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Effects of dietary superworm (*Zophobas morio*) oil on growth and reproductive performance of female zebrafish (*Danio rerio*)

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Abstract There was not different effect on the growth performances between female zebrafish (*Danio rerio*) after feed with fish oil and superworm oil (*Zophobas morio*). Feed conversion ratio, weight gain and specific growth were not significantly different between the two groups of fish fed the different type of oils. There was not statistically significant difference on the reproductive performances. Gonadosomatic index was not significantly different between two groups of fish fed the different type of oils. The relative expression of ovulation-inducing genes including *ptgs2a*, *sik1*, and *slc37a4a* were not significantly differed when compared between female zebrafish after feed with fish oil and superworm oil. Results suggested that superworm oil may be used in female zebrafish diet with no adverse effects on both growth and reproductive performance.

Keywords: Gene expression, qPCR, GSI, Ovulation-inducing genes

Introduction

Fish oil is an important source of essential fatty acids to maintain the regular health, growth, reproduction, and other biological functions. Dietary lipids containing fish oil is required for the production of omega-3-rich farmed fish. Fat in fish diets is very important to female fish productivity (Ohs *et al.,* 2013). It is an energy resource in the egg yolks, which will use this fat for their embryonic development. Hence, many fish agricultural companies have been applied high amount of fat dietary in their farm (Tocher, 2010). The global overfishing led to decline of the overall proportion of fish stocks. The decreased fish stocks would affect the production of fish oil for aquaculture. Other fat sources of insect oil is possible be an alternative fat source. Recently,

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there is no information available on the dietary insect oils, such as superworm oil in aquatic animals.

Zebrafish (*Danio rerio*) is an important organism in vertebrate reproductive study (Hoo *et al*., 2016; Klangnurak *et al*., 2018; Klangnurak and Tokumoto, 2017; Li and Ge, 2020; Tang *et al*., 2017; Tang *et al.*, 2018; Tokumoto *et al*., 2004; Tokumoto *et al*., 2011) because they are fast growth, short life cycle and high reproductive activity. Zebrafish breeding is stimulated by photoperiodic every 2-3 days and may contain several hundred eggs in a single clutch (Westerfield, 2000). Reproductive performance of zebrafish can be tracked in the fish proceed to ovulation via 11 genes involving during ovulation (Klangnurak *et al*., 2018; Klangnurak and Tokumoto, 2017).

The objective was to investigate the effect of fat from superworm oil towards female zebrafish growth and reproductive system. The growth performance and feed utilization efficiency were observed. The reproductive performance in quantitative RT-PCR was performed to investigate expression of ovulation-inducing gene of fish which were fed by fish oil dietary and superworm oil dietary and supported by gonadosomatic index.

Materials and methods

Experimental animals

Zebrafish (*D. rerio*) were received from our experimental aqualab at Department of Animal Production Technology and Fisheries, King Mongkut's Institute of Technology Ladkrabang. Fishes were raised in proper chamber with a recirculating water system which was maintained at 28.5 ˚C under a 14 h light: 10 h dark cycle (Westerfield, 2000) and fed a diet of brine shrimp in the morning and pellet (Tetra GmbH, Melle, Germany) in the evening. Female fishes were raised until they possessed full-grown immature oocytes before using in the experiment.

Experimental diets and formulation

Two experimental diets were formulated to be the isoprotein and isolipid by using different type of oils (Table1). All the feed ingredients were thoroughly mixed using a kitchen mixer and pelleted by pork grinder. The obtained pellets were dried in a hot air oven at 80˚C for 2 hour and then kept in room temperature. The pellets were pounded with a mortar thoroughly. Half of fine pellets were used for chemical composition analysis and another half was stored at –20˚C until use. Proximate analysis of all diets was performed following standard method from AOAC (1993).

Table 1. Feed formulations of the treatment diets

Ingredients (%)	Fish oil	Superworm oil
Fishmeal 60%	30	30
Fishmeal 55%	30	30
Soybean Meal	5	5
Wheat gluten	10	10
Potato starch	18	18
Vitamin mix	2	2
Fish oil	5	0
Soybean oil	0	0
Rice bran oil	0	0
Super worm oil	0	5
Total	100	100

Experimental design and fish husbandry

There were 2 treatments and triplicate groups for each treatment. Sixty fishes with approximately 0.31±0.05 g/fish were used. Ten fishes were individually weighted and randomly placed in each 30x13.5x30 cm^3 glass tank with 1.5L of water for a replicate. Fish were kept in close water system and maintained at 28.5 ˚C under a 14 h light: 10 h dark cycle during experiment. The fishes were fed twice a day at 8.00 and 16.00 until approach apparent satiation as determined by visualization.

A minimal water exchange of 40% was performed every week and any excess uneaten feed or debris were siphoned to remove every other day. Water quality parameters including temperature, ammonia nitrogen, nitrite, dissolved oxygen (DO), and pH were measured every 20 days using standard method and maintained at 26-29˚C, closed to 0 ppm ammonia nitrogen, 0-0.5 mg/l nitrite, 6-8 ppm DO and 7-8 pH which is suitable for zebrafish water system (Hammer, 2020).

Growth and body composition parameters

At the 40 days of the feeding experiment, the survived fishes were individually weighed and counted from each replicate to calculate survival rate and the growth performance including weight gain (WG g/fish), specific growth rate (SGR %/day), and feed conversion ratio (FCR) which calculated as described by Khieokhajonkhet and Surapon (2020). Growth parameters were compared between 2 treatments via T-test in SPSS software. The statistically significance was assigned with 95% confidence level.

Reproductive performance

Gonadosomatic Index (GSI)

Fishes were killed by spinal severance and opened haft of abdomen by scissors tip at the end of experiment. Ovaries were dissected and weighted from 5 females. The GSI was calculated from gonad weight (GW) and body weight (BW) as GSI = GW/BW*100. A GSI of all survival fishes in both treatments were pulled and compared with T-test in SPSS software. The statistically significance was assigned with a 95% confidence level.

Gene expression

Three ovaries were randomly selected from each replicate and treatment. Ovaries were placed in liquid nitrogen after dissected and weighted immediately. Total RNA was extracted from ovarian tissue using Presto™ DNA/RNA Extraction Kit (Geneaid Biotech Ltd., Taiwan) in accordance with the manufacturer's protocol. Total RNA was reverse transcribed using SensiFAST cDNA Synthesis Kit (Bioline Reagent Ltd., London) according to manufacturer's instruction to prepare cDNA.

Three ovulation-inducing genes were selected to examine reproductive performance including prostaglandin-endoperoxide synthase 2 (*ptgs2a*), salt inducible kinase1 (*sik1*) and solute carrier family 37member 4 gene (*slc37a4a*). The mRNA abundance of 3 selected genes was assessed by qPCR to confirm their expression level. A widely used reference gene, elongation factor 1 alpha (*ef1α*) was amplified using the same sample sets to validate the normalization procedure. The qPCR reactions were performed in 20 µl volume, containing 5 µl of 10 time-diluted cDNA, 1 µl of each primer (10 µM), and 10 µl of SYBR green PCR Master Mix (Roche Applied Science, Mannheim, Germany). Real time qPCR was conducted by LineGene K Plus Real-Time PCR Detection System (Hangzhou Bioer Technology, China). The qPCR reaction was 10 µl SYBR green PCR Master Mix, 1 µl of each primer (Klangnurak *et al*., 2018; Klangnurak and Tokumoto, 2017) and 5 µl of cDNA. The thermocycler was following 95℃ 1 minute, 40 cycles of 95℃ for 15 seconds, annealing temperature (Ta) at 60℃ for 30 seconds and final melting curve at 60-95℃ 1 second. The melting curve was observed to check the unspecific amplicons. The mRNA abundance of each target gene was calculated from a serially diluted cDNA and was normalized against the expression level of the reference gene. Triplicates were performed for each cDNA sample. The relative mRNA abundances by a reference gene were presented as the mean ± SE. The relative mRNA abundances of each gene were compared between 2 treatments with T-test in SPSS software. The statistically significance was assigned with a 95% confidence level.

Results

Chemical composition in food diet

The percentage of protein, fat, moisture, and ash content of superworm oil diet were not significant different when compared to fish oil diet (Table 2).

Table 2. Chemical composition of the treatment diets (Mean ± SD)

Chemical composition (%)	Fish oil	Superworm oil	P-value
Crude protein	46.36±0.47	49.20±0.33	≥0.05
Crude fat	10.81±0.01	8.10±0.03	≥0.05
Moisture	6.02±0.22	6.13±0.03	≥0.05
Ash	17.79±0.47	18.96±1.00	≥0.05

Zebrafish growth

After 40 days, zebrafish which fed fish-oil and superworm oil diet were shown all alive (Table 3). There were not significantly affected on the growth performances among the two groups of fish fed the different type of oils. Both experiments showed identical weight gain (0.05±0.00 g). Comparison of fish-oil and superworm oil was not statistically significance in specific growth (p-value=0.74) which was 0.35±0.01 and 0.21±0.01 %/d, respectively. FCR was not significant difference between both treatments (p-value=0.81). FCR from fish fed by fish-oil diet was 26.92±3.77 and fed with superworm oil was 25.52±4.89 (Table 3).

Table 3. Growth performance and feed utilization of zebrafish fed with experimental diets for 40 days (Mean ± SD)

Parameters	Fish oil	Super worm oil	P-value
Initial weight (g)	0.31±0.02	0.31±0.02	0.92
Final weight (g)	0.36±0.02	0.36±0.02	0.82
Weight gain (g)	0.05±0.00	0.05±0.00	0.71
SGR (%/d)	0.35±0.01	0.21±0.01	0.74
FCR	26.92±3.77	25.52±4.89	0.81
Survival rate (%)	100±0.00	100±0.00	-

Zebrafish reproductive

Gonadosomatic index was shown to be over 2 in both treatments (Figure1A) and statistical analysis of GSI was not significantly differed when

compared to two groups of fish fed the different type of oils at p-value ≥0.05. The highest mRNA expression level of both treatment is reported in *sik1* and the lowest in *ptgs2a* (Figure1B). The expression of all 3 selected genes were not significantly differed between 2 treatments (p-value ≥0.05).

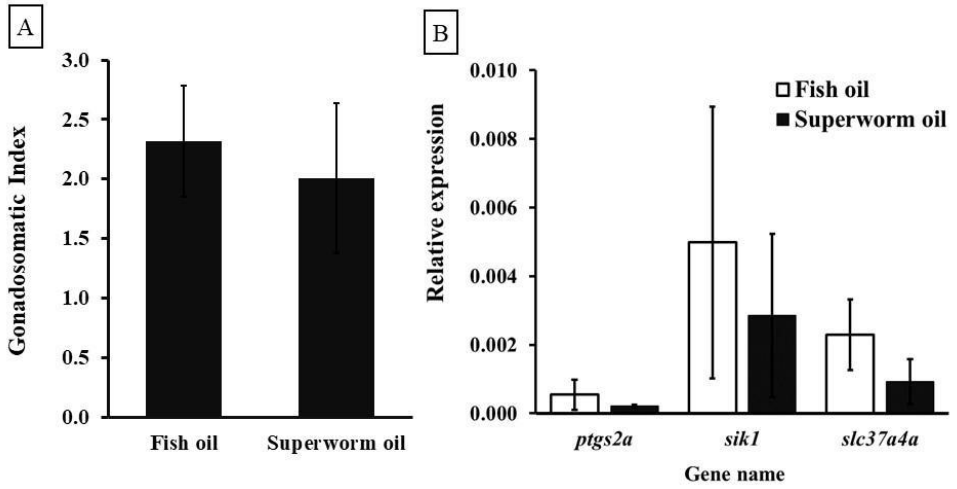

Figure 1. Reproductive performance on female zebrafish revel by GSI (A) and ovulation-inducing gene expression (B)

Discussion

Survival rate, growth and other feed utilization parameters revealed efficiency for applying superworm oil in aquatic animal dietary. FCR was very high when compared with other freshwater food fishes. Zebrafish is small fish and body size is increased slowly when exceeded to maximum growth. Female zebrafish in this study was a cluster of full-grown females which almost reached the maximum length. The reason why FCR was very high because the full-grown females still ate normally, while body weight was slowly increased. The zebrafish possessed highly FCR were found especially in the work using old adult zebrafish (Jaya-Rum *et al*., 2008).

Zebrafish females fed with fish oil and superworm oil as diet lipid sources was not differed in ovulation. Fat is essential for fish reproduction, egg fecundity, egg ovulation, egg hatching, and larvae development (Zhou *et al*., 2011). Reproductive performances were positively correlated with their lipid sources. Fish-oil is a common lipid source. Eel feed with fish-oil showed higher fecundity than pork oil and peanut oil diet because fish-oil diet had omega 3 EPA, 20:5n-3, DHA, 22:6n-3 and Arachidonic 20:4n-6 acid (ARA). The GSI

of the eel was low when compared to pork oil and peanut oil diet which contained less of those important fats (Zhou *et al.*, 2011). Three-spot gourami fish (*Trichopodus trichopterus*) feed with fish-oil treatment showed higher GSI comparing to other treatments (Berenjesaaki *et al*., 2014). In this study, zebrafish consumed superworm oil diet showed the same GSI with fishes that were fed by fish oil diet and supported by gene expression level. Three ovulation-inducing genes which expressed during ovulation and potentially involved in egg preparation for fertilization (Klangnurak *et al*., 2018; Klangnurak and Tokumoto, 2017). It showed the same level of expression in this study. Inclusion of superworm in fish diets become more interesting issue not only in zebrafish but also in other economic fish such as nile tilapia (*Oreochromis niloticus*), sea bream (*Sparus aurata*) and sea trout (*Salmo trutta*) (Rumbos and Athanassiou, 2021).

In conclusion, our findings suggested that the dietary superworm oil can replace the fish oil in the female zebrafish diet with no adverse effects on both growth and reproductive performances.

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