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Effects of sex on characteristics and expression levels of digestive enzymes in the adult guppy *Poecilia reticulata*

Karun Thongprajukaew^{1,2*} and Uthaiwan Kovitvadhi^{2,3}**Abstract**

Background: Characteristics (pH 2 to 12 and temperature of 25°C to 80°C) and expression levels of the main digestive enzymes, including amylase, lipase, total protease, trypsin, and chymotrypsin, were investigated in adult male and female guppies *Poecilia reticulata*. A completely randomized design was used during this experiment.

Results: Digestive enzyme studies indicated that sex had a direct effect on characteristic profiles ($n = 3$) and expression levels ($n = 10$) of all digestive enzymes. The most suitable conditions for studying digestive enzymes in general were pH 2 at 50°C for acidic amylase, pH 7 at 55°C for neutral amylase, pH 11 at 50°C for alkaline amylase, pH 7 at 30°C to 35°C for lipase, pH 2 at 55°C for acidic protease, pH 9 at 45°C for alkaline protease, pH 8 at 50°C for trypsin, and pH 9 at 50°C for chymotrypsin, regardless of sex. Specific activities of amylase, total protease, trypsin, and chymotrypsin were higher in females than males ($p < 0.001$), while the specific activity of lipase was higher in males than females. These findings were correlated with the higher growth capacity of females than males (except for lipase), as indicated by differences in body weight ($p < 0.001$), length ($p < 0.001$), and the activity ratio of trypsin (T) to chymotrypsin (C), i.e., the T/C ratio ($p < 0.007$).

Conclusions: Feeding habits of adult guppies differed between sexes, as indicated by a difference in the activity ratio between amylase and trypsin, i.e., the A/T ratio ($p < 0.04$). Information from digestive enzyme studies, as well as using optimal conditions of digestive enzymes to study *in vitro* nutrient utilization in guppies, might be important to understand the effects of sex on nutritional responses.

Keywords: Characteristics; Digestive enzymes; Guppy; *Poecilia reticulata*; Sex

Background

The guppy *Poecilia reticulata* is a small, beautiful, peaceful, lively, hardy fish. It is a popular freshwater aquarium fish around the world. Many strains of guppies have been created based on body color, color patterns, and fin shape (Nakajima and Taniguchi 2002). Moreover, in the same strain, differences in morphological appearances between sexes can clearly be observed, as female bodies are larger and rounder than those of males, while male fins are more colorful and larger than those of females. Recently, studies on digestive enzyme characteristics in

Siamese fighting fish *Betta splendens* indicated that female fish probably developed earlier than male fish when fed the same diet (Thongprajukaew et al. 2010a, b). This evidence led us to examine digestive enzymes as key markers of sexual dimorphism in that species (Thongprajukaew et al. 2013). A similar emphasis of sex on digestive enzymes was also found in the freshwater pearl mussel *Hyriopsis (Hyriopsis) bialatus* with the possibility of improving diet formulations (Areekijsee et al. 2004). These results indicated that differences in digestive strategies of nutrient utilization can be influenced by sex.

Utilization of nutrients in aquatic animals depends on the activity of their digestive enzymes (Areekijsee et al. 2006; Rungruangsak-Torrissen et al. 2006; Supannapong et al. 2008). Moreover, the digestive organ mass of fish positively contributes to the growth capacity (Thongprajukaew et al. 2011) by increasing the efficiencies of digestion,

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absorption, and utilization of nutrients. The use of digestive enzyme information for *in vitro* screening of feed ingredients was investigated in the freshwater pearl mussel (Areekijserree et al. 2006, Supannapong et al. 2008), spiny lobster *Panulirus argus* (Perera et al. 2010), and Siamese fighting fish (Thongprajukaew et al. 2013). Moreover, digestive enzyme activity was also used to determine growth (Sunde et al. 2001; Rungruangsak-Torrissen et al. 2006; Rungruangsak-Torrissen 2007; Thongprajukaew et al. 2011) and optimal feeding management (Chan et al. 2008; Xie et al. 2011).

Therefore, information on digestive enzymes can provide elementary knowledge to help understand the nutritional status of an aquatic animal. The objective of this study was to investigate the effect of sex on characteristics and expression levels of the main digestive enzymes including amylase, lipase, and proteolytic enzymes (total proteases, trypsin, and chymotrypsin) in the digestive tract of guppies. Information from this study may be important in determining future *in vitro* nutrient utilization of sex-specific feed formulations by guppies.

Methods

Fish culture and sample collection

Four-month-old male and female guppies were obtained from a private farm in Nakhon Pathom Province, Thailand. Fish sex was identified by morphological differences including body weight (BW), total length (TL), and color intensity. Fish were acclimatized in an aquarium (18-cm wide × 34-cm long × 19-cm high) with a 15-cm water level for 14 days. During acclimatization, fish were fed *ad libitum*, twice daily (at 0800 hours and 1700 hours), with a commercial diet for herbivorous fish containing 32% protein, 4% lipids, 5% fiber, and 10% ash. Seventy-five percent of the water content was changed every other day. Water quality measurements included temperature of $28.32^{\circ}\text{C} \pm 0.05^{\circ}\text{C}$, pH 7.51 ± 0.03 , and dissolved oxygen of 7.44 ± 0.04 mg/l. At the end of acclimatization, the fish were starved for 24 h prior to sampling to avoid the effect of diet ingestion on digestive enzyme activities. Fish were sacrificed by chilling in ice according to the Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes of the National Research Council, Thailand. BW and TL were individually measured before carefully collecting the digestive organs and the carcass. All tissues were then stored at -80°C until being analyzed.

Digestive enzyme studies

Digestive enzyme extraction

Digestive enzymes from male and female guppies were extracted from whole digestive organs using a micro-homogenizer (THP-220; Omni International, Kennesaw, GA, USA) in the presence of 50 mM Tris-HCl buffer (pH 8) containing 200 mM NaCl (1:3 w/v). The

homogenate was centrifuged at $15,000 \times g$ for 30 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 until the digestive enzyme assays were run. The protein concentration in the enzyme extract was determined according to Lowry et al. (1951) using bovine serum albumin as a standard curve.

Characterization and specific activities of digestive enzymes

pH and temperature characteristics of the main digestive enzymes were assayed according to the method described by Thongprajukaew et al. (2010a, b). Data for digestive enzyme assays are summarized in Table 1. pH profiles were studied at an ambient temperature using the following buffers: glycine-HCl for pH 2, citrate-phosphate buffer for pH 3 to 5, phosphate buffer for pH 6 to 8, $\text{NaHCO}_3\text{-Na}_2\text{CO}_3$ buffer for pH 9 to 10, $\text{Na}_2\text{HPO}_4\text{-NaOH}$ for pH 11, and KCl-NaOH for pH 12. Temperature profiles (25°C to 80°C) were determined at optimal pH values. For specific activities, all digestive enzymes were assayed at specified optimal conditions, as described in Table 2. The specific activities of digestive enzymes are expressed in units per milligram of protein with 1 unit of enzymes defined as 1 μmol of substrate released per minute. For total protease, the unit of enzyme activity was defined as the amount of enzyme causing an increase of 1.0 absorbance unit at 440 nm.

Determination of body compositions

Moisture and ash were respectively determined using an automatic moisture analyzer balance (MA 30; Sartorius, Göttingen, Germany) and a muffle furnace (ELF 11/14; Carbolite Limited, Hope Valley, UK), according to AOAC (2005). Protein concentrations were determined using TRIzol[®] reagent (Invitrogen, Carlsbad, CA, USA), as described in Rungruangsak-Torrissen (2007). The extinction coefficient for calculating protein concentrations was $E_{280} = 2.1$ mg/ml. Lipid contents were extracted using ethyl acetate, as described by Supannapong et al. (2008). All values were expressed on a wet weight basis.

Statistical analysis and calculations

The experiment was performed with a completely randomized design. Seventy fish of each sex ($n = 70$) were divided into three pooled samples ($n = 3$ from 60 fish) to characterize digestive enzymes, and ten fish ($n = 10$) were used to study body compositions and specific activities of digestive enzymes. All data are expressed as the mean \pm standard error of the mean. Significant differences between sexes were compared using an independent-sample *t* test. A condition factor (CF, g/cm^3) and digestosomatic index (DSI, %) were calculated from the respective equations, $100 \times (\text{live BW}/\text{TL}^3)$ and $100 \times (\text{digestive tract weight}/\text{BW})$, as described by Thongprajukaew et al. (2011).

Table 1 Digestive enzyme assays used in the experiment

Digestive enzyme	Substrate	Reference
α -Amylase (EC 3.2.1.1)	Soluble starch	Areekijsee et al. (2004)
Lipase (EC 3.1.1.3)	<i>p</i> -Nitrophenyl palmitate	Winkler and Stuckmann (1979)
Total proteases	Azocasein	Areekijsee et al. (2004)
Trypsin (EC 3.4.21.4)	<i>n</i> -Benzoyl-L-arginine- <i>p</i> -nitroanilide	Rungruangsak-Torrissen et al. (2006)
Chymotrypsin (EC 3.4.21.1)	<i>n</i> -Succinyl-ala-ala-pro-phenylalanine- <i>p</i> -nitroanilide	Rungruangsak-Torrissen et al. (2006)

Results

Effects of sex on growth indicators and body compositions

Significant differences in growth indicators were observed between sexes. Females had a higher BW ($p < 0.001$, Table 2), TL ($p < 0.001$), and CF ($p < 0.001$) than males by 2.54-, 1.26-, and 1.27-fold, respectively. The gastrointestinal weight of females was significantly higher than that of males ($p < 0.001$), by approximately 2.23-fold. However, the DSI did not differ between sexes ($p > 0.05$). As to body compositions, moisture and lipids significantly differed

between sexes ($p < 0.003$, Table 2). Females had a higher lipid content than males, while protein and ash did not differ between sexes ($p > 0.05$).

Effects of sex on digestive enzyme characteristics

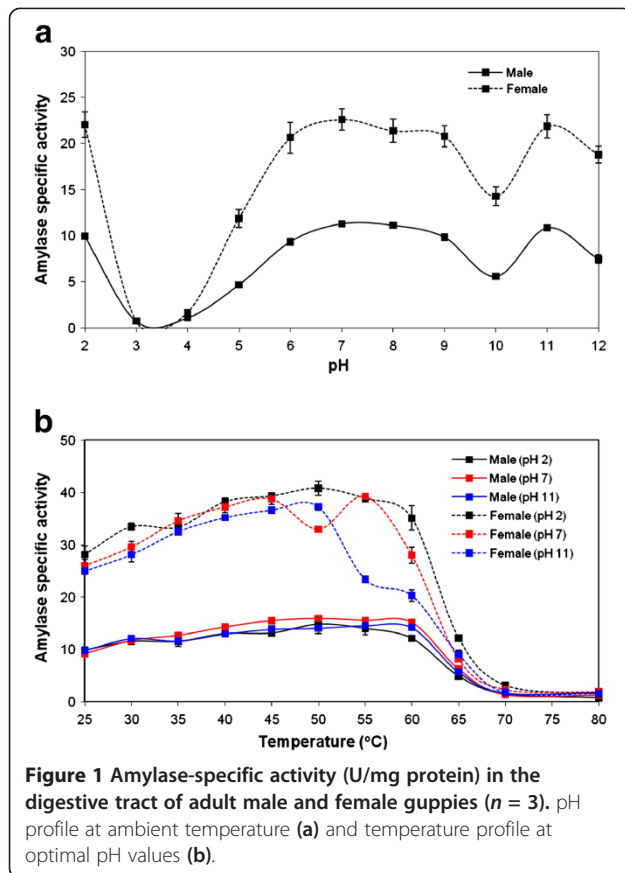
Characterization of amylase

Male and female guppies showed similar amylase characteristics at various pH values (Figure 1a). Optimal conditions for studying amylase in a neutral to alkaline solution ranged from pH 7 to 9, while extremely acidic and alkaline solutions were pH 2 and 11, respectively (Figure 1a). Optimal

Table 2 Growth parameters, body compositions, and digestive enzyme-specific activities of adult male and female guppies

Parameter	Male	Female	<i>p</i> value
Body weight (g), <i>n</i> = 70	0.35 ± 0.01	0.89 ± 0.02	<0.001 ^c
Total length (cm), <i>n</i> = 70	3.40 ± 0.02	4.27 ± 0.03	<0.001 ^c
Condition factor (g/cm ³), <i>n</i> = 70	0.89 ± 0.02	1.13 ± 0.01	<0.001 ^c
Digestive index, <i>n</i> = 70			
1. Gastrointestinal weight (g)	0.030 ± 0.01	0.067 ± 0.007	<0.001 ^c
2. Digestosomatic index (%)	9.02 ± 0.60	8.99 ± 0.65	0.961
Body composition (%), <i>n</i> = 10			
1. Moisture	76.82 ± 0.32	75.14 ± 0.19	0.002 ^c
2. Lipids	2.69 ± 0.09	3.16 ± 0.08	0.002 ^c
3. Protein	13.76 ± 0.59	11.47 ± 0.54	0.430
4. Ash	3.42 ± 0.08	3.69 ± 0.19	0.195
Digestive enzymes, <i>n</i> = 10			
1. Amylase (A) ^a			
1.1 Acidic amylase (pH 2, 50°C)	16.26 ± 0.58	39.51 ± 1.25	<0.001 ^c
1.2 Neutral amylase (pH 7, 55°C)	17.40 ± 1.17	36.86 ± 1.33	<0.001 ^c
1.3 Alkaline amylase (pH 11, 50°C)	16.39 ± 0.74	37.95 ± 0.95	<0.001 ^c
2. Neutral lipase (pH 7, 35°C) ^a	1.04 ± 0.09	0.42 ± 0.02	<0.001 ^c
3. Total proteases ^b			
3.1 Acidic protease (pH 2, 55°C)	111.69 ± 7.68	244.07 ± 7.23	<0.001 ^c
3.2 Alkaline protease (pH 9, 45°C)	74.70 ± 7.11	231.91 ± 18.94	<0.001 ^c
4. Trypsin (T) (pH 8, 50°C) ^a	0.61 ± 0.03	2.06 ± 0.19	<0.001 ^c
5. Chymotrypsin (C) (pH 9, 50°C) ^a	0.20 ± 0.01	0.38 ± 0.01	<0.001 ^c
6. T/C ratio	3.07 ± 0.05	5.39 ± 0.44	0.006 ^c
7. A/T ratio	30.29 ± 2.35	22.88 ± 0.26	0.035 ^c

^aExpressed in units per milligram (U/mg) of protein. ^bExpressed in milliunits per milligram (mU/mg) of protein. ^cDiffered significantly ($p < 0.05$) between sexes.



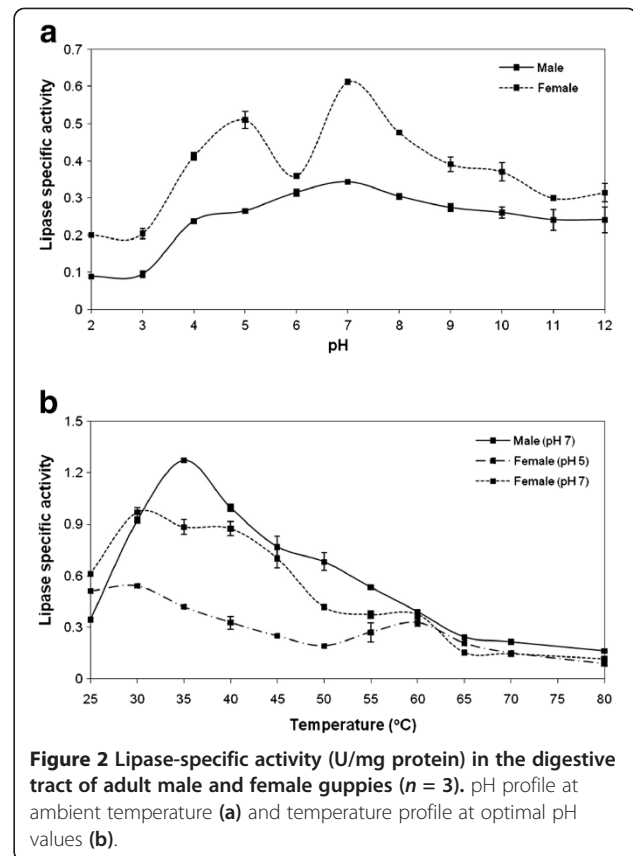
temperatures for all amylase isoforms in males were similar and in a range of 25°C to 60°C (Figure 1b), while those of females differed at 30°C and 40°C to 60°C for pH 2, 40°C to 45°C and 55°C for pH 7, and 40°C to 50°C for pH 11. Therefore, the most suitable conditions observed for studying amylase activity in guppies, regardless of sex, were pH 2 at 50°C for the acidic isoform, pH 7 at 55°C for the neutral isoform, and pH 11 at 50°C for the alkaline isoform.

Characterization of lipase

pH characteristics of lipase differed between the sexes (Figure 2a). Activity peaks of male and female lipases were mainly observed at pH 7 with optimal temperatures at 35°C and 30°C, respectively (Figure 2b). Another isoform of female lipase was found at pH 5 (Figure 2a) with temperature optima at 25°C to 30°C and 60°C (Figure 2b). The most suitable conditions observed for studying lipase activity in guppies, regardless of sex, were pH 7 and 30°C or 35°C.

Characterization of proteolytic enzymes

Acidic protease was similarly detected in male and female guppies. The optimal conditions were similar and were pH 2 (Figure 3a) and 55°C (Figure 3b). Alkaline proteases

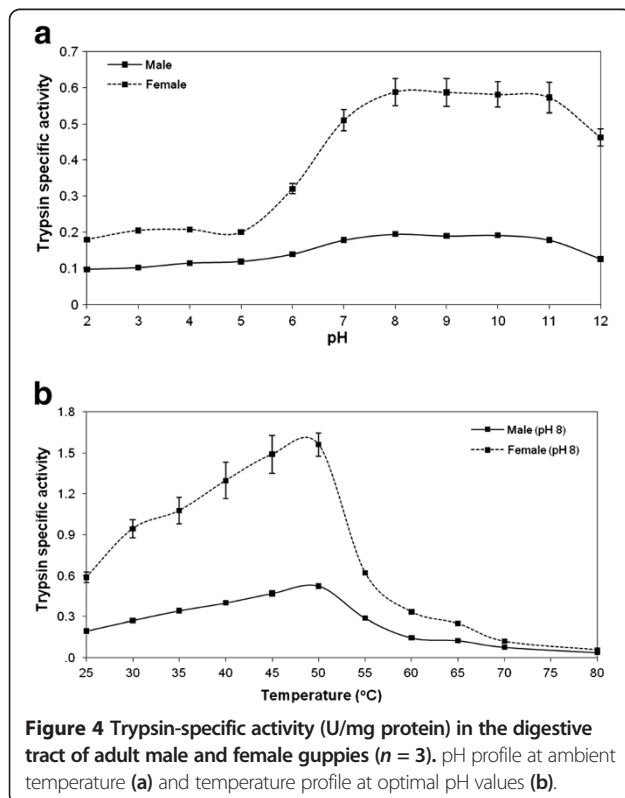
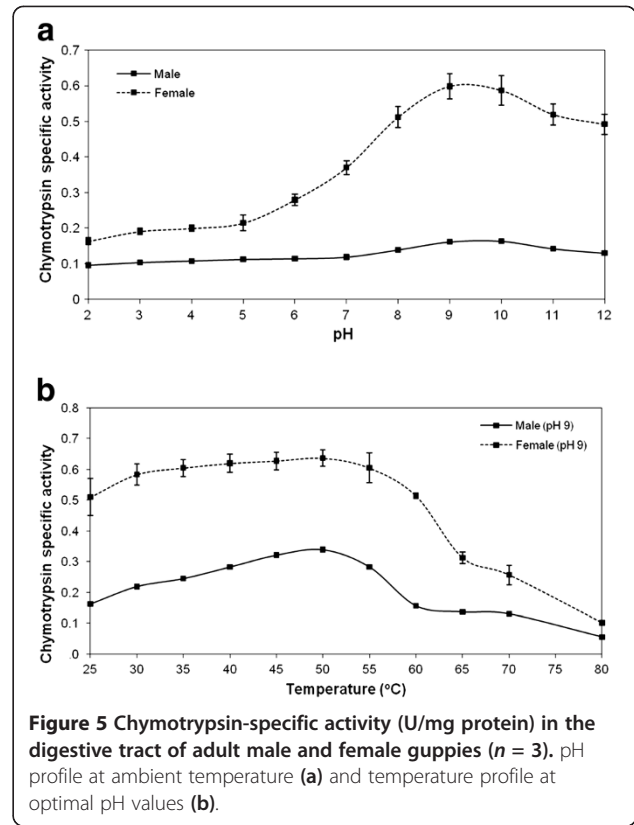
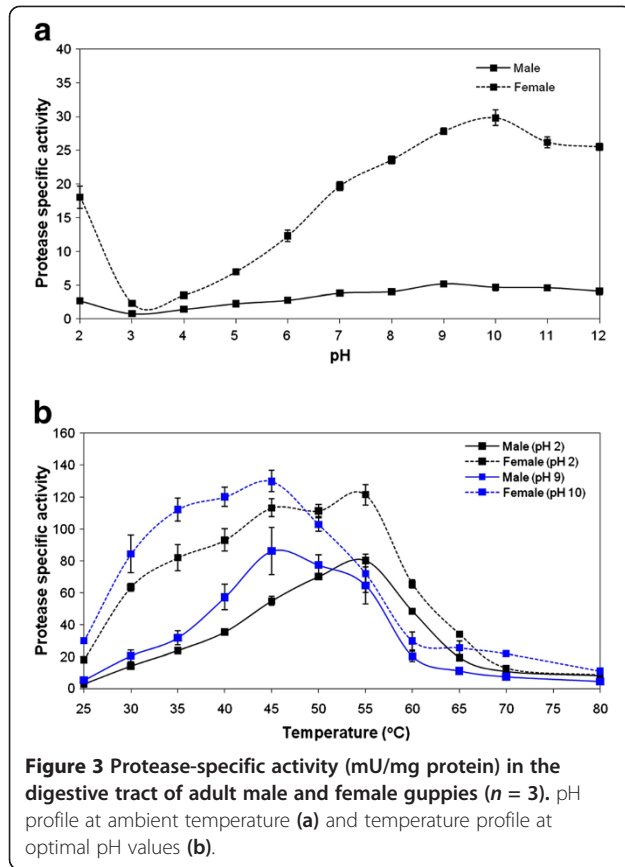


were found at pH 10 for females and at a pH range of 9 to 12 for males (Figure 3a), with similar maximal activities at 45°C (Figure 3b). Therefore, the most suitable conditions for studying total protease activity in guppies, regardless of sex, were pH 2 and 55°C for acidic protease and pH 9 at 45°C for alkaline protease.

Characteristic profiles of trypsin (Figure 4a,b) and chymotrypsin (Figure 5a,b) at various pH values and temperatures were similar between male and female guppies. A broad optimal pH of trypsin was detected at pH 8 to 11 for females and pH 7 to 11 for males (Figure 4a). For chymotrypsin, the optimal conditions were pH 9 to 10 (Figure 5a) and temperatures of 30°C to 55°C for females and 45°C to 50°C for males (Figure 5b). The most suitable conditions for studying trypsin and chymotrypsin activity in guppies, regardless of sex, were at pH 8 and 9 and 50°C, respectively.

Effects of sex on specific activities of digestive enzymes

Sex had a significant effect on specific activities of all digestive enzymes ($p < 0.001$, Table 2). Female guppies had higher specific activities of amylase, total protease, trypsin, and chymotrypsin than males, by approximately 2.29-, 2.65-, 3.38-, and 1.90-fold on average, respectively. On the other hand, lipase-specific activity of males was statistically higher than that of females ($p < 0.001$). For activity ratios of digestive enzymes, sex had a significant effect on the T/C



ratio ($p < 0.007$) and A/T ratio ($p < 0.04$) as females had a higher T/C ratio but a lower A/T ratio compared to males.

Discussion

Growth capacity and body composition

Different growth categories of guppies influenced by sex were reported by Nakajima and Taniguchi (2002). Moreover, those of other aquatic species, such as the Australian red claw crayfish *Cherax quadricarinatus* (Thompson et al. 2006) and Siamese fighting fish (Thongprajukaew et al. 2013), were also observed. Generally, in nature, this phenomenon occurs in many animal species in which morphometric differences are important for sexual attraction. However, differences in weight and length between sexes were not observed, such as in powan *Coregonus lavaretus* (Dörücü 2000) and *Opsariichthys bidens* (Sui et al. 2012). An increased digestive organ weight was reported to play a potent role in increasing growth (Thongprajukaew et al. 2011). This might illustrate higher efficiencies of the gastrointestinal system for digestion, absorption, and utilization of nutrients in females than in males.

A higher lipid content was observed in female bodies compared to males (Table 2). This is in agreement with positive correlations of concurrent increases in the lipid

content with fish size (Salam et al. 2001) and the lipid content with CF (Yousaf et al. 2011). A higher lipid content in the body of females might play a major role as a source of metabolic energy for growth and reproduction (Tocher 2003). This result is in general agreement with the effect of sex on body compositions reported in powan (Dörücü 2000), Russian sturgeon *Acipenser gueldenstaedtii* (Memid et al. 2006), Australian red claw crayfish (Thompson et al. 2006), and freshwater catfish *Wallago attu* (Yousaf et al. 2011).

Characterization of digestive enzymes

Neutral to alkaline isoforms of amylase with a pH range close to those in the pancreas and intestines (Chakrabarti et al. 1995) and in its natural habitat may be major enzymes for carbohydrate digestion in guppies. However, isoforms present in extreme conditions were also reported in other species, such as pH 11 at 50°C in a freshwater pearl mussel (Areekijserree et al. 2004; Supannapong et al. 2008) and Siamese fighting fish (Thongprajukaew et al. 2010b). Moreover, the optimal assayed conditions for amylase activity varied with fish species, such as pH 7.0 to 7.5 in seabream *Sparus aurata* and turbot *Scophthalmus maximus*, and pH 4.5 to 5.0 in redfish *Sebastes mentella*, with an optimal temperature range of 35°C to 45°C for the three species (Munilla-Moran and Saborido-Rey 1996).

The optimal condition for neutral lipase in guppies was similarly observed in red drum larvae *Sciaenops ocellatus* (Lazo et al. 2007) and juvenile Siamese fighting fish (Thongprajukaew et al. 2010b), while the acidic isoform of females was close to that of purified lipase, with optimal conditions of pH 5.5 at 35°C reported for Reba carp *Cirrhinus reba* (Islam et al. 2009). Optimal conditions for sex-specific lipase isoforms were reported by Areekijserree et al. (2002) and Thongprajukaew et al. (2010b). Other lipase isoforms with different optimal conditions were reported in many aquatic species, such as pH 8.5 and 35°C to 40°C for juvenile red claw crayfish *C. quadricarinatus* (López-López et al. 2003), pH 8.0 to 8.5 and 33°C to 35°C for grey mullet *Liza parsia* (Islam et al. 2008), and pH 8.5 and 50°C for gilthead seabream *S. aurata* (Nolasco et al. 2011).

The prominence of acidic protease at pH 2 might indicate the presence of pepsin-like enzymes in the stomach (Natalia et al. 2004). Various protease isoforms were investigated in different aquatic species (Areekijserree et al. 2004; Natalia et al. 2004; Supannapong et al. 2008; Thongprajukaew et al. 2010a). For alkaline proteases, the optimal conditions of trypsin in male and female guppies were similar to those of other fish species, such as arowana (Natalia et al. 2004), red drum *S. ocellatus* (Lazo et al. 2007), walleye pollock *Theragra chalcogramma* (Kishimura et al. 2008), and juvenile Siamese fighting fish (Thongprajukaew et al. 2010b). Similarly, the optimal

condition for chymotrypsin in guppies was also found in Atlantic salmon (Rungruangsak-Torrissen 2007) and arowana (Natalia et al. 2004). The alkaline condition of trypsin and chymotrypsin is close to the pH in the intestinal region (Natalia et al. 2004). The suggested conditions overlap with those of alkaline proteases, indicating the importance of trypsin and chymotrypsin for protein digestion in guppies.

Specific activity of digestive enzymes

Effects of sex on digestive enzyme-specific activities in guppies are in partial agreement with the phenomena observed in the freshwater pearl mussel (Areekijserree et al. 2004) and Siamese fighting fish (Thongprajukaew et al. 2013). Significantly higher specific activities of digestive enzymes (except lipase) in females might indicate the use of more energy for growth, as illustrated by a higher BW, TL, and CF, than in males. On the other hand, higher digestive enzyme activities might be important for sexual maturity, as observed in larger cod *Gadus morhua* (Rungruangsak-Torrissen et al. 2012). Moreover, findings indicate different strategies between sexes for nutrient utilization (digestion and absorption) through digestive enzymes. Significantly higher lipase-specific activity in males (in which nearly all enzymes were lower) might be attributed to it being an energy source when activities of carbohydrate- and protein-digesting enzymes are limited. The T/C ratio showed the same trend as in weight and length values in both sexes. A higher ratio indicated faster growth and better food utilization efficiency in many fish species (Sunde et al. 2001; Rungruangsak-Torrissen et al. 2006; Rungruangsak-Torrissen 2007; Chan et al. 2008). Therefore, a higher ratio in females probably indicates better growth capacity and food utilization compared to males. For carbohydrates, the A/T ratio was used as an effective marker to understand feeding habits (Hofer and Schiemer 1981; Gambao-Delgado et al. 2003). The significantly higher ratio in males might indicate better carbohydrate utilization or stronger omnivorous feeding habits than females. Moreover, the association of this ratio with lower weight and length might be helpful to increase agility, as observed in fast swimming behavior, which allows males to better compete for food and other resources than females, as well as for mating performance. Maintaining energy in males by reducing their size with a higher A/T ratio is probably required. Therefore, differences in carbohydrate contents between male and female diets should be of interest.

Conclusions

Sex had a direct effect on pH and temperature characteristics of all digestive enzymes. The optimal conditions for studying digestive enzymes in the guppy were pH 2 at 50°C for acidic amylase, pH 7 at 50°C for neutral amylase, pH 11

at 50°C for alkaline amylase, pH 7 at 30°C to 35°C for lipase, pH 2 at 55°C for acidic protease, pH 9 at 45°C for alkaline protease, pH 8 at 50°C for trypsin, and pH 9 at 50°C for chymotrypsin, regardless of sex. Specific activities of amylase, total proteases, trypsin, chymotrypsin, and the T/C ratio were higher in females than in males. This correlated with morphometric measurements of BW, TL, CF, and gastrointestinal weight. These findings indicate a potent effect of sex on digestive enzyme strategies for digestion, absorption, and utilization of nutrients in guppies, as indicated by alterations in specific activities of all digestive enzymes and the A/T ratio. Therefore, sex-specific diets based on digestive enzyme activity and *in vitro* nutrient utilization should be of interest for this species.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KT carried out the measurement of growth, analysis of body composition, and characterization of digestive enzymes and drafted the manuscript. UK participated in the design of the study, financial support, and discussion. Both authors read and approved the final manuscript.

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