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Full Length Research Paper

Evaluation of growth performance and nutritional quality of diets using digestive enzyme markers and *in vitro* digestibility in Siamese fighting fish (*Betta splendens* Regan, 1910)

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Digestive enzymes and their effects on *in vitro* digestibility of feeds and feedstuffs, as well as on growth performance quality were studied in Siamese fighting fish (*Betta splendens* Regan, 1910). The specific activities of total protease, amylase, trypsin and chymotrypsin increased during development and were higher in females than in males at maturation ($P < 0.05$). The activity ratio of trypsin to chymotrypsin corresponded to fish growth, and showed lower values in females than in males. White muscle levels of RNA decreased during development, with females having higher levels than males, while the protein levels increased with no difference between sexes. In the oocytes, trypsin-like and chymotrypsin-like specific activities were very low, and the concentrations of RNA, protein and protein/lipid ratio were higher than in the muscle. For *in vitro* digestibility, wheat gluten, soybean meal and fish meal were among good protein sources while the meals from peanut, fish and soybean were good carbohydrate sources. Golden apple snail meat was a good source for both protein and carbohydrate. The crude enzyme extracts from different growth stages and sexes had different abilities to digest the same feeds and feedstuffs. This will make it possible to preliminarily study the authenticated nutritional quality of raw materials for future feed formulations for *B. splendens*.

Key words: *Betta splendens*, digestive enzymes, feedstuff, *in vitro* digestibility, muscle quality, oocyte quality, Siamese fighting fish.

INTRODUCTION

The Siamese fighting fish (*Betta splendens* Regan, 1910), widely distributed throughout Southeast Asia, is one of the most popular species for freshwater aquarium. There are two domesticated forms; ornamental fish with long fins, and sport fish with short and rounded fins for improved fighting ability (Meejui et al., 2005). Long-finned males are very important economically, providing the highest income among the exported ornamental fishes of Thailand (Wiwatchaisaet, 2000). The development of

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economical and palatable dry feeds with optimized nutrient content is essential. However, there is no report on the nutrient requirements and their digestibilities for this species.

Studies on the main digestive enzymes have demonstrated changes in isoforms and activity profiles at different developmental stages of Siamese fighting fish, based on normal feeding habits (Thongprajukaew 2010). The nutritional requirements of the fish should be closely linked to the digestive physiology and growth during development, and protein digestion is the key factor for food utilization and growth (Rungruangsak-Torrissen et al., 2006). Trypsin specific activity and the protease activity ratio of trypsin to chymotrypsin (T/C ratio) have been used as enzymatic markers for growth rate and feed efficiency in association with amino acid absorption and transport (Sunde et al., 2004). Therefore, digestion of protein should rely primarily on the presence of pancreatic proteases such as trypsin (associated with growth) and chymotrypsin (associated with limited or reduced growth), as illustrated by Rungruangsak-Torrissen et al. (2006). This knowledge of the role of the key enzyme trypsin has made it possible to compare the nutritional qualities of feeds and feed raw materials for different growth stages, using *in vitro* protein digestibility technique standardized by trypsin activity which has been studied in relation to growth (*in vivo* trials) in different fish species (Rungruangsak-Torrissen, 2007). For carbohydrate, it has been investigated in herbivore (freshwater mussel) and shown that among different nutrient digestibilities; carbohydrate is the secondary factor after protein for nutritional quality of feeds (Areekijseree et al., 2006). *In vitro* lipid digestibility has not been shown to be associated with feed efficiency and the quality of the lipid itself (Areekijseree et al., 2006). Growth is not only associated with protein digestive efficiency, but also with muscle (filet) and gamete qualities. Changes in protein metabolism in the muscle and oocytes have been investigated in different species, and it was found that the levels of RNA and protein in these tissues are affected by different feedings. In addition, the level of trypsin-like specific activity in oocytes is associated with maturation rate; whereas females with higher maturation rates showed lower oocyte trypsin-like specific activity.

The objectives of this study were to examine whether changes in the levels of the main digestive enzymes (total protease, amylase, trypsin, and chymotrypsin) could affect the digestive efficiency and growth performance quality at different developmental stages and of different sexes of Siamese fighting fish feeding on natural foods. Analysis was conducted using: (1) the T/C ratio for indication of digestive efficiency and growth; (2) the activity ratio of amylase to trypsin (A/T ratio) as an indicator for carnivorous feeding habits (Hofer and Schiemer, 1981); (3) the levels of RNA, RNA/protein ratio, and protein/lipid ratio in the muscle and oocytes, as well as oocyte trypsin-like specific activity, for indications of a quality of growth performance; and (4) *in vitro* digestibility of feeds and feed raw materials for evaluation of protein and carbohydrate utilizations, a method which is simple, economical, and less time-consuming than *in vitro* growth trials. The results from *in vitro* digestibility will provide a basic knowledge for selecting appropriate feed raw materials for future feed formulations, whereas their effects on growth performance quality (including digestive enzyme expressions and qualities of muscle and oocytes) of Siamese fighting fish can be further studied.

MATERIALS AND METHODS

Fish rearing and preparation

Betta splendens were randomly collected at three different stages: 10 days old (completely developed digestive tract and having predatory behavior); 1.5 months old (sexually identified for individual rearing); and 3 months old (maturation stage). The experiment was performed in a completely randomized design (CRD) at a Thai fish farm in Nakorn Pathom province, which is a main production area in Thailand of fish for export. The experiment began in three cement tanks (75 cm diameter × 35 cm height). In each tank, 3 days old newly hatched larvae were pooled from five females (~400 larvae per female) and reared in 25 L of water. The water volume was increased every few days, up to 150 L within one week when the fish were 10 days old after hatching. The fish were reared at the same water volume until they were 1.5 months old. They were then reared individually in 200 mL glass bottles until maturation at 3 months old. The fish were cultured under natural light regime at an ambient temperature range of 27 to 29°C, pH 7.0 to 7.2, and 5.0 to 5.3 mg/L of dissolved oxygen. During the first week of feeding, the larvae were fed with cooked chicken egg yolk dissolved in water, followed by paramecium and then water flea (*Moina* sp.), twice daily. During 10 days old to 1.5 months old, the fish were fed with water flea; after that they were fed with mosquito larvae until maturation at 3 months old. They were starved for 2 h prior to sampling. Both males and females were studied at 1.5 and 3 months old. Females were identified by visual observation of an ovipositor, appearing as a small white spot in the anal region. At each stage and for each sex, 30 individuals were measured for average body weight and total length; five pooled samples (four fish per sample) were used for digestive enzymes study; and 20 pooled samples were used for white muscle and oocyte studies. In addition, three pooled samples (100 fish per sample) from each sex at 1.5 and 3 months old were used for determining *in vitro* digestibility.

Animal ethics

The use of Siamese fighting fish as an animal model was performed in accordance with the “Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes”, National Research Council, Thailand. The fish were sacrificed by chilling in ice before the white muscle, oocytes and digestive tracts were carefully collected.

Digestive enzyme study

Enzyme extraction

Enzyme extracts from *B. splendens* were prepared from the whole

body of 10 days old juveniles, from the digestive area of 1.5 months old fish, and from the digestive tracts of 3 months old adults. The procedure was performed according to Rungruangsak-Torrissen (2007). Briefly, the samples were homogenized on ice in 50 mM Tris-HCl buffer pH 8 containing 200 mM NaCl (1:1 w/v). The homogenate was centrifuged at 4°C at 10000 × g for 20 min; then the supernatant was collected and kept at -80°C for later determination. Total protein content of each crude enzyme extract was determined according to Lowry et al. (1951).

Protease specific activity

Total protease activity was assayed by measuring the increase in cleaved short-chain polypeptides using azocasein as substrate, according to the study of Areekijseree et al. (2004). The 100 mM phosphate buffer pH 8 and temperature 50°C were chosen as the most suitable condition for total protease activity in Siamese fighting fish (Thongprajukaew, 2010). Total protease specific activity was expressed as the increase in absorbance at 440 nm h^{-1} mg $protein^{-1}$.

Amylase specific activity

Amylase activity was determined by measuring the increase in reducing sugar from starch solution using 3,5-dinitrosalicylic acid (DNS), according to the study of Areekijseree et al. (2004). The 100 mM phosphate buffer pH 8 and temperature 50°C were chosen as the most suitable condition for amylase activity in Siamese fighting fish (Thongprajukaew, 2010). The amylase specific activity was expressed as μmol maltose produced h^{-1} mg $protein^{-1}$.

Trypsin and chymotrypsin specific activities

Trypsin and chymotrypsin activities were determined by initial reaction rates at optimal temperatures according to the method described by Rungruangsak-Torrissen (2007), using BAPNA (*N*-α-Benzoyl-*DL*-arginine-*p*-nitroanilide HCl) and SAPNA (*N*-succinyl-*L*-ala-*L*-ala-*L*-pro-*L*-phe-*p*-nitroanilide) as specific substrates, respectively. The 100 mM phosphate buffer pH 8 and temperature 50°C were chosen as the most suitable condition for trypsin and chymotrypsin activities in Siamese fighting fish (Thongprajukaew, 2010). Both trypsin and chymotrypsin specific activities were expressed as μmol *p*-nitroanilide produced h^{-1} mg $protein^{-1}$.

Muscle and oocyte qualities

RNA and protein concentrations in white muscle and oocyte samples were determined as described in Rungruangsak-Torrissen (2007). The samples were extracted with monophasic solution of phenol and guanidine isothiocyanate (TRIzol® reagent; Invitrogen, Carlsbad CA, USA). The extinction coefficients for calculating RNA and protein concentrations are *E260* = 40 µg RNA $ml^{-1}$and *E280* = 2.1 mg protein ml^{-1}. For lipid determination, the samples were dried, ground and then extracted using ethyl acetate as described by Rungruangsak-Torrissen et al. (2009). Protein synthesis capacity and protein growth were expressed as RNA/protein ratio and protein/lipid (P/L) ratio, respectively. All values were expressed on wet weight basis.

Oocyte maturation

Enzyme extracts from the oocytes were obtained as described in enzyme extraction. Trypsin-like and chymotrypsin-like activities were determined by initial reaction rates at optimal temperatures using BAPNA and SAPNA as specific substrates, respectively according to the method described by Rungruangsak-Torrissen (2007). The pH and temperature profiles were studied to determine optimal conditions for further investigation. The pH profiles were performed at ambient temperature using 100 mM buffers of various pHs (6 to 11). The buffers used were phosphate buffer for the pH range 6 to 8, $NaHCO_3$-Na_2CO_3 buffer for the pH range 9 to 10, and Na_2HPO_4-NaOH for pH 11. For the temperature profile study, the reaction mixture was performed at optimal pH and at various temperatures (20 to 80°C). Both trypsin-like and chymotrypsin-like specific activities were expressed as μmol *p*-nitroanilide produced h^{-1} mg $protein^{-1}$.

Biochemical composition of diets

Live diets, commercial feeds with specific formulations for Siamese fighting fish at juveniles and mature stages, and feedstuffs were used. They were dried for 24 h at 105°C before analyses of other biochemical compositions, such as protein, lipid, fiber, and ash, as described by the AOAC (2005). The values were expressed on dry matter basis. Carbohydrate values or nitrogen free extract (NFE) were calculated by the difference.

In vitro digestibility

Protein and carbohydrate digestibilities of different feeds and feedstuffs using fish crude enzyme extracts standardized by enzyme activity were determined using the method described by Areekijseree et al. (2006). The feeds and feedstuffs were gently dried at 60°C for 48 h to control moisture level, and then ground. Blanks without crude enzyme extracts were used as controls to measure the levels of amino acids and sugar liberated from the feed ingredients. Protein digestibility (based on trypsin activity) was determined by measuring the increase in liberated reactive amino groups of cleaved peptides. *In vitro* protein digestibility was expressed as μmol *DL*-alanine equivalent g dried $feed^{-1}$ trypsin $activity^{-1}$. Carbohydrate digestibility (based on amylase activity) was determined by measuring the increase in reducing sugar. *In vitro* carbohydrate digestibility was expressed as μmol maltose g dried $feed^{-1}$ amylase $activity^{-1}$.

Statistical analysis

All analyses were performed using SPSS version 14 software (SPSS Inc., Chicago, USA). Mean and standard error of mean were calculated throughout. Statistical analysis at 95% significance level was determined using one-way ANOVA, and multiple comparisons were analyzed using Duncan's multiple range test (DMRT).

RESULTS

Fish growth

Growths of *B. splendens* were higher during the first 1.5 months (6 folds on average) compared to the last 1.5 months (2 folds on average), regardless of sex (Table 1). Males grew faster than females, indicating by higher total

length and body weight at the same stage. During the last 1.5 months, both total length and body weight of males increased 1.8 and 2.9 folds, while in females they increased 1.3 and 2 folds, respectively (Table 1). Almost entirely female samples (> 90%) at 3 months old were mature.

Development of digestive enzymes

Specific activities of total protease, amylase, trypsin and chymotrypsin increased with age, and showed significant differences between sexes ($P < 0.05$) only at maturation (Table 1). Regression analyses between these enzyme specific activities and growth parameters (total length and body weight) indicated significantly positive relationships during development in both males and females ($P < 0.05$) (Table 2). Regardless of sex, these significant relationships were also observed, except for between total protease specific activity and total length and between chymotrypsin specific activity and all growth parameters (Table 2). According to the correlation coefficient values, females seemed to show more variations in relation to total length and less variation in relation to body weight, compared to males (Table 2). Interestingly, while the enzyme specific activities in females were similar to or higher than in males, the protease activity ratios of trypsin to chymotrypsin (T/C ratio) in females were lower than in males, and became significant at maturation in accordance with fish growth parameters (Table 1). The correlations between T/C ratio and growth parameters were negative and significant relationships were only observed in females (Table 2). The feeding marker of the activity ratio of amylase to trypsin (A/T ratio) fluctuated during development, and was not statistically different ($P > 0.05$) between sexes (Table 1). It also showed negative relationships with growth parameters during development, albeit insignificant (Table 2).

Muscle quality during development

The levels of RNA and RNA/protein ratio in the white muscle decreased with age, and females had significantly higher levels than males ($P < 0.05$) at all studied stages (Table 1). On the other hand, protein and lipid levels in the white muscle increased with age, and the levels were similar between sexes, except at maturation when significantly higher lipid contents ($P < 0.05$) were observed in females (Table 1). This resulted in significantly lower ratio of protein to lipid (P/L ratio) in the white muscle of females at maturation (Table 1). RNA concentrations were negatively correlated with trypsin expression ($r = -0.79$, $P < 0.01$), total length ($r = -0.83$, $P < 0.01$) and body weight ($r = -0.81$, $P < 0.01$), regardless of sex. A positive relationship between RNA concentration and T/C ratio was observed in females ($r = 0.73$, $P < 0.05$). Protein contents were positively correlated with trypsin expression ($r = 0.80$, $P < 0.01$), total length ($r = 0.82$, $P < 0.01$), and body weight ($r = 0.82$, $P < 0.01$), regardless of sex. In contrast, a negative relationship between protein content and T/C ratio was observed in females ($r = -0.73$, $P < 0.01$). Similar to RNA, RNA/ protein ratios were negatively correlated with trypsin expression ($r = -0.79$, $P < 0.01$), total length ($r = -0.82$, $P < 0.01$) and body weight ($r = -0.72$, $P < 0.01$). Interestingly, only the T/C ratio showed positive correlation with the muscle RNA/protein ratio ($r = 0.63$, $P < 0.01$), regardless of sex.

Oocyte quality

Characterization of trypsin-like and chymotrypsin-like enzymes in the oocytes

Oocyte trypsin-like specific activity showed optimum pH 10 (Figure 1A) where at least two optimal activities at temperature ranges of 20 to 30°C and 50 to 70°C were observed (Figure 1B). Similarly, chymotrypsin-like specific activity exhibited optimum pH 10 (Figure 2A) with at least two activity peaks at temperature ranges of 20 to 30°C and 70 to 80°C (Figure 2B). According to the characteristics observed, the optimum pH 10 and temperature 20°C were chosen for studying trypsin-like and chymotrypsin-like activities in the oocytes of Siamese fighting fish.

Oocyte quality at maturation

The levels of trypsin-like and chymotrypsin-like specific activities in the oocytes were rather low (Table 1). The concentrations of RNA (2.3 folds), protein (3.1 folds) and lipid (1.3 folds) were higher in the oocytes than in the white muscle (Table 1). These resulted in higher P/L ratio (2.3 folds) and lower RNA/protein ratio (0.8 fold) in the oocytes, compared to the white muscle (Table 1).

In vitro digestibility during development

The feeds and feedstuffs chosen for the *in vitro* digestibility study and their biochemical compositions are shown in Table 3. The enzyme extracts obtained from the different life stages of Siamese fighting fish showed different abilities to digest the same materials, and the digestion ability decreased with age for both protein (Figure 3) and carbohydrate (Figure 4). Moreover, differences in digestion between males and females were observed (Figures 3 and 4), especially in carbohydrate digestion (Figure 4).

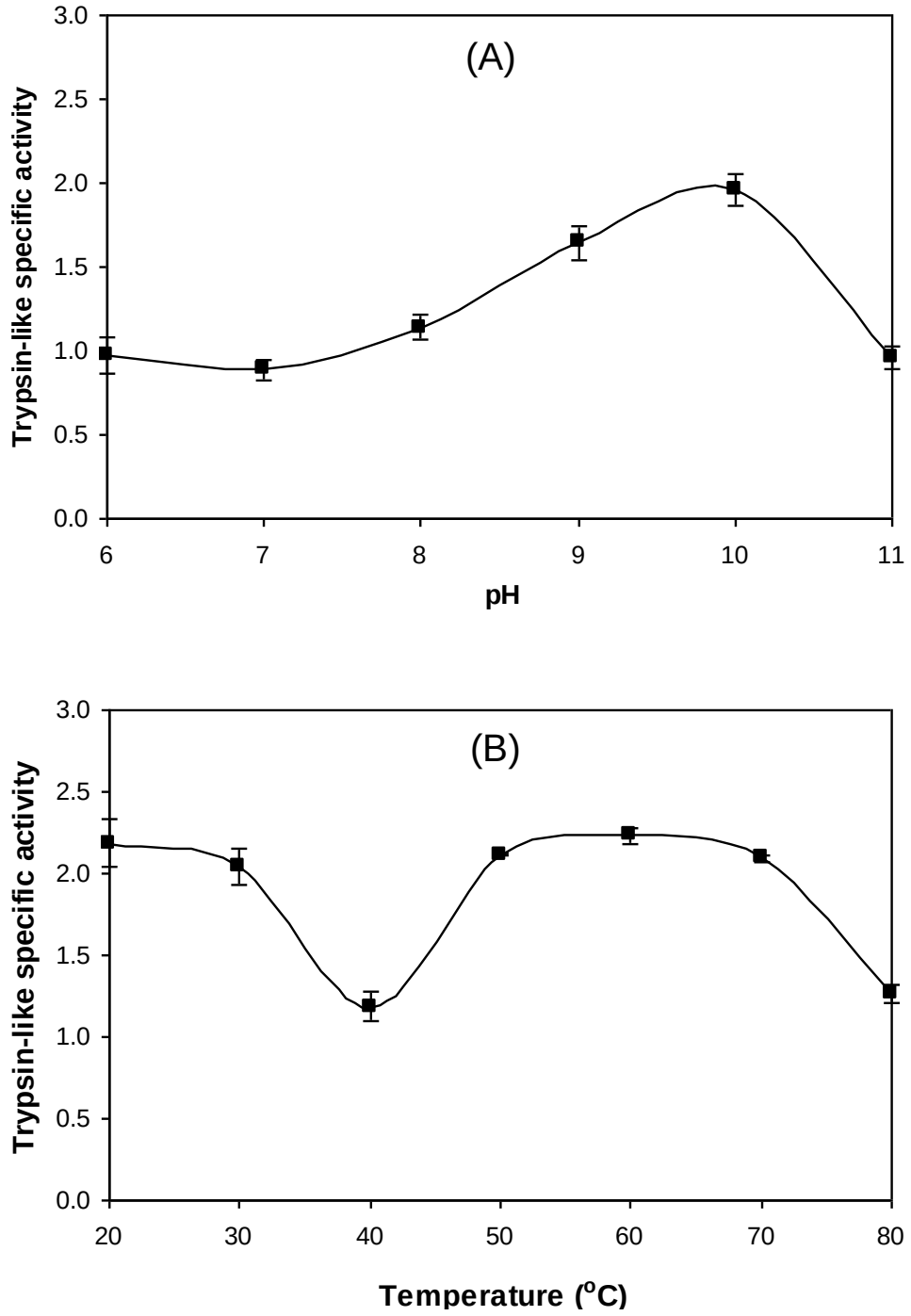

Figure 1. Trypsin-like specific activity (μmol *p*-nitroanilide produced h^{-1} mg $protein^{-1}$) in the oocytes of 3 months old females (*n* = 3), pH profile at ambient temperature (A) and temperature profile at pH 10 (B).

In vitro protein digestibility

Among the selected feeds and feed raw materials, wheat gluten had the highest protein digestibility at all three representative stages ($P < 0.05$) (Figure 3). Interestingly, golden apple snail (*Pomacea canaliculata*) meat could be an alternative source of protein for 10 days old juveniles, as it had a relatively high protein digestibility (Figure 3A). In addition, ranking from high to low digestibility, the meals from soybean, fish, meat and bone, peanut, and corn gluten, which are common ingredients in feed formulations, also showed high protein digestibility (Figure

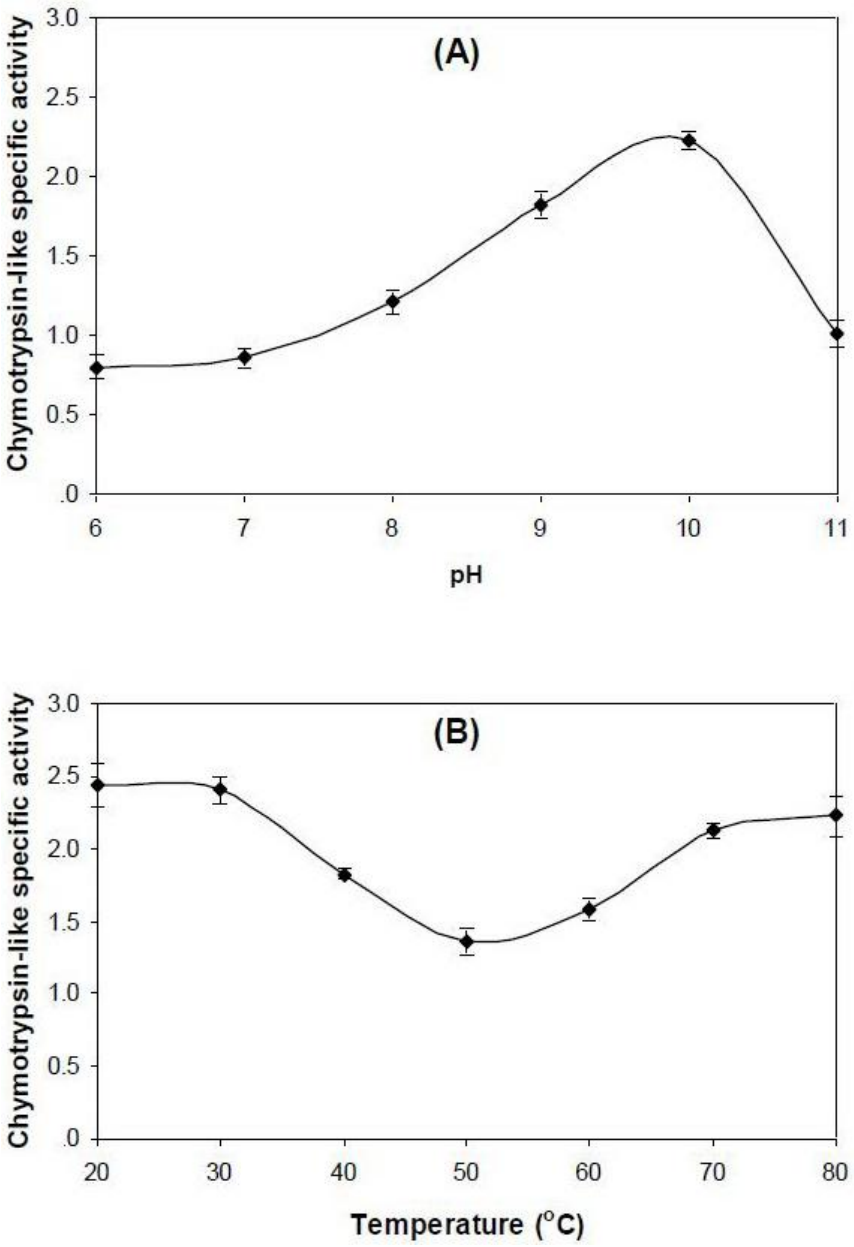

Figure 2. Chymotrypsin-like specific activity (μmol *p*-nitroanilide produced h^{-1} mg $protein^{-1}$) in the oocytes of 3 months old females (n = 3), pH profile at ambient temperature (A) and temperature profile at pH 10 (B).

3A). The protein sources suitable for 1.5 months old fish (Figure 3B) were similar to 10 days old juveniles (Figure 3A), regardless of sex. Females showed significantly higher protein digestion than males ($P < 0.05$) for *Spirulina* sp. meal, commercial feed formulation 2, blood meal, and white sorghum (Figure 3B). Similar suitable protein sources were also observed for 3 months old adults, but with no difference in protein digestion between sexes (Figure 3C). Blood meal could also be an alternative protein source at maturation stage (Figure 3C). Surprisingly, natural diets (water flea, mosquito larva, and chicken and duck egg yolks) had lower protein digestibility than golden apple snail meat, the main feedstuffs, and the commercial feeds (Figure 3).

In vitro carbohydrate digestibility

The digestibility of carbohydrate using the enzyme extracts exhibited low levels of digested products from

Table 1. Biological parameters at different stages of Siamese fighting fish. The values with asterisk (*) indicated significant differences between males and females in each stage (P < 0.05).

Parameter	10 days old	1.5 months old		3 months old		Pooled SEM
		Male	Female	Male	Female	
Growth parameters (*n* = 30)						
Length (mm)	6.63	30.31	27.33	53.79*	34.49*	0.20
Weight (mg)	48.75	413.33*	329.00*	1,190.48*	648.57*	8.26
Enzyme parameters (*n* = 5)						
[a]Total protease specific activity	0.30	3.19	3.18	5.42*	13.01*	0.29
[b]Amylase specific activity [A]	18.52	67.39	72.93	136.41*	169.76*	0.99
[c]Trypsin specific activity [T]	2.10	11.29	10.72	14.42*	22.40*	0.54
[c]Chymotrypsin specific activity [C]	1.79	13.89	14.67	14.41*	34.48*	0.29
T/C ratio	1.17	0.81	0.73	0.96*	0.69*	0.01
A/T ratio	9.58	6.03	6.81	9.11	7.41	0.26
White muscle parameters (*n* = 20)						
RNA (μg g^{-1})	6,198	3,607*	4,407*	1,830*	2,223*	33
Protein (mg g^{-1})	33.22	70.66	67.66	149.97	134.09	1.68
Lipid (mg g^{-1})	16.19	13.42	14.89	32.12*	42.58*	0.32
RNA/protein ratio (μg g^{-1})	186.74	48.52*	67.21*	12.35*	16.67*	0.75
Protein/lipid ratio (mg mg^{-1})	2.07	5.29	4.41	4.60*	3.33*	0.07
Oocyte parameters (*n* = 20)						
[c]Trypsin-like specific activity	-	-	-	-	2.18	0.06
[c]Chymotrypsin-like specific activity	-	-	-	-	2.44	0.06
RNA (μg g^{-1})	-	-	-	-	5,145	172
Protein (mg g^{-1})	-	-	-	-	412	23.07
Lipid (mg g^{-1})	-	-	-	-	55.64	1.58
RNA/protein ratio (μg g^{-1})	-	-	-	-	13.20	0.63
Protein/lipid ratio (mg mg^{-1})	-	-	-	-	7.65	0.40

[a] Expressed as increase in absorbance at 440 nm h^{-1} mg $protein^{-1}$, [b] Expressed as μmol maltose produced h^{-1} mg $protein^{-1}$, [c] Expressed as μmol *p*-nitroanilide produced h^{-1} mg $protein^{-1}$.

the selected feeds and feed raw materials (Figure 4). This could be affected by sex and developmental stages (Figure 4). The highest digestibility of carbohydrate was found in peanut meal for 10 days old juveniles (Figure 4A). In addition, white sorghum, water flea, corn, tapioca chip, wheat bran, and mosquito larva (ranked from higher to lower digestibility) were among the highly digestible carbohydrate sources for this early stage (Figure 4A). Males at 1.5 months old showed high carbohydrate digestibility for commercial feed formulation 5 > peanut meal > commercial feed formulation 6 > fish meal > soybean meal, with higher digestibility than females of the same age (Figure 4B). An indication that mosquito larvae are the ideal carbohydrate source for females during growth was observed, as they showed the highest carbohydrate digestibility by enzyme extracts from females with significantly higher (P < 0.05) than male enzyme extracts (Figure 4B). For fish at maturation, males showed high carbohydrate digestibility for peanut meal > golden apple snail meat > commercial feed formulation 2 > commercial feed formulation 5 > fish meal 58 > commercial feed formulation 8 > shrimp shell (Figure 4C). Females at maturation showed high carbohydrate digestibility for peanut meal > fish meal 58 > commercial feed formulation 8 > commercial feed formulation 5 > soybean meal > commercial feed formulation 2 (Figure 4C). At maturation, females exhibited higher carbohydrate digestibility than males for most of the feeds and feedstuffs selected (Figure 4C).

DISCUSSION

Specific activities of total protease, trypsin and chymotrypsin increased with age in Siamese fighting fish, and they were significantly different between sexes at maturation (P < 0.05) (Table 1). Proteolytic activity has been early reported to play an important role in carnivorous and omnivorous species (Hofer and Schiemer, 1981). Among acidic and alkaline proteases,

Table 2. Regression analysis (n = 15) between growth parameters (total length and body weight) and expression of each enzyme parameter in Siamese fighting fish during development of males, females, and regardless of sex. Significant correlation coefficients (r) are shown by the P values < 0.05 (**bold values**).

Enzymes	Sex	Total length		Body weight	
		r	P	*r*	P
Total protease	Regardless of sex	0.44	0.054	0.49	**0.034**
	Male	0.96	**0.000**	0.92	**0.000**
	Female	0.83	**0.002**	0.98	**0.000**
Amylase (A)	Regardless of sex	0.77	**0.000**	0.78	**0.000**
	Male	1.00	**0.000**	0.99	**0.000**
	Female	0.92	**0.000**	0.99	**0.000**
Trypsin (T)	Regardless of sex	0.60	**0.007**	0.59	**0.008**
	Male	0.91	**0.000**	0.92	**0.001**
	Female	0.90	**0.000**	0.99	**0.000**
Chymotrypsin (C)	Regardless of sex	0.46	0.050	0.42	0.076
	Male	0.83	**0.001**	0.86	**0.006**
	Female	0.93	**0.000**	1.00	**0.000**
T/C ratio	Regardless of sex	-0.30	0.214	-0.16	0.501
	Male	-0.42	0.170	-0.54	0.349
	Female	-0.94	**0.000**	-0.90	**0.003**
A/T ratio	Regardless of sex	-0.04	0.879	-0.32	0.679
	Male	-0.17	0.595	-0.28	0.808
	Female	-0.60	0.067	-0.68	0.156

only the alkaline protease trypsin is the key for feed efficiency and growth (Rungruangsak-Torrissen et al., 2006), while the alkaline protease chymotrypsin is the key for limited or reduced growth (Rungruangsak-Torrissen et al., 2006; Chan et al., 2008). This has made the protease activity ratio of trypsin to chymotrypsin (T/C ratio) the key factor for digestive efficiency and growth performance in aquatic animals (Sunde et al., 2004; Rungruangsak-Torrissen, 2007; Rungruangsak-Torrissen et al., 2006, 2009).

In Siamese fighting fish, acidic proteases are dominant in early life stage while alkaline proteases are dominant in later stages (Thongprajukaew, 2010). Trypsin contributes approximately 40 to 50% of overall protein digestion activity in carnivorous species (Eshel et al., 1993), and plays a crucial role in regulating the activity of pancreatic proteases (Stryer, 1988). Amylase specific activity also increased with age in Siamese fighting fish, similar to the observations in other species by Lazo et al. (2007), and the expressions were also significantly different between sexes at maturation (P < 0.05) (Table 1). In spite of significantly higher female digestive enzymes (either for protein or carbohydrate digestions) at maturation, the T/C ratio was significantly lower in females in association with slower growth, compared to the males (P < 0.05) (Table 1). Both T/C and A/T ratios showed negative correlations with total length and body weight due to reduction in growth rate at older stage, but significant relationships were only observed in the female T/C ratios (Table 2). On the other hand, all studied digestive enzymes showed positive relationships with total length or body weight or both growth parameters, and could be affected by sexes (Table 2). The results indicated that the T/C ratio was the most reliable key parameter for growth efficiency, independent of the digestive enzyme specific activity levels, as described by Rungruangsak-Torrissen et al. (2009). The A/T ratio was not significantly correlated to growth parameters (P > 0.05) (Table 2) and not associated with the levels of carbohydrate or protein in the diets (Tables 1 and 2), similar to the observation by Gamboa-Delgado et al. (2003) but in contrast to the suggestions by Hofer and Schiemer (1981) and Hidalgo et al. (1999). Higher digestive enzyme expressions could be associated with higher consumption or higher utilization or both, but the indication of food utilization for somatic growth efficiency (regardless of protein or lipid growth) is the T/C ratio, designated as digestive efficiency (Rungruangsak-Torrissen et al., 2006, 2009).

During development, the levels of white muscle RNA

Table 3. Biochemical compositions of feeds and feedstuffs used for *in vitro* digestibility studies. The commercial feeds are specific feed formulations for Siamese fighting fish, collected from a Thai aquatic animal market. The formulations 3 and 4 are used for rearing juveniles, and the formulations 1 and 2 and 5 to 8 are used for rearing maturing fish. The analyses were performed as described by the AOAC

Feed and feedstuff	Moisture g kg^{-1}	g kg^{-1} on dry matter basis				
		Protein	NFE	Lipid	Fiber	Ash
Natural feeds						
Pomacea canaliculata meat	808.2	660.1	177.8	26.5	2.1	133.5
Moina sp.	856.4	557.1	279.9	62.0	47.4	53.6
Mosquito larva	734.2	526.3	242.0	127.5	27.8	76.4
Chicken egg yolk	480.6	302.7	86.3	566.0	nd	45.0
Duck egg yolk	417.4	283.2	24.0	654.1	nd	38.7
Commercial feeds						
Formulation 1	81.3	618.5	110.3	131.4	13.9	125.9
Formulation 2	45.8	533.5	216.7	134.4	5.4	110.0
Formulation 3	55.9	518.1	203.4	151.1	4.4	123.0
Formulation 4	66.1	517.0	273.8	95.0	9.6	104.6
Formulation 5	71.4	512.0	333.3	44.3	10.9	99.5
Formulation 6	81.3	390.9	477.9	50.4	6.5	75.0
Formulation 7	74.8	337.4	540.8	37.9	9.1	74.8
Formulation 8	67.8	338.2	526.2	37.9	22.2	75.5
Feedstuffs						
Animal sources						
Blood meal	59.8	903.9	71.4	4.3	3.6	16.8
Fish meal 59	89.4	592.5	64.0	93.5	7.5	242.5
Fish meal 58	73.8	585.4	71.0	80.2	7.9	255.5
Meat and bone meal	46.0	400.6	163.6	85.3	2.1	348.4
Shrimp shell	732.6	491.4	49.0	23.9	161.2	274.5
Plant and algal sources						
Wheat gluten	82.7	836.6	150.0	6.5	nd	6.9
Corn gluten meal	75.1	520.7	422.3	30.8	5.2	21.0
Spirulina sp. meal	87.4	461.4	390.5	2.3	4.2	141.6
Soybean meal	99.6	395.2	455.9	34.7	41.5	72.7
Peanut meal	68.2	385.3	424.2	51.8	67.9	70.8
Coconut meal	63.0	200.9	536.7	100.3	89.3	72.8
Wheat bran	100.5	140.2	709.5	33.0	71.2	46.1
Rice bran	98.3	115.9	596.7	150.7	57.3	79.4
White sorghum	120.9	115.3	811.7	23.0	22.2	27.8
Wheat flour	120.6	111.4	874.2	3.8	3.4	7.2
Corn	109.1	74.2	841.9	45.3	25.9	12.7
Steam broken rice	104.4	68.1	903.4	13.2	1.7	13.6
Tapioca chip	92.4	27.5	904.7	5.0	15.8	47.0

NFE = nitrogen free extract, nd = not detected.

decreased while protein increased, resulting in decreasing RNA/protein ratio (Table 1), similar to the observations in other species by Mathers et al. (1993) and Peragon et al. (2001). Increased white muscle protein concentrations during successive growth have also been observed in rainbow trout, *Oncorhynchus mykiss* (Rungruangsak-Torrissen et al., 2009). This indicates that more protein is retained at older age, and *B. splendens* females had higher protein synthesis and turnover rate (RNA concentration and RNA/protein ratio)

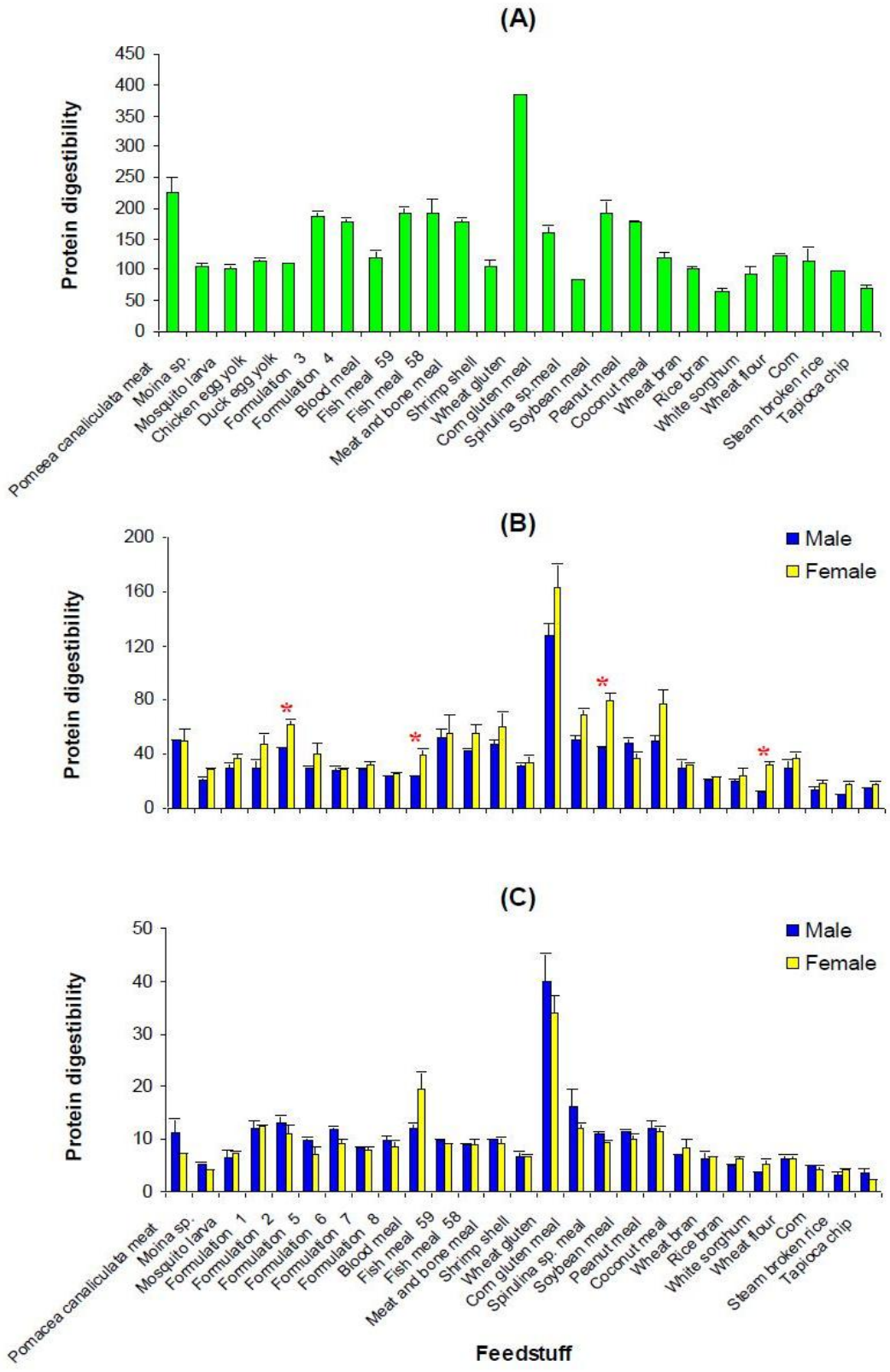

Figure 3. *In vitro* protein digestibility (μmol *DL*-alanine equivalent g dried feed^{-1} trypsin activity^{-1}) of feeds and feedstuffs using dialyzed crude enzyme extract from 10-day-old juveniles (A), males and females of 1.5-month-old (B), and males and females of 3-month-old (C). The bars with asterisks indicate significant differences between males and females ($P < 0.05$).

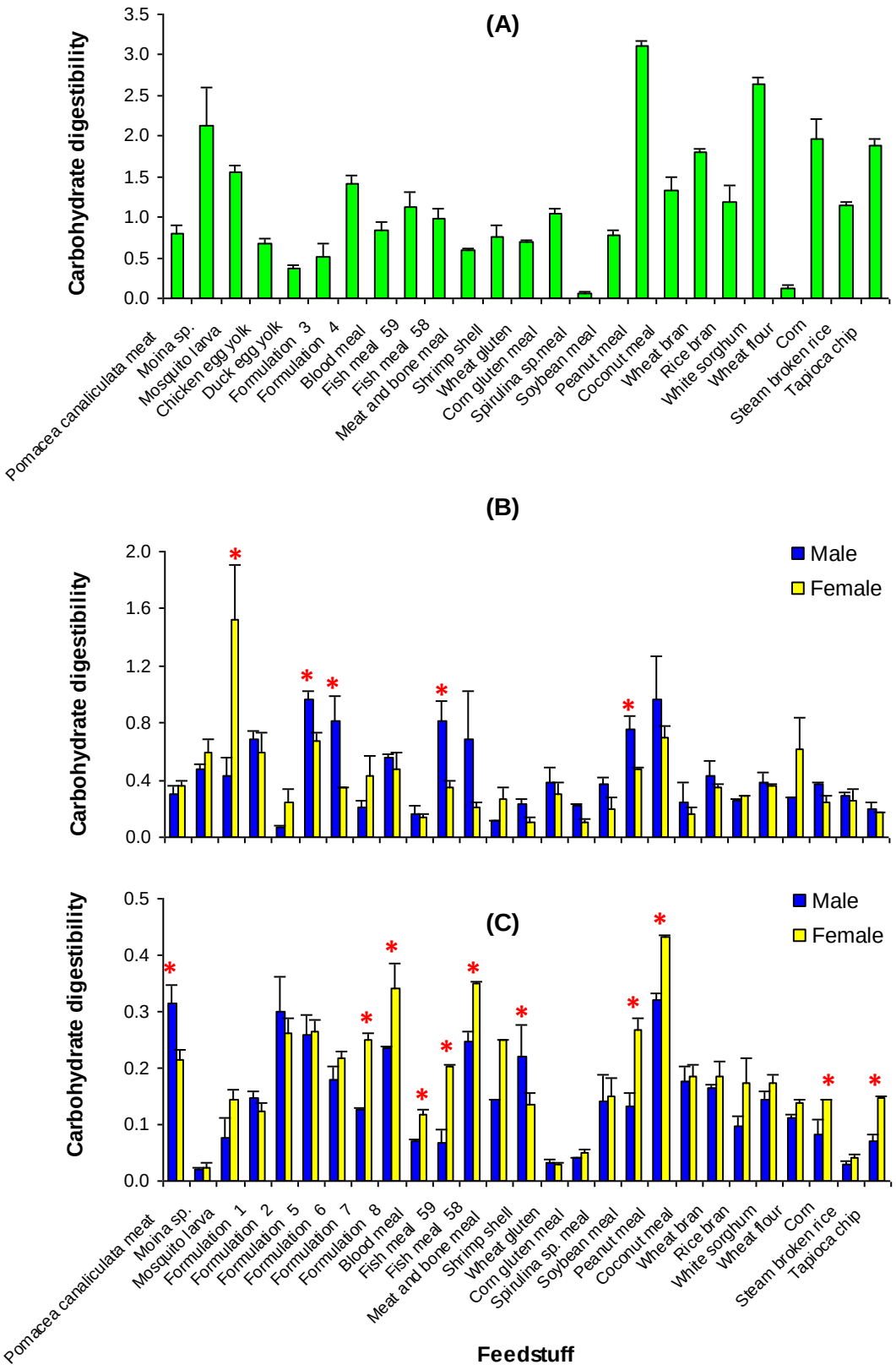

Figure 4. *In vitro* carbohydrate digestibility (μmol maltose g dried feed^{-1} amylase activity^{-1}) of feeds and feedstuffs using dialyzed crude enzyme extract from 10 days old juveniles (A), males and females of 1.5 months old (B), and males and females of 3 months old (C). The bars with asterisks indicate significant differences between males and females ($P < 0.05$).

than males (Table 1). White muscle protein and lipid concentrations were similar between sexes during development, except at maturation when lipid concentrations were significantly higher in females, resulting in significantly lower P/L ratio in female white muscle ($P < 0.05$) (Table 1). This indicates a higher energy requirement during maturation in females than males. Male Siamese fighting fish exhibit more aggressive and predatory behaviors than females (Jaroensutasinee and Jaroensutasinee, 2001); this may also result in a higher protein content and P/L ratio in male white muscle. At maturation, females showed a higher level of protein synthesis and turnover rate in the oocytes than in the white muscle (Table 1), as indicated by the levels of RNA or RNA/protein ratio or both. The specific activities of trypsin-like and chymotrypsin-like in the oocytes were at very low levels, similar to the levels of digestive trypsin and chymotrypsin in 10 days old juveniles (Table 1). However, these protease specific activity levels in the oocytes cannot be compared directly to those in the larval digestive tract which would increase after feeding. Sveinsdottir et al. (2006) reported increases of both protease expressions in Atlantic cod (*Gadus morhua*) embryos during segmentation (organogenesis) period, followed by a decrease to very low levels at hatching and early larval period (about 0.1 fold for trypsin-like and 0.5 fold for chymotrypsin-like specific activities, compared to non-fertilized eggs). This means that the levels of trypsin-like and chymotrypsin-like expressions in Siamese fighting fish embryos prior to hatching should be lower than the levels observed in the oocytes in Table 1. The expression levels observed in the 10 days old juveniles in Table 1 should have resulted from the new development of trypsin and chymotrypsin in the digestive system at first feeding period. The low protease specific activity of trypsin-like enzyme (probably also chymotrypsin-like enzyme) in the oocytes at maturation stage in Siamese fighting fish should be a common.

Both protein and lipid are important for egg development and newly hatched larvae. Lipid in the eggs appears to be a main metabolic fuel during egg stage and early yolk-sac stage in European sea bass (*Dicentrarchus labrax*), while protein seems to be mobilized for energy at late yolk-sac stage (Rønnestad et al., 1998). Both protein and lipid levels in Siamese fighting fish decreased after hatching, as the levels were 12.4 and 3.4 folds in the oocytes, respectively compared to 10 days old juveniles (Table 1). This indicates that the level of protein in the eggs should be relatively more important than the lipid level, resulting in significantly higher P/L ratio in the oocytes than in the 10 days old juveniles ($P < 0.05$) (Table 1). Serine proteases play an important role in yolk formation and degradation during embryogenesis (Hiramatsu et al., 2002; Sveinsdottir et al., 2006). Trypsin and chymotrypsin from the Siamese fighting fish digestive system, assayed at an optimal pH 10, showed optimal activities at temperature 30 to 35°C and 40°C, respectively (Thongprajukaew, 2010). On the other hand, both trypsin-like and chymotrypsin-like activities in the oocytes showed optimal conditions at pH 10 and 20 to 30°C, with respectively low activities at 40 and 50°C (Figures 1 and 2). The differences in temperature profiles of the two serine proteases between the oocytes (current work) and the digestive system (Thongprajukaew, 2010) in Siamese fighting fish indicate different forms of the serine proteases between these tissues. It is interes-ting to note that both trypsin and chymotrypsin in the digestive tract with an optimal pH 10 showed higher specific activities at 20 to 30°C in females at maturation, compared to immature females and males at all stages (Thongprajukaew, 2010). This probably indicates that the trypsin and chymotrypsin in the digestive system with activity characteristics at pH 10 and low tempe-ratures of 20 to 30°C (Thongprajukaew, 2010), similar to trypsin-like and chymotrypsin-like in the oocytes (current work), may influence maturation in female Siamese fighting fish. Surprisingly, the relatively high activities of the oocyte enzymes were observed at high temperatures of 70 to 80°C (Figures 1B and 2B). This phenomenon has also been observed in cod oocytes (unpublished data), and in the hepatopancreas of cuttlefish (*Sepia officinalis*), showing trypsin with optimal activity but less stability at 70°C (Balti et al., 2009).

The developmental variations in trypsin and amylase expressions has made it possible to use them to standardize *in vitro* digestibility values for protein and carbohydrate, respectively in feedstuffs for suitable future feed formulations for specific stages and sexes (Figures 3 and 4). The most appropriate protein source for all stages was wheat gluten (Figure 3) with protein content of 837 g kg^{-1} on dry matter basis (Table 3). Another interesting source for 10 days old juveniles was golden apple snail, a pest against plants in agricultural ecosystems and an intermediate host of aquatic parasites. Golden apple snail meat had highly digestible protein value (Figure 3) with protein content of 660 g kg^{-1} on dry matter basis (Table 3), and with relatively high essential amino acid index and essential fatty acid profile (Bombeo-Tuburan et al., 1995). It could be a good replacer for other animal protein sources in formulated diets because of low price, and its gonad and egg contain high content of the main carotenoids, astaxanthin and carotenoprotein (Dreon et al., 2007), which may improve survival rate of juveniles and coloration in Siamese fighting fish. Soybean meal and fish meal were the other good feed raw materials for all stages. Soybean meal is widely used as the most effective alternative for fish meal in artificial feeds, because of its well-balanced amino acid profile and reasonable price. Chou et al. (2004) reported that fish meal could be replaced up to 40% by soybean meal without causing reduction in growth and protein utilization in cobia (*Rachycentron canadum*). Sales (2008) demonstrated similarity in apparent protein digestibility values between fish meal (56 to 99%) and soybean meal

(50 to 99%).

The other suitable protein sources for the older stages were corn gluten, *Spirulina* sp. meal, and blood meal. Corn gluten meal is expensive, and caused low fiber and yellow color in salmonids (Hardy, 1996), which may also limit its use in feeds for Siamese fighting fish. *Spirulina* sp. contains high levels of essential amino acids, vitamins, and color enhancing pigments (Peirettie and Meineri, 2008), and has 461 g kg^{-1} in protein content on dry matter basis (Table 3) with moderate levels of digestible products (Figure 3B and C). These microalgae have no cell wall, resulting in improved digestion and absorption (Nandeesha et al., 1998), and *Spirulina* sp. meal has been identified as a potential protein source (Nandeesha et al., 2001). Blood meal is not an ideal main protein source because of very low isoleucine and glycine (Khawaja et al., 2007), although its digestibility values (55 to 99%) is relatively high (Sales, 2008). Moreover, during on-growing (1.5 months old), females showed significantly higher protein digestibility for *Spirulina* sp. and white sorghum than males (Figure 3B), which may indicate the presence of certain amino acids and other nutrients suitable for females in these feedstuffs.

Carbohydrate digestibility is low in carnivorous species whereas amylase expression is important, but it plays as secondary role in selecting appropriate feed ingredients having equally digestible protein. Areekijseree et al. (2006) reported protein digestibility to be the key factor for determining feed quality in an aquatic herbivore (freshwater mussel), followed by carbohydrate digestibility as secondary factor. A good alternative carbohydrate source for Siamese fighting fish rearing at all stages was peanut meal (Figure 4). White sorghum and water flea were the other alternative feed raw materials for 10 days old juveniles (Figure 4). The other suitable alternatives were fish meal and soybean meal for 1.5 months old males and adult females, mosquito larva and probably wheat flour for 1.5 months old females, and golden apple snail meat for adult males (Figure 4). Despite the lower carbohydrate content in feed raw materials from animal sources compared to plant sources (Table 2), animal feedstuffs demonstrated high *in vitro* carbohydrate digestibility (Figure 4). Using these animal protein sources will also provide good carbohydrate sources.

Siamese fighting fish at various growth stages and of different sexes had different abilities to digest the same raw materials and commercial feeds (Figures 3 and 4). Atlantic salmon with genetically different trypsin phenotypes have different abilities to digest the same fish meals (Bassompierre et al., 1998). Therefore, utilizing *in vitro* digestibility techniques using fish crude enzyme extracts from specific growth stages and sexes, and standardized by trypsin or amylase activity, is important in screening feedstuffs for developing formulated feeds with high nutritional qualities for optimizing growth performance quality. The work on feed formulations for *in vitro* and *in vivo* studies to optimize coloration and growth performance quality in Siamese fighting fish is currently under way.

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