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Effects of red monascal rice supplementation on growth, digestive function and oocyte maturation in Siamese fighting fish (*Betta splendens* Regan, 1910)

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Evaluation of red monascal rice supplementation on growth, digestive function and oocyte maturation were investigated in Siamese fighting fish (*Betta splendens*). Completely randomized design with different dietary levels of red monascal rice (0.00, 0.25, 0.50, 1.00 and 2.00%) was conducted for six weeks. The growth of fish fed a control diet was not statistically different ($P > 0.05$) from a diet containing 0.25% of red monascal rice. However, significantly lower values ($P < 0.001$) were observed in fish fed more than 0.50% red monascal rice, in a dose-dependent manner. Muscle RNA concentrations were higher in fish fed control diet than in fish fed red monascal rice, while protein concentration and RNA/protein ratio were similar. Body composition and fatty acid profiles (saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, total omega-3 and total omega-6) were unchanged among dietary treatments. Digestive functions were perturbed by decreasing the specific activities of amylase, total protease, trypsin and chymotrypsin ($P < 0.001$), and increasing the specific activity of lipase ($P < 0.005$). Reproductive consummation of oocytes was significantly different in fish fed red monascal rice, due to increasing RNA concentration and RNA/protein ratio ($P < 0.003$) and decreasing specific activities of trypsin- and chymotrypsin-like enzymes ($P < 0.03$). These findings suggest the toxicological effects of red monascal rice by interfering with growth, digestive function and oocyte maturation in Siamese fighting fish.

Key words: Digestive enzyme, fatty acid, growth, muscle, oocyte, red monascal rice, Siamese fighting fish.

INTRODUCTION

Red monascal rice is a product of ordinary rice fermented with the fungal genus *Monascus*. It has been widely used as a food additives for coloring meat (Bakosova et al.,

2001), fish (Takatsuki et al., 1988) and chicken eggs (Wang and Pan, 2003). Monascal rice provides at least six pigments-yellow (ankaflavin and monascin), orange

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Abbreviations: SGR, Specific growth rate; DSI, digestosomatic index; GSI, gonadosomatic index; MOFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

(monascorubrin and rubropunctanin) and red (monascorubramine and rubropunctamine), as well as other secondary metabolites, antioxidant compounds (Yang et al., 2006), hypertensive agents and antihypercholesterolemic agents (Su et al., 2003; Wang and Pan, 2003).

However, a toxic chemical, citrinin, is often a by-product of fermentation, depending on culture conditions; for example, fermentation of rice by foodstuff-relevant *Monascus* sp. produces up to 2.5 g kg⁻¹ citrinin, while liquid culture has reached as high as 56 mg kg⁻¹ (Eisenbrand, 2006). This compound has been found to induce reproductive abnormalities in male gametes (Qingqing et al., 2012), and to reduce the rate of oocyte maturation and fertilization (Chan, 2008); it also has a teratogenic effect (Chan and Shiao, 2007; Singh et al., 2007a; Chan, 2008), and has induced maternal toxicity in pregnant rats (Singh et al., 2007b). Moreover, it has been associated with cytotoxicity (Liu et al., 2005) and the activation of apoptosis by the mitochondrial pathway (Yu et al., 2006).

Recently, *in vitro* screening of feedstuffs for the liberation of various pigments based on crude enzyme digestion, standardized with trypsin activity, indicated that red monascal rice is an appropriate source for promoting coloration in Siamese fighting fish (Thongprajukaew et al., 2012). Moreover, supplementation of aquaculture feed with red monascal rice for rearing juvenile Siamese fighting fish has been reported (Thongprajukaew et al., 2011). Safety evaluation of red monascal rice for use as a food supplement has been studied in chickens (Wang and Pan, 2003), rats (Chan and Shiao, 2007; Kumari et al., 2009) and rabbits (Wei et al., 2003). However, *in vivo* observation of red monascal rice supplementation in aquatic animals has not yet been performed.

The objective of the present study was to investigate the effects of red monascal rice supplementation on growth, digestive function and oocyte maturation in aquatic species. Unique combinations of biochemical parameters were used as indicators: digestive enzyme specific activities (amylase, lipase, total protease, trypsin and chymotrypsin); oocyte qualities (trypsin-like enzyme specific activity, chymotrypsin-like enzyme specific activity, RNA, protein and RNA/protein ratio); and muscle qualities (RNA, protein, lipid, RNA/protein ratio and protein/lipid ratio). Siamese fighting fish (*Betta splendens* Regan, 1910) was chosen as a representative model because they generate the highest income among sales of exported ornamental fish in Thailand. The findings of this study could provide additional information regarding red monascal rice supplementation as a food additive for rearing aquatic species.

MATERIALS AND METHODS

Diet preparation and biochemical compositions

The ingredients of experimental diets and their biochemical compo-

sition are shown in Table 1. Mixtures of specified feedstuffs (fish meal, soybean meal, wheat gluten, squid meal and wheat flour) were modified using microwave irradiation to enhance digestive enzyme hydrolysis. The diets were produced by mixing the modified feed mixtures with red monascal rice ("Mona" rice; Fame Biotech Co, Ltd., Thailand) in different concentrations (0.00, 0.25, 0.50, 1.00 and 2.00%), together with other additives and sufficient water (30%) to achieve an appropriate moisture content. The glutinous mixtures were passed through a hand pelletizer, dried at 60°C for 3 h, and then stored at 4°C until used for feeding. For biochemical composition analysis, the diets were dried at 105°C for 24 h before determining protein, lipid, fiber, and ash contents, in accordance with AOAC standard method (2005). Nutritional values of the diet were expressed as percentage on a dry matter basis. Carbohydrate contents were calculated by the difference.

Fish husbandry and sample collection

Solid red females, 1.5 months old, were obtained from a private farm in Nakhon Pathom province, Thailand. The fish were individually acclimatized in a plastic aquarium (8 cm diameter × 11 cm height) for 10 days before starting the experiment. A control diet (without red monascal rice) was fed two times daily during fish acclimatization. The fish (0.40 ± 0.01 g initial weight and 3.44 ± 0.05 cm initial length) were distributed into individual aquariums (7 cm width × 7 cm length × 15 cm height, with a 10 cm water level). The experiment was conducted for six weeks under a 12-h light/12-h dark cycle. Twenty (20) fish in each dietary treatment were fed *ad libitum* at 8:00 a.m. and 6:00 p.m. At the end of the experiment, the fish were starved for 24 h prior to sampling to avoid metabolic interference after diet ingestion. All fish were sacrificed by chilling in ice according to "Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes," National Research Council, Thailand.

Fifteen (15) fish ($n = 15$) were used for measurement of growth, muscle quality, digestive enzyme specific activity and oocyte quality. Body weight and total length were individually measured before carefully collecting the epaxial white muscle, digestive tracts, and oocytes. Pooled body samples from fish dissection were used to analyze biochemical composition (three pooled fish per sample, $n = 5$). Four fish from each treatment ($n = 4$) were randomly sampled for fatty acid determination. All tissues were then stored at -80°C until determinations were performed. Parameters for measuring growth and development of female fish were calculated by the following formulae:

$$\text{Condition factor (CF, g cm}^{-3}\text{)} = 100 \times (\text{live body weight}/\text{total length}^3)$$

$$\text{Digestosomatic index (DSI, \%)} = 100 \times [\text{digestive tract weight}/\text{body weight}]$$

$$\text{Gonadosomatic index (GSI, \%)} = 100 \times [\text{oocyte weight}/\text{body weight}]$$

$$\text{Specific growth rate (SGR, \% day}^{-1}\text{)} = 100 [e^g - 1]$$

Where, $g = (\ln W_t - \ln W_0)/(t - t_0)$; W_t = mean weight at month t ; W_0 = mean weight at month t_0 .

Weight gain (WG, g) = final body weight - initial body weight

Water quality management

Water was changed at a rate of 75% every other day. Water qualities were measured weekly using a water analyzer (556 MPS

Table 1. Feedstuff ingredients and biochemical composition of experimental diets with different supplementation levels of red monascal rice.

Ingredient and composition	Red monascal rice (%)				
	0.00	0.25	0.50	1.00	2.00
Ingredient (%)					
Fish meal	24	24	24	24	24
Soybean meal	20	20	20	20	20
Wheat gluten	11	11	11	11	11
Squid meal	5	5	5	5	5
Wheat flour	31	30.75	30.50	30	29
Lecithin	1	1	1	1	1
Fish oil	2	2	2	2	2
Soybean oil	1	1	1	1	1
Mineral mixture*	0.05	0.05	0.05	0.05	0.05
Vitamin mixture**	0.25	0.25	0.25	0.25	0.25
Vitamin C	0.1	0.1	0.1	0.1	0.1
Fermented red rice	0.00	0.25	0.50	1.00	2.00
Cellulose	5.1	4.6	4.6	4.6	4.6
Composition (%)					
Moisture	8.1	8.6	7.7	6.6	6.5
Crude protein	39.1	41.6	40.5	42.7	40.1
Crude lipid	4.5	3.5	3.2	3.7	4.2
Nitrogen free extract	33.5	32.3	33.9	32.3	34.5
Crude fiber	3.5	3.2	3.8	3.5	3.6
Ash	11.3	10.8	10.9	11.2	11.1
Gross energy (kJ/g)	16.8	16.8	16.7	17.1	17.1

*Mineral mixtures in 1 kg of feed contained 30 mg iron, 20 mg zinc, 25 mg manganese, 5 mg copper, 5 mg iodine and 0.2 mg selenium. **Vitamin mixtures in 1 kg of feed contained 4,000 IU vitamin A, 2,000 IU vitamin D₃, 50 mg vitamin E, 10 mg vitamin K, 20 mg thiamine, 20 mg riboflavin, 20 mg pyridoxine, 200 mg calcium panthothenate, 150 mg niacin, 2 mg biotin, 5 mg folic acid, 0.2 mg vitamin B₁₂, 400 mg inositol and 200 mg ethoxyquin.

Multi Probe System, YSI Inc., Yellow Springs OH, USA). During the whole experimental periods the average temperature was $28.44 \pm 0.07^\circ\text{C}$, pH was 7.45 ± 0.02 , and dissolved oxygen was $7.56 \pm 0.05 \text{ mg L}^{-1}$. Ammonia content ($0.0044 \pm 0.0001 \text{ ppm}$) was measured according to APHA, AWWA and WPCF (1998). All analysis was performed in triplicate.

Digestive enzyme activities

Digestive enzyme extraction

Digestive enzymes were extracted from whole digestive organs and oocytes in the presence of 50 mM Tris-HCl buffer pH 8 containing 200 mM NaCl, using a micro-homogenizer (THP-220; Omni International, Kennesaw GA, USA). The homogenate was then centrifuged at $13,000 \times g$ for 20 min at 4°C . The lipid portion on the upper layer of the supernatant was carefully removed. The supernatant was collected and then kept at -80°C for digestive enzyme assay. Protein concentration in the enzyme extract was determined according to Lowry et al. (1951) using bovine serum albumin as a standard curve.

Digestive enzyme assays

Specified optimal conditions for studying digestive enzyme activities

in Siamese fighting fish were used as described in Thongprajukaew et al. (2011). Amylase activity was determined based on Areekijserree et al. (2004) using starch solution as a substrate. Amylase specific activity was expressed as μmol maltose produced $\text{h}^{-1} \text{ mg protein}^{-1}$. Lipase activity was analyzed based on Winkler and Stuckmann (1979) using *p*-nitrophenyl palmitate as a substrate. The specific activity of lipase was expressed as μmol *p*-nitrophenol produced $\text{h}^{-1} \text{ mg protein}^{-1}$. Total protease activity was assayed using azocasein as a substrate, based on Areekijserree et al. (2004). The specific activity of total protease was expressed as $\text{mU mg protein}^{-1}$. One unit (U) of total protease activity was defined as the amount of enzyme producing an increase of 1.0 absorbance unit at 440 nm. Trypsin and trypsin-like activities from the digestive tract and oocyte extracts, respectively, were assayed using *N*-benzoyl-*L*-arginine-*p*-nitroanilide (BAPNA) as a substrate. Chymotrypsin and chymotrypsin-like activities from the digestive tract and oocyte extracts, respectively, were assayed using *N*-succinyl-*ala*-*ala*-*pro*-*phe*-*p*-nitroanilide (SAPNA) as a substrate. The assays of these serine proteases were performed according to Rungruangsak-Torrissen (2007), with specific activities expressed as μmol *p*-nitroanilide produced $\text{h}^{-1} \text{ mg protein}^{-1}$.

Muscle, body and oocyte compositions

Moisture of the body was determined using an automatic infrared

moisture analyzer (MA 30; Sartorius, Göttingen, Germany). Concentrations of RNA and protein in the muscle, body and oocytes were determined using TRIzol[®] reagent (Invitrogen, Carlsbad CA, USA), as described in Rungruangsak-Torrissen (2007). The extinction coefficient for calculating RNA is $E_{260} = 40 \mu\text{g RNA ml}^{-1}$, and for protein is $E_{280} = 2.1 \text{ mg protein ml}^{-1}$. Lipid contents were extracted using ethyl acetate, as described by Supannapong et al. (2008). Ratios of RNA/protein and protein/lipid were calculated from the amounts of RNA, protein and lipid from the same sample. All values were expressed on wet weight basis.

Fatty acid determination

Fish samples ($n = 4$) were dried using a freeze dryer (Heto FD3; Heto-Holten Allerød, Denmark) under dark conditions for 48 h before analyzing fatty acid profiles. Lipids for fatty acid analysis were extracted according to Kates (1986). Fatty acid methyl esters were prepared using sodium methoxide, as described by Gandhi and Weete (1991). Fatty acid profiles were analyzed using a gas chromatography (GC-14A, Shimadzu, Kyoto, Japan) equipped with a stainless steel packed SS column and flame ionization detector. Injector temperature was increased from 205 to 250°C at a rate of 2°C min⁻¹. Detector temperature was maintained at 275°C. Nitrogen was used as a carrier gas at 2 kgf cm⁻². Menhaden oil methyl esters (National Marine Fisheries Service, Seattle WA, USA) were used as internal standards.

Statistical analysis

All data were expressed as mean \pm standard error of mean (SEM). Significant differences among averages were ranked using Duncan's Multiple Range Test (DMRT) at a 95% significance level. Relationships among selected parameters were calculated using Pearson's product moment correlation.

RESULTS

Growth, body compositions and muscle quality

Survival rate of fish in all dietary treatments was 100% (Table 2). All growth indicators, including total length, body weight, weight gain and specific growth rate (SGR), were similar between fish fed control diet and those fed the diet with 0.25% red monaschal rice (Table 2, $P > 0.05$). Nearly all parameters of growth were significantly affected by red monaschal rice supplementation ($P < 0.05$), and were decreased in a dose-dependent manner (except the condition factor).

Body biochemical composition, including moisture, lipid, protein, protein/lipid ratio and total minerals, were not statistically different (Table 2, $P > 0.05$) among dietary treatments. Protein concentration and RNA/protein ratio in the white muscle of untreated and treated fish were similar, while the RNA concentration showed a significant decrease ($P = 0.008$) in fish fed red monaschal rice when compared to the control (Table 2).

Digestive indices and digestive enzyme specific activities

Digestive tract weight was significantly lower in fish fed the diet containing 2.00% red monaschal rice ($P < 0.05$), while the digestosomatic index (DSI) fluctuated among dietary treatments (Table 3). Supplementation of red monaschal rice had significant effects on the specific activities of digestive enzymes for protein ($P < 0.001$), carbohydrate ($P < 0.001$) and lipid ($P = 0.004$). Specific activities of amylase, total protease, trypsin and chymotrypsin were significantly decreased ($P < 0.05$), while specific activity of lipase was significantly increased, in the presence of red monaschal rice ($P < 0.05$).

Activity ratios of amylase to trypsin (A/T ratio) and trypsin to chymotrypsin (T/C ratio) were similar among dietary treatments ($P > 0.05$), whereas the slope of trypsin to chymotrypsin (slope T/C ratio calculated by the slope of regression between chymotrypsin (X-axis) and trypsin (Y-axis) specific activities of the same sample according to Rungruangsak-Torrissen et al. (2009) was significantly decreased in treated groups when compared with control ($P = 0.002$).

Reproductive indices and oocyte qualities

The maturation rate of fish in all dietary treatment was 100% (Table 4), as indicated by the presence of oocyte in all collected fish. Oocyte weight progressively decreased ($P < 0.05$) in a dose-dependent manner, while the gonadosomatic index (GSI) slightly decreased (pooled data, $P < 0.05$) when fish received red monaschal rice. Protein synthesis (RNA concentration) and protein turn-over (RNA/protein ratio) in the oocytes were significantly increased with administration of red monaschal rice. Supplementation of red monaschal rice had potent effects on specific activities of trypsin-like ($P < 0.03$) and chymotrypsin-like ($P < 0.008$) enzymes; there was a slight decrease in enzyme activities with red monaschal rice doses of 0.25 and 0.50%, and a dramatic decrease at doses of 1.00 and 2.00%.

Relationship between growth and digestive capacities

Specific growth rate (SGR) of fish showed positive correlations with digestive tract weight and specific activities of amylase, total protease, trypsin and chymo-trypsin (Table 5). However, specific activity of lipase and the T/C ratio were negatively correlated with SGR. The changes of SGR, digestive tract weight, specific activities of all digestive enzymes, and A/T ratio were closely related. For muscle qualities, RNA concentration showed positive correlations with SGR, protein concentration, and specific

Table 2. Effect of different dietary levels of red monascol rice on survival rate, growth, body composition and muscle quality of female Siamese fighting fish. Analysis was conducted using individual fish from each treatment.

Parameter	P	Red monascol rice (%)				
		0.00	0.25	0.50	1.00	2.00
Survival rate (%)	-	100	100	100	100	100
Growth (n = 15)						
Total length (cm)	< 0.001	4.78 ± 0.06 ^a	4.84 ± 0.06 ^a	4.65 ± 0.06 ^{ab}	4.49 ± 0.07 ^{bc}	4.37 ± 0.12 ^c
Body weight (g)	< 0.001	1.21 ± 0.04 ^a	1.22 ± 0.03 ^a	1.09 ± 0.03 ^b	1.04 ± 0.04 ^b	1.01 ± 0.05 ^b
Weight gain (g)	< 0.001	0.81 ± 0.04 ^a	0.82 ± 0.03 ^a	0.69 ± 0.03 ^b	0.64 ± 0.04 ^b	0.61 ± 0.05 ^b
Condition factor (CF, g cm ⁻³)	0.037	1.10 ± 0.02 ^a	1.08 ± 0.03 ^a	1.08 ± 0.03 ^a	1.15 ± 0.03 ^{ab}	1.22 ± 0.06 ^b
Specific growth rate (SGR, % day ⁻¹)	< 0.001	3.15 ± 0.10 ^{ab}	3.20 ± 0.07 ^a	2.86 ± 0.07 ^{bc}	2.72 ± 0.11 ^c	2.61 ± 0.15 ^c
Body composition (n = 5, %)						
Moisture	0.104	76.12 ± 0.95	76.34 ± 0.76	77.94 ± 0.68	78.68 ± 0.65	77.37 ± 0.34
Protein	0.926	13.51 ± 0.56	12.97 ± 0.89	12.92 ± 0.43	12.77 ± 0.65	12.80 ± 0.79
Lipid	0.918	3.62 ± 0.42	3.82 ± 0.41	3.77 ± 0.32	3.56 ± 0.40	3.41 ± 0.23
Protein/lipid ratio	0.938	3.90 ± 0.53	3.46 ± 0.30	3.51 ± 0.43	3.77 ± 0.59	3.80 ± 0.33
Ash	0.389	4.12 ± 0.19	4.33 ± 0.48	4.07 ± 0.28	4.34 ± 0.20	4.62 ± 0.03
Muscle quality (n = 15)						
RNA (µg g ⁻¹)	0.008	2,460 ± 42 ^a	2,270 ± 45 ^b	2,283 ± 48 ^b	2,298 ± 40 ^b	2,216 ± 64 ^b
Protein (mg g ⁻¹)	0.922	300 ± 13	299 ± 13	287 ± 9	295 ± 12	291 ± 12
RNA/protein ratio (µg mg ⁻¹)	0.496	8.38 ± 0.31	7.74 ± 0.28	8.04 ± 0.24	7.98 ± 0.39	7.70 ± 0.23

Data with different superscripts in each row indicate significant differences ($P < 0.05$). Probabilities with significant values in each measurement parameter are indicated by bold letters ($P < 0.05$).

activities of amylase, total protease, trypsin and chymotrypsin; while RNA/protein ratio showed positive and negative relationships with A/T ratio and protein concentration, respectively (Table 5).

Relationship between growth and reproductive parameters

Increased oocyte weight occurred concurrently with increase of body weight and decrease of

RNA concentration (Table 6). Oocyte weight showed a negative relationship with protein synthesis and protein turnover. Trypsin-like specific activity was highly correlated with chymotrypsin-like enzyme activity.

Both protease enzymes showed positive correlations with protein synthesis capacity (RNA and RNA/protein ratio). However, no significant relationship was observed between protein concentration and other reproductive parameters.

Fatty acid profiles of fish

The main fatty acids found in the bodies of female Siamese fighting fish were C16: 0, C18: 1n9 and C18: 2n6 (Table 7). Fatty acid content was relatively higher in fish fed red monascol rice compared to fish fed control diet ($P > 0.05$). Total amounts of saturated fatty acid (SFA) monounsaturated fatty acid (MOFA), polyunsaturated fatty acid (PUFA), $\Sigma n-3$ and $\Sigma n-6$ were similar among

Table 3. Effect of different dietary levels of red monaschal rice on digestive growth and digestive enzyme expressions in female Siamese fighting fish. Analysis was conducted using individual fish from each treatment.

Parameter	P	Red monaschal rice (%)				
		0.00	0.25	0.50	1.00	2.00
Digestive growth (n = 15)						
Digestive tract weight (g)	0.001	0.11 ± 0.00 ^a	0.11 ± 0.00 ^a	0.10 ± 0.01 ^a	0.10 ± 0.00 ^a	0.08 ± 0.00 ^b
Digestosomatic index (DSI, %)	0.103	8.84 ± 0.32	8.74 ± 0.37	9.35 ± 0.48	9.63 ± 0.47	8.06 ± 0.43
Digestive enzyme (n = 15)						
Amylase [A] ^a	< 0.001	359.45 ± 23.86 ^a	274.33 ± 23.90 ^b	265.38 ± 19.83 ^b	191.17 ± 14.07 ^c	199.51 ± 19.78 ^c
Lipase ^b	0.004	4.17 ± 0.12 ^a	4.40 ± 0.15 ^{ab}	4.36 ± 0.16 ^{ab}	4.87 ± 0.28 ^{bc}	5.25 ± 0.31 ^c
Total protease ^c	< 0.001	70.81 ± 3.27 ^a	53.57 ± 4.81 ^b	56.41 ± 4.06 ^b	45.15 ± 3.93 ^c	42.96 ± 4.67 ^c
Trypsin [T] ^d	< 0.001	15.71 ± 1.03 ^a	11.64 ± 0.97 ^b	11.67 ± 0.86 ^b	9.70 ± 0.90 ^{bc}	8.40 ± 0.93 ^c
Chymotrypsin [C] ^d	< 0.001	128.20 ± 6.23 ^a	98.03 ± 8.83 ^b	100.18 ± 6.96 ^b	80.42 ± 8.19 ^{bc}	78.19 ± 7.79 ^c
A/T ratio	0.722	23.71 ± 1.84	24.47 ± 1.66	23.80 ± 1.70	21.47 ± 2.44	25.25 ± 1.91
T/C ratio	0.126	0.12 ± 0.00	0.12 ± 0.00	0.11 ± 0.00	0.12 ± 0.00	0.12 ± 0.01
Slope T/C ratio	-	0.1409	0.1037	0.1047	0.1070	0.0950

Data with different superscripts in each row indicate significant differences ($P < 0.05$). Probabilities with significant values in each measurement parameter are indicated by bold letters ($P < 0.05$). ^aexpressed as $\mu\text{mol maltose h}^{-1} \text{mg protein}^{-1}$; ^bexpressed as $\mu\text{mol } p\text{-nitrophenol h}^{-1} \text{mg protein}^{-1}$; ^cexpressed as $\text{mU mg protein}^{-1}$; ^dexpressed as $\mu\text{mol } p\text{-nitroanilide h}^{-1} \text{mg protein}^{-1}$.

Table 4. Effect of different dietary levels of red monaschal rice on reproductive growth and oocyte quality in female Siamese fighting fish. Analysis was conducted using individual fish from each treatment ($n = 15$).

Parameter	P	Red monaschal rice (%)				
		0.00	0.25	0.50	1.00	2.00
Reproductive growth (n = 15)						
Maturation (%)	-	100	100	100	100	100
Oocyte weight (g)	0.002	0.24 ± 0.01 ^a	0.25 ± 0.01 ^a	0.21 ± 0.01 ^{ab}	0.19 ± 0.01 ^b	0.18 ± 0.02 ^b
Gonadosomatic index (GSI, %)	0.095	20.23 ± 0.95	20.01 ± 0.79	19.07 ± 1.07	17.68 ± 0.79	16.98 ± 1.26
Oocyte quality (n = 15)						
RNA ($\mu\text{g g}^{-1}$)	0.002	5,210 ± 209 ^c	5,208 ± 209 ^c	5,359 ± 186 ^{bc}	6,055 ± 190 ^{ab}	6,443 ± 420 ^a
Protein (mg g^{-1})	0.078	290 ± 13	316 ± 15	279 ± 11	294 ± 13	269 ± 5
RNA/protein ratio ($\mu\text{g mg}^{-1}$)	0.002	18.37 ± 0.94 ^{ab}	17.19 ± 1.05 ^a	19.81 ± 1.29 ^{ab}	20.93 ± 0.94 ^{bc}	24.06 ± 1.61 ^c
Trypsin-like specific activity*	0.026	0.40 ± 0.01 ^a	0.38 ± 0.02 ^{ab}	0.38 ± 0.02 ^{ab}	0.33 ± 0.02 ^b	0.33 ± 0.02 ^b
Chymotrypsin-like specific activity *	0.007	0.44 ± 0.02 ^a	0.41 ± 0.02 ^a	0.40 ± 0.02 ^{ab}	0.34 ± 0.02 ^b	0.35 ± 0.02 ^b

Data with different superscripts in each row indicate significant differences ($P < 0.05$). Probabilities with significant values in each measurement parameter are indicated by bold letters ($P < 0.05$). *expressed as $\mu\text{mol } p\text{-nitroanilide h}^{-1} \text{mg protein}^{-1}$.

Table 5. Pearson correlation coefficient (r) among SGR, digestive enzyme expressions and muscle qualities (RNA, protein and RNA/protein ratio) in female Siamese fighting fish at the end of the experiment. Data were calculated from fish in all treatments ($n = 75$).

Parameter	SGR	DTW	Amylase	Lipase	Total protease	Trypsin	Chymotrypsin	A/T ratio	T/C ratio	RNA	Protein
SGR	1										
DTW	0.520**	1									
Amylase	0.470**	0.231	1								
Lipase	-0.478**	-0.324**	-0.382**	1							
Total protease	0.428**	0.470**	0.690**	-0.468**	1						
Trypsin	0.489**	0.537**	0.682**	-0.488**	0.950**	1					
Chymotrypsin	0.491**	0.530**	0.650**	-0.536**	0.962**	0.945**	1				
A/T ratio	-0.065	-0.359**	0.311**	0.242*	-0.381**	-0.434**	-0.415**	1			
T/C ratio	-0.279*	-0.159	-0.199	0.477**	-0.370**	-0.228	-0.464**	0.147	1		
RNA	0.263*	0.066	0.357**	-0.171	0.259*	0.291*	0.251*	0.037	-0.071	1	
Protein	0.068	0.138	-0.014	-0.186	0.121	0.124	0.128	-0.201	0.046	0.394**	1
RNA/protein ratio	0.108	-0.109	0.252*	0.077	0.037	0.064	0.033	0.237*	-0.090	0.211	-0.804**

SGR, specific growth rate; DTW, digestive tract weight. Significant correlation coefficients between measurement parameters are indicated by bold values ($*P < 0.05$, $**P < 0.01$).

Table 6. Pearson correlation coefficient (r) between body weight and oocyte parameters in female Siamese fighting fish at the end of the experiment. Data were calculated from fish in all treatments ($n = 75$).

Parameter	Body weight	Oocyte weight	Trypsin-like	Chymotrypsin-like	RNA	Protein
Body weight	1					
Oocyte weight	0.796**	1				
Trypsin-like	0.182	-0.122	1			
Chymotrypsin-like	0.212	-0.046	0.920**	1		
RNA	-0.508**	-0.738**	0.322**	0.259*	1	
Protein	0.133	0.150	-0.231	-0.137	-0.073	1
RNA/protein ratio	-0.045	-0.570**	0.385**	0.266*	0.773**	-0.666**

Significant correlation coefficients between measurement parameters are indicated by bold values ($*P < 0.05$, $**P < 0.01$).

Table 7. Fatty acid compositions in carcasses of female Siamese fighting fish. Analysis was conducted from four fish ($n = 4$) in each treatment.

Fatty acid (%)	Significance	Red monascal rice (%)				
		0.00	0.25	0.50	1.00	2.00
Fatty acid (% total lipid)	0.119	97.13 ± 0.95	98.35 ± 0.09	98.55 ± 0.35	99.03 ± 0.34	97.78 ± 0.29
C14:0	0.256	1.63 ± 0.05	1.68 ± 0.05	1.85 ± 0.09	1.70 ± 0.09	1.75 ± 0.06
C15:0	0.179	0.25 ± 0.05	0.45 ± 0.05	0.40 ± 0.06	0.45 ± 0.05	0.45 ± 0.05
C16:0	0.359	28.70 ± 0.59	29.35 ± 0.90	31.63 ± 0.70	30.03 ± 0.78	29.55 ± 1.69
C16:1	0.149	2.37 ± 0.24	2.78 ± 0.17	3.35 ± 0.27	3.15 ± 0.33	3.25 ± 0.29
C18:0	0.322	6.35 ± 0.75	6.48 ± 0.51	5.53 ± 0.22	5.05 ± 0.25	5.73 ± 0.67
C18:1n9	0.647	28.73 ± 1.14	27.35 ± 1.20	28.95 ± 1.59	30.70 ± 2.16	28.23 ± 1.45
C18:2n6	0.094	17.23 ± 0.82	17.35 ± 0.46	15.05 ± 0.37	17.85 ± 1.01	17.20 ± 0.63
C18:3n3	0.139	1.05 ± 0.06	0.90 ± 0.07	0.85 ± 0.03	0.80 ± 0.13	0.78 ± 0.05
C18:4n3	0.041	0.98 ± 0.09 ^{ab}	0.93 ± 0.03 ^{ab}	0.80 ± 0.04 ^b	1.08 ± 0.07 ^a	0.85 ± 0.05 ^b
C20:1n9	0.018	0.60 ± 0.00 ^a	0.38 ± 0.00 ^{bc}	0.48 ± 0.05 ^{ab}	0.45 ± 0.03 ^{bc}	0.33 ± 0.06 ^c
C20:2n6	0.152	0.53 ± 0.05	0.68 ± 0.05	0.60 ± 0.07	0.53 ± 0.02	0.63 ± 0.02
C20:3n6	0.007	1.78 ± 0.19 ^a	2.08 ± 0.11 ^a	1.75 ± 0.21 ^a	1.10 ± 0.14 ^b	1.60 ± 0.07 ^a
C20:4n6	0.114	1.35 ± 0.10	1.33 ± 0.11	1.20 ± 0.11	0.98 ± 0.15	1.38 ± 0.06
C22:4n3	0.002	0.43 ± 0.02 ^a	0.45 ± 0.03 ^a	0.37 ± 0.03 ^b	0.30 ± 0.00 ^c	0.30 ± 0.00 ^c
C22:4n6	0.866	0.78 ± 0.05	0.80 ± 0.04	0.83 ± 0.06	0.73 ± 0.15	0.83 ± 0.03
C22:5n3	0.477	0.37 ± 0.03	0.40 ± 0.00	0.30 ± 0.00	0.28 ± 0.09	0.25 ± 0.05
C22:6n3	0.564	5.00 ± 0.44	5.33 ± 0.27	4.85 ± 0.54	4.35 ± 0.53	5.13 ± 0.21
Σ SFA	0.235	36.80 ± 0.62	37.73 ± 0.45	39.30 ± 0.76	37.00 ± 0.93	37.25 ± 1.09
Σ MUFA	0.512	30.95 ± 1.22	30.50 ± 1.30	32.78 ± 1.70	34.30 ± 2.44	31.78 ± 1.22
Σ PUFA	0.428	29.38 ± 1.43	30.13 ± 1.07	26.43 ± 1.31	27.73 ± 2.18	28.73 ± 0.75
Σ n-3	0.519	7.73 ± 0.45	7.90 ± 0.44	7.00 ± 0.56	6.73 ± 0.85	7.10 ± 0.24
Σ n-6	0.300	21.65 ± 0.99	22.23 ± 0.68	19.43 ± 0.77	21.00 ± 1.38	21.63 ± 0.61

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; nd = not detected. Data with different superscripts in each row indicate significant difference ($P < 0.05$). Significant values in each fatty acid are indicated by bold letters ($P < 0.05$).

all dietary groups ($P > 0.05$).

DISCUSSION

Effect of red monascal rice supplementation on growth

Growth performance studies indicated the accep-

tance of red monascal rice at 0.25% whereas the higher levels resulted in a significant decrease in growth, as indicated by the reduction of nearly all growth indicators (except condition factor) (Table 2). This observation is in agreement with the report of Wang and Pan (2003), which found the greatest reduction of body weight and egg production, and increases of feed consumption and feed conversion, in laying hens fed with 8% red mona-

scal rice.

Wei et al. (2003) reported significant decreases in weight gain in rabbits when the concentration of red monascal rice was increased. However, Kumari et al. (2009) observed no significant differences in food intake, body weight and relative organ indices in rats fed diets with acute doses of red monascal rice. The progressively increased condition factor with the dose of red

monascal rice indicated an alteration of the morphometric relationship between weight and length. Fish fed with monascal rice decreased weight and length. The skeletal growth (length) of the fish fed monascal rice decreased faster than their weight (9.9 to 16.5% vs 2.7 to 8.6%). Moisture content of the fish body increased when fish received red monascal rice (Table 2). This might lead to a reduction of body strength, which is in agreement with the poorer performances for movement and display in treated fish (behavioral observation) when compared with the control.

The correlation coefficient indicated that SGR of fish was positively related to RNA concentration in white muscle ($r = 0.263$, $P < 0.05$). This finding explains the lower growth of fish when RNA synthesis is prohibited. Toxicological effects of citrinin, a secondary metabolite produced by several fungal species including *Monascus* sp. and *Penicillium* sp., have been reported, including increasing apoptosis and decreasing the number and viability of treated cells (Liu et al., 2005; Chan and Shiao, 2007). Citrinin increased DNA fragmentation in HL-60 cells (Yu et al., 2006), which might lead to a reduction of the muscle RNA (Table 3) thereby inducing lower cell proliferation (Chan and Shiao, 2007).

This phenomenon suggests a lower growth rate of treated fish. All observations indicate a retarding effect from chronic administration of red monascal rice on the growth in female.

Effect of red monascal rice supplementation on digestive function

Digestive processes of the fish were statistically affected ($P < 0.002$) by supplementation levels of red monascal rice, as also indicated by lowering digestive tract weight (Table 3). A positive correlation between body weight and gastrointestinal weight was observed in mature female ($r = 0.523$, $P < 0.01$). This phenomenon indicates that fish growth is driven by digestive capacities. This illustrates a tendency for suppressing digestion, absorption and utilization of nutrients in fish fed monascal rice. Toxicological evaluation of red monascal rice for interfering with digestive functions was clearly observed, using digestive enzymes as indicators.

Decreased specific activities of amylase and proteolytic enzymes in fish fed red monascal rice (Table 3) indicated digestive dysfunctions in the utilization of carbohydrate and protein, respectively. This correlated with a reduction of growth and development, as shown by lowered expression levels of serine proteases, trypsin and chymotrypsin (Chan, 2008); this governed a decrease of the slope T/C ratio (Rungruangsak-Torrissen et al., 2009), a key digestive enzyme factor for predicting growth and feed utilization in aquatic animals. Increased lipase specific activity in treated fish might be due to the

use of lipids as a main energy source when carbohydrate and protein digestion is inhibited. Perturbation of lipid metabolism by supplementation of red monascal rice could affect the maturation of females, as shown by a negative correlation coefficient between lipase specific activity and oocyte weight ($r = -0.414$, $P < 0.01$). These findings are in agreement with previous reports on the reduction of cholesterol and triglycerides in animals after dietary supplementation with monascal rice (Wang and Pan, 2003; Wei et al. 2003). Moreover, upregulation of lipase activity might increase total fatty acids in the bodies of treated fish (Table 7). However, some fatty acid might be obtained from the monascal rice (Juzlova et al., 1996).

Effect of red monascal rice supplementation on fish reproduction

Red monascal rice induced a significant reduction in the reproductive indices by decreasing oocyte weight and GSI (Table 4). Effects of the mycotoxin citrinin on inhibiting the maturation of oocytes, fertilization efficiency and fetal development have been investigated (Chan and Shiao, 2007; Chan, 2008). Moreover, citrinin plays a potent role in inducing maternal toxicity (Singh et al., 2007b) and teratogenic effects in Wistar rats (Singh et al., 2007a). This is similar to its toxic effect on the male reproductive system, increasing the number of abnormal spermatozoa and decreasing the number of live spermatozoa (Qingqing et al., 2012). However, in a study on dietary supplementation of monascal rice in rat (Kumari et al., 2009), histopathological examination found no differences in the relative weights of sex organs (testes and ovaries).

Increased oocyte RNA and RNA/protein ratio in fish fed red monascal rice indicated a slower development of oocytes by increasing genetic materials and protein turnover rate for cell proliferation and differentiation in the earlier developmental stage. Similar results were confirmed by negative correlations between oocyte weight and RNA ($r = -0.738$, $P < 0.01$, Table 6), and between oocyte weight and RNA/protein ratio ($r = -0.570$, $P < 0.01$).

Significantly, lower specific activities of trypsin-like and chymotrypsin-like enzymes were observed in fish that received monascal rice. Both enzymes are reported to play an integral role in yolk formation and degradation (Hiramatsu et al., 2002), and embryogenesis and hatching (Sveinsdottir et al., 2006). Hydrolytic activities of serine proteases govern the abundance of amino acids for regulating osmotic pressure in oocytes and maintaining the buoyancy capacity of newly hatched juvenile fish (Finn et al., 2002). Sveinsdottir et al. (2006) discussed that increases in trypsin-like and chymotrypsin-like activities at first feeding may be possible to increase

larval survival of Atlantic cod (*Gadus morhua*). However, variation of trypsin-like enzyme in fish oocytes has been found to occur due to differences in diet quality (Rungruangsak-Torrissen, 2007). Therefore, these findings point to the reproductive effect of red monascal rice, by interfering with oocyte development in female Siamese fighting fish.

Conclusions

Supplementation of red monascal rice resulted in a significant reduction in growth, digestive function and oocyte maturation in female Siamese fighting fish, as indicated by the negative alterations of various biochemical parameters. These findings indicate an adverse effect of red monascal rice supplementation on somatic and reproductive differentiation in female Siamese fighting fish. They provide sufficient data for deciding on the merits of using red monascal rice as a food additive for rearing Siamese fighting fish.

Furthermore, the use of these biochemical parameters for measuring the effect of red monascal rice in higher animals should be of interest.

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REFERENCES

- AOAC (2005). Official Methods of Analysis of AOAC International. 18th eds. Association of Official Analytical Chemists, Maryland.
- APHA, AWWA, WPCF (1998). Standard Methods for the Examination of Water and Wastewater. 20th eds. American Public Health Association, American Water Works Association, Water Pollution Control Federation, Washington, DC.
- Areekijseree M, Engkagul A, Kovitvadi U, Thongpan A, Mingmuang M, Pakkong P, Rungruangsak-Torrissen K (2004). Temperature and pH characteristics of amylase and proteinase of adult freshwater pearl mussel, *Hyriopsis (Hyriopsis) bialatus* Simpson 1900. *Aquaculture* 234:575-587.
- Bakosova A, Mate D, Laciakova A, Pipova M (2001). Utilization of *Monascus purpureus* in the production of foods of animal origin. *Bull. Vet. Inst. Pulawy* 45:111-116.
- Chan WH (2008). Effects of citrinin on maturation of mouse oocytes, fertilization, and fetal development *in vitro* and *in vivo*. *Toxicol. Lett.* 180:28-32.
- Chan WH, Shiao NH (2007). Effect of citrinin on mouse embryonic development *in vitro* and *in vivo*. *Reprod. Toxicol.* 24:120-125.
- Eisenbrand G (2006). Toxicological evaluation of red mould rice. *Mol. Nutr. Food Res.* 50:322-327.
- Finn RN, Ostby GC, Norberg B, Fyhn HJ (2002). *In vivo* oocyte hydration in Atlantic halibut (*Hippoglossus hippoglossus*); proteolytic liberation of free amino acid, and ion transport, are driving forces for osmotic water influx. *J. Exp. Biol.* 205:211-224.
- Gandhi SR, Weete JD (1991). Production of the polyunsaturated fatty acids arachidonic acid and eicosapentaenoic acid by the fungus *Pythium ultimum*. *J. Gen. Microbiol.* 137:1825-1830.
- Hiramatsu N, Ichikawa N, Fukada H, Fujita T, Sullivan CV, Hara A (2002). Identification and characterization of proteases involved in specific proteolysis of vitellogenin and yolk proteins in salmonids. *J. Exp. Zool.* 292:11-25.
- Juzlova P, Rezanka T, Martinkova L, Kren V (1996). Long-chain fatty acids from *Monascus purpureus*. *Phytochem.* 43:151-153.
- Kates M (1986). Techniques in lipidology: Isolation, analysis and identification of lipids. 2nd eds. Elsevier, Amsterdam.
- Kumari HPM, Naidu KA, Vishwanatha S, Narasimhamurthy K, Vijayalakshmi G (2009). Safety evaluation of *Monascus purpureus* red mould rice in albino rats. *Food Chem. Toxicol.* 47:1739-1746.
- Liu BH, Wu TS, Su MC, Chung CP, Yu FH (2005). Evaluation of citrinin occurrence and cytotoxicity in *Monascus* fermentation products. *J. Agric. Food Chem.* 53:170-175.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Qingqing H, Linbo Y, Yunqian G, Shuqiang L (2012). Toxic effects of citrinin on the male reproductive system in mice. *Exp. Toxicol. Pathol.* 64(5):465-469.
- Rungruangsak-Torrissen K (2007). Digestive efficiency, growth and qualities of muscle and oocyte in Atlantic salmon (*Salmo salar* L.) fed on diets with krill meal as an alternative protein source. *J. Food Biochem.* 31:509-540.
- Rungruangsak-Torrissen K, Sunde J, Berg AE, Nordgarden U, Fjellidal PG, Oppedal F (2009). Digestive efficiency, free amino acid pools and quality of growth performance in Atlantic salmon (*Salmo salar* L.) affected by light regimes and vaccine types. *Fish Physiol. Biochem.* 35: 255-272.
- Singh ND, Sharma AK, Dwivedi P, Patil RD, Kumar M (2007a). Citrinin and endosulfan induced teratogenic effects in Wistar rats. *J. Appl. Toxicol.* 27:143-151.
- Singh ND, Sharma AK, Dwivedi P, Patil RD, Kumar M (2007b). Citrinin and endosulfan induced maternal toxicity in pregnant Wistar rats: pathomorphological study. *J. Appl. Toxicol.* 27:589-601.
- Su YC, Wang JJ, Lin TT, Pan TM (2003). Production of the secondary metabolites γ -aminobutyric acid and monacolin K by *Monascus*. *J. Ind. Microbiol. Biotechnol.* 30:41-46.
- Supannapong P, Pimsalee T, A-komol T, Engkagul A, Kovitvadi U, Kovitvadi S, Rungruangsak-Torrissen K (2008). Digestive enzymes and *in vitro* digestibility of different species of phytoplankton for culture of the freshwater pearl mussel, *Hyriopsis (Hyriopsis) bialatus*. *Aquacult. Int.* 16: 437-453.
- Sveinsdottir H, Thorarensen H, Gudmundsdottir A (2006). Involvement of trypsin and chymotrypsin activities in Atlantic cod (*Gadus morhua*) embryogenesis. *Aquaculture* 260:307-314.
- Takatsuki K, Suzuki S, Ushizawa I, Shoji T (1988). Confirmation of *Monascus* pigment colored on the skin of fresh fishes. *Jap. J. Toxicol. Environ. Health* 34: 350-358.
- Thongprajukaew K, Kovitvadi S, Kovitvadi U, Rungruangsak-Torrissen K (2012). Pigment deposition and *in vitro* screening of natural pigment sources for enhancing pigmentation in adult male Siamese fighting fish (*Betta splendens* Regan, 1910). *Aquacult. Res.* doi:10.1111/are.12009.
- Thongprajukaew K, Kovitvadi U, Kovitvadi S, Somsueb P, Rungruangsak-Torrissen K (2011). Effects of different modified diets on growth, digestive enzyme activities and muscle compositions in juvenile Siamese fighting fish (*Betta splendens* Regan, 1910). *Aquaculture* 322-323:1-9.
- Wang JJ, Pan TM (2003). Effect of red mold rice supplements on serum and egg yolk cholesterol levels of laying hens. *J. Agric. Food Chem.* 51:4824-4829.
- Wei W, Li C, Wang Y, Su H, Zhu J, Kritchevsky D (2003). Hypolipidemic and anti-atherogenic effects of long-term cholestin (*Monascus*

- purpureus*-fermented rice, red yeast rice) in cholesterol fed rabbits. J. Nutr. Biochem. 14:314-318.
- Winkler UK, Stuckmann M (1979). Glycogen, hyaluronate and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. J. Bacteriol. 138:663-670.
- Yang JH, Tseng YH, Lee YL, Mau JL (2006). Antioxidant properties of methanolic extracts from monascal rice. LWT- Food Sci. Technol. 39:740-747.
- Yu FY, Liao YC, Chang CH, Liu BH (2006). Citrinin induces apoptosis in HL-60 cells via activation of the mitochondrial pathway. Toxicol. Lett. 161:143-151.