4) of both groups was low and there was no obvious effect of the emulsifier. The presence of C18:3(n-3) FA in diets Table(4.1) contained linseed Twenty times more than fish oil diets and seventy times more than sunflower diets,, therefore, increasing the percentage of C18:3(n-3) in the fish faeces group S0%L6% +PGPR Table(4.3). Despite the fish fed control and control+PGPR diet were high digestibility (%) of C20:4(n-6), C20:5(n-3), 22:5(n-3), and C22:6 (n-3) FAs Table(4.4) but in their faeces were high than other groups. The interaction effects of PGPR were clear with sunflower oil with than fish and linseed oil because the fish of the S6%L0%+PGPR group was high digestibility (%) of most fatty acids Table(4.4) and had fewer percentages in faeces Table(4.3), compare to with fish of S6%L0% group was low digestibility (%) of most fatty acids Table(4.4) and had high percentages in faeces Table(4.3). The utilization of dietary (n-3) fatty acids to compose body lipids is highly in cold water fish both marine and freshwater, for that the digestibility and transfer of this fatty acids is highly than other fatty acids groups and when compare between rainbow trout and carp muscle the (n-3) fatty acids is high in rainbow trout this means; its presence in the required quantities is necessary in the feed (Steffens and Wand , 2005; and Trushenski and Rombenso, 2020). When look at (Table 4.2 and Table 4.4) find that; the digestibility of (n-3) fatty acids and accumulated in zebrafish body was high similar to cold water marine and freshwater fish and the effective of PGPR was clear with C18:3(n-3) and C20:3(n-3) than other types of (n-3) fatty acids.

According to Fowler *et al*., (2019) Found; that zebrafish body lipids range from27% to 39.2% on dry matter base when fed with commercial and laboratory zebrafish diets. In Figure(4.1) the total body lipids that composed zebrafish adipose tissuse effected by deitary oil type with PGPR show in groups control+PGPR and S0%L6%+PGPR were excelsior ($p<0.05$) than in rest of groups, this explain the role of PGPR as an emulsifier in improving the utilization of dietary fatty acids in the synthesis of tissue fats, and thus preserving an estimated amount of gross energy in the fish body (Bontempo *et al*., 2018). When looking at Figure (4.2), it is

noted that the percentage of ∑SFAs was generally low in diets containing sunflower and linseed oils in contrast to fish oil diets. The ∑SFAs were excelsior ($p<0.05$) in fish depasture control+PGPR and S0%L6% (PGPR) regimes Figure(4.2) than those on other diets ($p<0.05$). In general, the fish fed experimental regimes containing PGPR tended to have higher percentages of ∑SFAs in their tissues, meaning that the emulsifier increased the availability and sludging of this FAs group of fish muscles. From Table(4.2) the percentage C16:0 is the highest percentage of the rest of the ∑SFAs in fish tissues, and in control+PGPR and S0%L6%+PGPR group, it reaches the highest percentage($p<0.05$) unlike its presence in the tissues of other fish groups. A similar tendency was the case for the ∑MUFAs in fish tissues Figure(4.3), where dietary exogenous PGPR significantly increased the ∑MUFAs of the whole body ($p<0.05$) draw a parallel between their un-supplemented likes of. Although the feeds containing linseed oil had a significantly higher propotion of ∑HUFAs and ∑PUFAs with ($p<0.05$) Figure(4.4), the zebrafish fed S0%L6%+PGPR showed a further significant increase the ∑HUFAs and ∑PUFAs in body lipids. The 6.7g/100g of linseed oil in zebrafish diet rasie level of EPA/ARA ratio and total PUFAs, then increase it in muscles, liver ,ovaries and eggs(Jaya-Ram *et al*., 2008). Altogether; there is a close interaction among the dietary emulsifier PGPR and major FAs groups stored in the body of zebrafish. Freshwater fish tissues are high in C16:0, ∑SFAs, ∑MUFAs, and ∑n-6 PUFA than marine fish, while the percentage of ∑n-3 PUFA is high in marine tissues than in Freshwater fish (Łuczyńska *et al*., 2014). The DHA and EPA in generally is high amounts in fish body as n-3 HUFAs than AA and other fatty acids, and had importance during spawning season it composed cell and tissues of gonads and considerable amount of eggs lipids especially in marine fish like seabream and other species (Rodriguez *et al*., 1998; Sargent *et al*., 1999; and Cejas *et al*., 2003). İn (Figure4.4) and (Table 4.1) reflect the high transport of dietary n-3 HUFAs to compose body lipids and enhance the reproductive process in form of the increase eggs size and raise the fertilized and hatching rate (%), this it appears clearly in diets groups contain (PGPR) and It may be explained by the effects of PGPR substance on this group of fatty acids (n-3 HUFAs) on their metabolism after absorption.

The presence of PGPR with 0.06% level in the zebrafish diets had not opposite effects on fish wellnes and growth rate, For this reason; the paradoxes survival rate slightly nerrow among the zebrafish groups ($p>0.05$) (Figure4.5), it can be claimed that (PGPR) is not poisonous or harmful side effects for fish. This claim is consistent with the signs of a number of quondam

enquiries (Roy *et al*., 2010; Mortensen *et al*., 2017; Wilson and Smith, 1998; Wilson *et al*., 1998,). Generally the results in of growth, feed utilization parameters and total body constituent were significantly influenced by dietary composition. This reflects the effects of dietary lipid types and whether they contain PGPR as an emulsifier or not. The fish in group S0%L6%+PGPR recorded the highest final live body weight and weight gain ($p<0.05$) among groups Figure(4.6) and Figure(4.7), when comparing the total types of fatty acids, group S0%L6%+PGPR contains the highest percentage ($p<0.05$) of ∑MUFAs and ∑PUFAs without the rest of the groups Figure(4.3) and Figure(4.4). And another thing from Table (2.4), find that the type of fatty acid (n-3) (C18:3(n-3), C20:3(n-3), C20:5(n-3), C22:5(n-3), and C22:5(n-3)) is a higher percentage($p<0.05$) in the tissues of group S0%L6% (PGPR) fish than all the tissues of fish of other groups. But this results does not agree with what Francis *et al*., (2006) obtained that; the introduced of linseed oil with 8.5% partial replacement with fish oil in the murray cod (*Maccullochella peelii peelii*.) diet did not give a differentiation ($p> 0.05$)in growth rate compared to the control diet containing fish oil 17% This may demonstrate the beneficial effects of the dietary use of linseed oil as a partial substitute for fish oil with supplemental PGPR. Linseed oil includes over 85% unsaturated fatty acids, which were reported to have circumspect ploys in the growth of freshwater fish species such as rainbow trout, coho salmon, and chum salmon (Takeuchi and Watanabe, 1982; Gruia *et al*., 2012). The S0%L6%+PGPR group resulted in a higher SGR in zebrafish than S0%L6% ($p<0.05$) Figure(4.8), which may be due to a higher influence of dietary PGPR on the utilization of dietary linseed oil compared with fish and sunflower oils. There were no significant effects of dietary PGPR on the feed intake of zebrafish Figure(4.9)in the present experiment ($p>0.05$). The amounts of lipid consumed and digested were significantly higher in groups containing linseed oil (6%) ($p<0.05$). In general, dietary emulsifiers play a positive role in the enhancement of lipid digestibility and feed efficiency (Drażbo *et al*., 2019). Dietary addition of PGPR in control+PGPR diet group (100% fish oil) numerically enhanced the amounts of digested lipid consumption in S0%L0% group. Inspite of there was similiar ($p>0.05$) increase in the digestibility of fats in the groups content PGPR Figure(4.10), while it was increment ($p<0.05$) in the digestibility of the feed as a whole than groups without PGPR, and however According to previous studies; The positive effect of emulsifying additives is not limited to dietary lipids , but is also reflected in the efficiency of feed utilization in general (Lai, 2018; San Tan *et al*., 2016; Bontempo *et al*., 2018). The values of

feed conversion and protein efficiency ratios (Figure4.11) for S0%L0% (PGPR) and S0%L6% (PGPR) groups were significantly better than those for S6%L0%. Although dietary supplemental PGPR in the experimental diets resulted in numerically better feed conversion and protein efficiency ratio, it could be speculated that the presence of dietary emulsifiers could increase the benefiting of ingredients by fish to build tissues and promote the growing process.

The lipids structure of sperm plasma membrane has similar to mammalian animal sperm, and it considers the main compound of teleost fish spermatozoa (Bell *et al*., 1997a). Köprücü *et al*., 2015 mention that; the addition of 2% of n-3 PUFA in rainbow trout diet effects positively milt volume, sperm concentration, start sperm motility, and total continuation time of sperm motility. In spawning season have a positive relationship with increasing DHA and other PUFAs than MUFAs and SFAs in fillet and gonads tissues of anchovy and sardine (Pacetti *et al*., 2013). Mehdinejad *et al*., (2013) found that the spermatozoa motility variables were more affected by C20:3n-3 and (∑ n-3) compare with other fatty acids. In Table(4.1) there is a clear differences between diet groups in content of n-3 PUFA% (C18:3(n-3), C20:3(n-3), C20:5(n-3), C22:5(n-3), and C22:6(n-3)), and the fish oil diets (90g/kg) (Control and S0%L0%(PGPR) diet group) had high level of most this FAs ($p<0.05$) rest of groups, but the presence of these fatty acids (n-3 PUFA) in the fish tissues Table(4.2) were high percentages significantly ($p<0.05$) in fish tissues fed fish oil and linseed diet groups especially groups with PGPR, in spite of sperm performance of this groups (Figure4.12 and Figure4.13) was not high in motility and progressive SKPs, but arrived to longer total motility time (Figure4.14) and high fertilized eggs and hatching rate (%) Figure (4.15) ($p<0.05$).

Lahnsteiner et al., (2009) found that; saturated fatty acids were higher in the spermatozoa of rainbow trout especially C16:0 and C18:0.The VCL and VSL of Senegalese sole (Solea senegalese) sperm effected with adjunct of PUFAs in the diet (Beirão, 2011). Meinelt *et al*., (1999) mentioned that; the FAs compound and level of n-6 FAs in zebrafish broodstock diet affected sperm quality and spawning performance, and from Table(4.1)find that; the sunflower diets content highly level ($p <0.05$) of C18:2(n-6) as n-6 FAs this reflect positively on the total sperm motility and progressive motility SKPs of fish spermatozoa of S6%L0% and S6%L0%+PGPR group as the average for three times (6th , 9th , and 12th sec.) in Figure(4.12) and Figure(4.13) was highly ($p <0.05$) than other groups. The S0%L6%+PGPR

group had high values ($p < 0.05$) of sperm motility and progressive motility parameters than S0%L6%group, Perhaps the PGPR had a role by raising the percentage of stored HUFAs and PUFAs (Figure(4.4)) in the body of fish, which part of it moves to the gonads when synthesis sperm. In Figure(4.14) there slightly egual in the time that sperm spent in motility ($p>0.05$), from the beginning point to the vibration point in all groups, but when comparing from the beginning until the complete point between groups found that the sperm of S0%L6%, control+PGPR, and S0%L6%+PGPR groups had longer total time than other. When comparing the progressive motility (VCL) and total sperm motility time Figure (4.13 and Figure (4.14); the sperm of group S06%L0%, and S6%L0%(PGPR) groups were higher speed at the first 6th time Figure(4.13) than other groups significantly ($p < 0.05$), and at the same time had good perseverance to keep motility until for longer time Figure(4.14) significantly ($p < 0.05$) until complete stop point. The total amount of C18:1(n-9), C18:0, C18:2(n-3), and C18:3(n-3) in sunflower diets Table (4.1) was more than other diets and perhaps a part of it was converted as precursors to C20:3n-3 and other ($\sum$ n-3) fatty acids through desaturation and elongation biosynthesis Pathway (Tocher, 2003).

The FAs composition in cobia broodstock diets directly influenced egg fatty acid composition (Nguyen *et al*., 2010). Huynh et al., 2007 found that; PUFA was the high amount in the milt and ovary in Pacific herring fish at spawning season and n-3 fatty acids with DHA was higher twice time in gonads than flesh tissues. In the rainbow trout female diet; an alternative of 75% of fish oil with vegetable oil did not have adverse effects on fecundation and hatching rate (Agh *et al*., 2019). In spawning season have a positive relationship with increasing DHA (C22:6 (n-3)) and other PUFAs than MUFAs and SFAs in fillet and gonads tissues of anchovy and sardine (Pacetti *et al*., 2013). The fish fed sunflower oil diet groups content below than 1% of C22:6 (n-3) inside their tissues Table (4.2) significantly ($p<0.05$) than other fish groups, which reduced the fertilized eggs and hatching rate (%) Figure (4.15), and when compare between tow sunflower oil diet groups find that; S6%L0%+PGPR (48.63± 15.3 and 48.63± 15.3) highly with significant different than S6%L0% group (33.1±16.1and 32.32±15.32 %). In Table (4.4) appear that; indirect effect of emulsifier material in enhance the fatty acids digestibility generally and this resulting on raise of the fertilized egg and hatching rate in S6%L0%+PGPR group (Figure 4.15) . Jaya-Ram *et al*., (2008) found that the zebrafish fed diet contained linseed oil and squid oil in a ratio of (1:1), which enhanced the total egg production, relative fecundity, and hatching rate (%). The introduction of crude

palm oil as vegetable oil with a percentage of 9.16% in the diet of female Nile tilapia led to; premature first spawning activity, extended duration of broodfish fecundity, enlargement of the gonads, increased number released of eggs, raise hatching rate, and reduce cases of larval distortions (Ng and Wang, 2011). The fertilizability and hatchability (%) of yellowfin sea bream broodstock was (77.61± 3.2 and 43.55± 2.7) when replacement 50 % of fish oil with sunflower in diet (5g/100g: 5g/100g) (Zakeri *et al*., 2011). Ghosi Mobaraki *et al*., (2020) found that; there is no significant difference when used 40 and 80g/kg of soybean oil in pearl gourami diet on egg and yolk diameter length significant compare with fish oil (80g/kg). From first sight in Figure (4.16), the diet groups included PGPR had longer egg and yolk diameter, and larvae lengths significantly ($p < 0.05$) than other groups. Introduced the arachidonic acid in Blue gourami diet with 1% show longest oocyte diameter (µm) (861.66 ± 45.18) significantly ($p<0.05$), while Introduced it with 1.5% given a high Hatching rate (%) (84.10 ± 10.41) significantly ($p<0.05$) (Asil *et al*., 2017). When looking to the percentage of arachidonic acid in Table(4.1) find is high in control and control+PGPR diet groups, and the fish of these groups in Figure(4.15) had high fertilized egg and hatching rate (%) and the fish of S0%L0%+PGPR longest egg and yolk diameter length Figure (4.16) significantly ($p < 0.05$). Although arachidonic acid is the low percentage in diets contained linseed oil (approximately half of the contents of fish oil diets) Table(4.1) but fish in S0%L6% group is a high percentage of fertilized egg and hatching rate (%) and fish of S0%L6%+PGPR group had longest egg and yolk diameter length significantly ($p < 0.05$). It can be explained with compare the percentage values of arachidonic acid in fish tissues Table(4.2) of that fish fed fish oil diets and linseed oil diets; there is narrow differentiation and this is mean that the synthesis of C20:4n-6 in tissues of linseed oil fish groups was high to compensate the lack in feed. Fed zebrafish females on diet involving 9% corn oil increased the C20:4n-6 level in ovaries, while an olive oil diet (9%) had a low level of highly unsaturated fatty acids (HUFAs) had an adverse effect on egg and yolk diameter (Araújo *et al*., 2017). The embryo development Figure(4.17) at 9hpf ((90%-epiboly stage) was more progress in S6%L0%+PGPR and S0%L6%+PGPR groups and at 35hpf (prim-22 stage) control embryo had less development significantly($p<0.05$) than other groups, for that PGPR had positively affected the lengthening of the larvae. But at 25 and48hpf (prim-6 and long pec stage) there is no significant difference ($p<0.05$) in embryo development between all groups, Araújo *et al*., (2017) mentioned that the C20:4n-6 level in ovaries and embryo, and had a positive effect on

embryo development. Despite the diet groups included fish oil (9%) Table (4.1) were high in percentage of, 20:4n-6 (Control (0.10957548± 0.00414849%) and S0%L0%(PGPR)(0.11161648± 0.00256143%)) with significant different ($p<0.05$) than other diets groups, but sunflower and linseed oil diet groups (6%) so rich in C18:2(n-6);(S6%L0% (5.1690161± 0.110778%) and S6%L0%+PGPR (4.547361± 0.344978%)) and C18:3(n-3); (S0%L6% (3.708751± 0.0473938%) and S0%L6%+PGPR (3.9273214± 0.171327%)) for that; perhaps it could transfer to, C20:4n-6 through desaturation and elongation processes inside female fish tissues to utilizing in eggs synthesis and embryo development after fertilization.

From Figure (4.15), Figure (4.16), Figure (4.17) It could been noted that zebrafish female generally in groups fed diet with PGPR had a good reproductive performance than males in same group and female in groups fed diet without PGPR and this similar to results effect of PGPR on zebrafish growth performance; fish female was high LBW, WG and SGR than zebrafish female fed rations without added PGPR (Figure 4.6, Figure 4.7, Figure 4.8).

At conclusion of this study results can get that; there are no adverse effects of PGPR on outgrowth and venereal achievement of zebrafish. The effect of PGPR to improving the fatty acids digestibility was more a clear when added to the sunflower oil diet saturated fatty acids (C14:0, C16:0, C17:0, C18:0, C20:0, C22:0) and unsaturated fatty acids (C16:1(n-7), C20:3(n-3), C20:4(n-6), C20:5(n-3), C24:1, C22:5(n-3), and C22:5(n-3)). PGPR as an emulsifier in zebrafish had a positive impact on the utilization of protein and lipids the close relationship between dietary PGPR and vegetable oils concerning promoting the exploitation of their fatty acids by fish clearly shows that dietary emulsifiers can increase lipid availability to fish. The presence of ∑ HUFAs and ∑PUFAs in the fish diet with suitable levels supports the growth process better than ∑SFAs and ∑MUFAs. The fish female in groups fed diets content PGPR was show better physical increase (LBW, WG and SGR) and reproductive performance(fertilized eggs and hatching rate (%), and egg, yolk diameter, and larvae length (μm)) than males in the same groups and female in other groups. It can be suggested the introduction of PGPR as an emulsifier feed additive into the diets of various commercial inland and salty water fish species would be profitable. Yet, this topic remains to be studied in future studies.

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Articles and Publications

1-Ahmed M. H. , Keskin İ., Özkorucuklu S., Ekici A., 2022 Study on Effect of Using Magnetized Water in Dilution the Milt of Black Sea Trout (*Salmo trutta labrax*) on Sperm Motility. *Aquaculture Research*, 53 (17) 6324-6332,

2- Ahmed M. H., Ekici A., 2023 Effects of Emulsifier Material and Vegetable Oils on Zebrafish (*Danio rerio*) Growth Performance. *Aquaculture Studies*, 23, AQUAST1052