Midgut Protease Activity During Larval Development of *Anastrepha obliqua* (Diptera: Tephritidae) Fed With Natural and Artificial Diet

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Abstract

In this study, we examined the activity of two serine proteases (chymotrypsin and trypsin) and two metalloproteases (carboxypeptidases A and B) during larval development in *Anastrepha obliqua* fed natural (mango fruit) and artificial (formulation used in mass-rearing) diets. Proteolytic activity of chymotrypsin, trypsin, carboxypeptidase A, and carboxypeptidase B was detected in the midgut of different instars of *A. obliqua* and was strongly affected by the pH and diet type. The protein content of the natural and artificial diets was similar. Enzymatic activity was higher in the midgut of the larvae fed the natural diet than in larvae fed the artificial diet. The activity of the endopeptidases (chymotrypsin and trypsin) was lower than those of the exopeptidases (carboxypeptidases A and B). The pH of the midgut varied from acidic to neutral. The results indicate that in the midgut of the larvae reared on both types of diet, the level of carboxypeptidase activity was approximately 100-fold greater than the level of chymotrypsin activity and 10,000-fold greater than the level of trypsin. In conclusion, carboxypeptidase A and B are the main proteases involved in the digestion of proteins in the larvae of *A. obliqua*. The natural diet showed a high bioaccessibility. A clear tendency to express high activities of chymotrypsin and trypsin was observed by the third instar. Our research contributes to the planning and development of novel bioaccessibility assays to understand the nutrition processing of *A. obliqua* larvae under mass-rearing conditions for sterile insect technique.

Key words: carboxypeptidases, serine proteases, digestion, midgut pH, mass-rearing diet

The West Indian fruit fly, *Anastrepha obliqua* Macquart (Diptera: Tephritidae), is one of the main pests of the mango (*Mangifera indica* L.) (Sapindales: Anacardiaceae) and the mombin purpurea (*Spondias purpurea* L.) (Sapindales: Anacardiaceae) (Hernández-Domínguez, Chiapas, Mexico, for at least 174 generations (Artiaga-López et al. 2004, Orozco-Dávila et al. 2017). Although *A. obliqua* can be successfully mass-reared, life history traits indicate a low fitness for these insects (larval and pupal weight, flight ability, and male competitiveness) (Hernández et al. 2009, Meza-Hernández et al. 2004, Rull et al. 2012). Experiments show that increment in protein content of 1.5–4.5% for *A. obliqua* and 5–20% for *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) did not result in an increase in the pupal weight and adult eclosion (Zucoloto 1987; Hernández et al. 2010). In contrast, when the larvae developed with a different type of yeast as the protein source, the weight of the pupa and sexual competitiveness increased (Hernández et al. 2016). A similar pattern was observed for insects developed on different host fruits (Kotkar et al. 2009). This means that if the diets are nutritionally different, the expected effects on the traits of mass-reared insects should be different, indicating that the bioaccessibility could also be different. The bioaccessibility is the fraction of a component that is released...
from the food matrix in the gut during digestion. Traditionally, the quality control of the ingredients for the larval diet is defined by physicochemical analysis (protein content, lipids, and carbohydrates) without considering the bioaccessibility and bioavailability of the components after their ingestion (Pascacio-Villafán et al. 2015, 2016).

Bioaccessibility models provide a convenient alternative to animal and human models by rapidly screening food ingredients with different compositions and structures in a short time. These models are based on the composition of the digestive fluids (enzyme contents, enzyme composition, pH, buffers, and temperature) must be adjusted according to sample characteristics and the biological model of interest (Hur et al. 2011). Several studies have indicated that food with the same nutrient composition but different molecular structures can have different levels of bioaccessibility and thus different nutritional values (Rinaldi et al. 2014). The mass-rearing of fruit flies uses artificial food made by mixing raw ingredients (i.e., torula yeast, corn cob fractions, sucrose, citric acid, methyl paraben, sodium benzoate, and water) with different structures and compositions of the host fruit. Torula yeast contains approximately 50% protein in dry weight and is considered the main nutritional component and source of protein in the larval diet for mass-rearing fruit flies to apply SIT (Orozco-Davila et al. 2017). However, its nutritional value has only been determined by the total protein and amino acid composition without considering protein digestibility (Hernández et al. 2016). Protease activity in the raw extracts of the larval gut has been described in different species of fruit flies (Lemos et al. 1992, Silva et al. 2006, Xiao-Zhen and Ying-Hong 2007, Ping-Ping et al. 2014). In larvae, digestive proteases catalyze the release of peptides and amino acids from the larval diet. Amino acids are essential to maintain the viability and development of insects (Chang 2004).

Knowledge of the type and activity of the enzymes involved in the hydrolysis of the protein could allow for the development of bioaccessibility assays as a useful tool to understand the mechanism of insect nutrition. Therefore, the main goal of this study was to evaluate enzymatic activity of carboxypeptidase (A and B), trypsin, and chymotrypsin during larval development in A. obliqua reared on an artificial diet and mango fruit. The results are discussed to improve the diet for mass-rearing fruit fly larvae for SIT.

Material and Methods

The present study was conducted in the Colonization and Rearing Laboratory of the Development Methods Unit of the Moscafrut Program Facilities in Metapa de Domínguez, Chiapas, Mexico, and the Department of Ecology of Fruit Flies of the El Colegio de la Frontera Sur in Tapachula, Chiapas, Mexico.

Insects

The A. obliqua eggs used to sow on artificial diet and the adults used to infest mango fruits were obtained from a mass-reared population that was raised under artificial conditions for more than 174 generations in the Moscafrut facility at Metapa de Domínguez in Chiapas, Mexico (Artiaga-López et al. 2004).

Experimental Design

The effect of diet on enzymatic activity was determined in larvae that had been raised on one of the following two types of food: a natural diet (mango fruit cv. ‘Ataulfo niño’) and an artificial diet.

The artificial diet was prepared in accordance with the methods described by Artiaga-López et al. (2004) and Dominguez et al. (2010), which consisted of mixing and hydrating the following: 18% corn cob fraction (Mt. Pulaski Products Inc.; Chicago, IL), 8.66% maize flour (Maíz Industrializado del Sureste, S.A. de C.V; Arriaga, Chiapas, México), 9.0% sugar (Ingenio de Huixtla; Huixtla, Chiapas, Mexico), 6.33% Torula yeast (Lake States, Div. Rhinelander Paper Co.; Rhinelander, WI), 0.18% methyl paraben (Malinckrodt Specialty Chemicals Co.; St. Louis, MO), 0.33% sodium benzoate (Cia. Universal de Industrias, S.A. de C.V., México), 0.1% guar gum (Tic Gums, Inc.; Belcamp, MD), 0.43% citric acid (Anhidro Acidulantes FNEUM, Mexana, S. A. de C.V.; Morelos, México), and 56.97% water. The first, second, and third instar of multiple newly hatched larvae were raised on this diet. Larvae were separated from the artificial diet on days 2, 4, and 7 after they were initially sown to obtain first, second, and third instar larvae, respectively (as determined by preliminary testing).

The natural diet consisted of mango cv. ‘Ataulfo niño’ fruits that were collected from orchards in Tapachula, Chiapas, Mexico. The fruits were washed with water and kept until they were fully ripe with a uniform yellow color. To obtain second and third instar, five mangoes were exposed to 200 mated flies for 8 h in 50 x 50 x 50 cm wooden cages covered with a white mesh. The larvae were separated from the mangoes 8 and 12 days after infestation to obtain second and third instar, respectively (according to previous results, unpublished data). The first instar was obtained on day 2 after the newly hatched larvae were sown in liquefied mango pulp containing 1% methyl paraben and 1% sodium benzoate. The artificial and natural diets were stored at 25°C ± 1, 60% humidity, and a 12/12 photoperiod (light/dark).

The physiological maturation times for first, second, and third instar was previously determined in larvae fed natural and artificial diets by dissecting 50 individuals per day. Physiological maturity was determined based on cephalopharyngeal skeleton development (Elson-Harris 1988) using a stereoscope (BestScope BLM-340, International Limited; Beijing, China).

Protein Content in Natural and Artificial Diet

The protein content of artificial diet was determined 0 (without infestation), 2, 4, and 7 days after the newly hatched larvae were sown on the diet. The protein content of the natural diet was determined in mango fruits 0 (without infestation), 6, 8, and 12 days after infestation (corresponding to the first, second, and third instar, according to preliminary trials). To perform these analyses, six 50-g samples of each treatment were obtained for each diet type on each sampling day. Each sample was placed in a pot to dry (DGH 9070 Oven) at 80°C for 72 hours. Then, 0.1–0.5 g of a dry sample was digested by adding 2.0 ml of NaOH and incubating the mixture at 60°C for 60 min. Subsequently, this solution was centrifuged at 14,000 rpm (Eppendorf 5424 Centrifuge) for 15 min. The supernatant was neutralized using a 1 N HCl solution. Posteriorly, 1 ml of buffer solution (pH 9.2) was added, and distilled water was then added until the total volume was 10 ml. Depending on the protein content in the dry sample, the factor dilution was from 400- to 800-fold to adjust it within the calibration range curve. A calibration curve was constructed using standard bovine serum albumin (BSA, 0.5–30 µg/ml), and the absorbance was read at 562 nm using a Multiskan GO spectrophotometer (Thermo Fisher Scientific; Finland).

The bicinconinic acid (BCA) method was used to calculate the amount of protein (Walker 2002, Lowry et al. 1951). A total of 125 µl of each sample solution was mixed with 125 µl of BCA reagent (Sigma–Aldrich 2017). The solution was then placed in a water bath (Thermo Precision, 280 Series) for 30 min at 37°C. Five readings were taken for each of the six samples.
Preparation of Enzyme Extract
The crude gut homogenate of the first, second, and third instar of each diet treatment was used as an enzyme solution. Larvae were washed with distilled water and subjected to chilling pulses to reduce the movement of larvae and to facilitate their manipulation. The guts of the second and third instar were removed by placing the larvae under a stereo microscope, cutting their abdominal region and extracting their midgut in a Petri dish containing a solution of 0.8% NaCl (pHv) at 4°C (Broadway and Duffy 1985). The extraction of the gut homogenates of the first instar was performed using decapsulated larvae according to methods described in previous studies performed using beetles (Overney et al. 1997, Girard et al. 1998). Six replicates were performed, each one consisting of a pool of 20 larvae midguts for the first and second instar and 10 larvae midguts for the third instar.

The midguts of each pool were placed in Eppendorf tubes containing 0.5 ml of a saline solution (0.8%) and macerated. This solution was then centrifuged for 15 min at 6,000 rpm (Blahovec et al. 2006). The supernatants were stored at −20°C until they were used for the enzymatic assays (Overney et al. 1997, Girard et al. 1998).

Protein Content in Midgut Homogenates
The method used to determine the protein content of the gut homogenates was the same as that used to determine the protein content of the diets using the BCA (Lowry et al. 1951, Walker 2002).

Measurement of pH Inside the Gut
The conditions for the enzymatic activity trials considered the pH of the midgut of the third instar reared on the artificial diet since one of the main goals was defining the prevailing pH conditions in the midgut for larval mass-rearing.

A vital pH indicator stain was mixed at 1% (p/v) with either artificial diet or mango pulp. The following pH indicators were applied separately: eosin yellowish (pH 0–3.0), bromophenol blue (pH 3.0–4.6), bromoresol green (pH 3.8–5.4), and bromothymol blue (pH 5.8–7.6) (Ossa-O et al. 2000, Overend et al. 2016).

The mixes of the pH indicator and diet (i.e., natural or artificial) were placed in plastic containers with 500 g capacities. Each container was sown with 0.1 ml (~1500 eggs) of newly hatched larvae. The larvae remained in the diet-pH indicator mixture until the pH was determined. Thirty larval guts were dissected from larvae fed artificial diets. The procedure used for gut extraction is described in the previous section. Photographs were obtained using a BestScope BLM-340 stereoscope (Bestscope International Limited).

Enzymatic Assays
The enzymatic activity was determined at pH 3.0, 4.5, and 6.0 in the first, second, and third instar midgut homogenates obtained from insects reared on natural and artificial diet.

Chymotrypsin Activity
N-benzoyl-l-tyrosine ethyl ester (BTEE) was used as a specific substrate to determine the level of chymotrypsin activity (EC 3.4.21.1) (Wirnt 1974). The reaction mixture consisted of 1.32 ml of tris-HCl buffer 80 mM, 1.40 ml BTEE 1.18 mM (dissolved in methanol, 63% (v/v)), 0.08 ml CaCl2, and 0.2 ml of gut homogenate.

Trypsin Activity
Benzoyl arginine p-nitroanilide (BApNA) was used as a specific substrate to determine the level of trypsin activity (EC 3.4.21.4) (Erlanger et al. 1961).

Chymotrypsin Activity
The reaction mixture consisted of 2.68 ml tris-HCl buffer 100 mM/CaCl2 10 mM, 120 µl BApNA 12.4 mM (dissolved DMSO 12.5 mM), and 200 µl of gut homogenate.

Carboxypeptidase A Activity
Hippuryl-l-phenylalanine was used as a specific substrate to determine the level of carboxypeptidase A (EC 3.4.17.1) activity (Folk and Schirmer 1963). The reaction mixture consisted of 2.9 ml of hippuryl-l-phenylalanine 1.0 mM in Et-OH (diluted in a solution of 25 mM tris-HCl buffer and 500 mM NaCl) and 0.1 ml of gut homogenate.

Carboxypeptidase B Activity
Hippuryl-l-arginine was used as a specific substrate to determine the level of carboxypeptidase B activity (EC 3.4.17.2) (Hass and Derr 1979). The reaction mixture consisted of 2.9 ml of 1 mM hippuryl-l-arginine in 25 mM HCl buffer and 0.1 ml of gut homogenate.

Spectrophotometer Reading
Absorbance readings were measured every 30 s for 5 min (Multiskan GO spectrophotometer, Thermo Fisher Scientific, Finland). Absorbance readings for chymotrypsin were obtained at 256 nm (ΔA256/min), for trypsin at 410 nm (ΔA410/min), and for carboxypeptidase A and for carboxypeptidase B at 234 nm (ΔA234/min). One unit of activity (U) was defined as the amount that enzyme activity increased per minute. The blank solution replaced the gut homogenate with saline solution. The absorbance of each treatment was measured considering at least three different pools, with each one considered a replicate. The absorbance values were used to estimate gut activity and were expressed as activity units per gut (U).

Statistical Analysis
Differences in protein content between the eight treatments resulting from the type of diet (natural and artificial) and the time after the infestation (0, 6, 8, and 12 days/0, 2, 4, and 7 days) were determined using a Kruskal–Wallis test (α < 0.05). The difference in protein content in the gut between the six treatments including the type of diet (natural and artificial) and the first, second, and third instars was compared using a Kruskal–Wallis test (α < 0.05). The protein content of the natural and artificial diets was compared with a Wilcoxon test (Mann–Whitney U-test), using the statistical program InfoStat (2016; version 2016e).

Differences in the enzymatic activity of larvae guts between the six treatments resulting from the first, second, and third instars reared on natural and artificial diets were compared using analysis of variance, and the means were separated by a Tukey HSD test. Previously, Bartlett’s test (Zar 1999) for unequal variances was carried out, and the data were log-transformed when they showed heterogeneity (P < 0.05) (Underwood 2005). All analyses were performed using the statistical program JMP (version 5.0.1) Statistical Discovery Software (SAS Institute 2003).

Results
Measurement of pH Inside the Gut
The larval midgut has an acidic pH. According to the degree of acidity, larval midguts were divided into the following three regions: the anterior-midgut has a pH between 3.0 and 5.0, the mid-midgut has a pH between 3.0 and 5.0, and the posterior-midgut has a pH higher than 5.0 but lower than 7.6 according to the range of biological indicators used in this study (Fig. 1). The pH conditions selected to estimate enzymatic activity were pH 3.0, 4.5, and 6.0.
Protein Content in Natural and Artificial Diets
The freshly prepared artificial diet had a higher protein content than the natural diet without infestation and 2 d after the infestation (W = 24.00; P = 0.015). Unexpectedly, no difference was observed for the protein content of the natural and artificial diets 4 and 8 d after the infestation (Fig. 2). The type of diet (natural and artificial) and the time after the infestation (0, 2, 4, and 7 days) were significant predictors of protein content (H = 28.280; df = 7; P < 0.001). The natural diet without larvae (mangoes not infested) was the treatment with the lowest protein content, while the artificial and natural diets that contained 7-d-old larvae had the highest protein content.

Protein Content in Midgut Homogenates
The protein content in the midguts did not show significant differences between the larvae developed on artificial and natural diets for the first (W = 34.00; P = 0.222), second (W = 34.00; P = 0.222), or third instars (W = 32.00; P = 0.421). The third instar showed the highest protein content, while the lowest protein levels were observed in the larval midgut of the first instar (H = 26.35; df = 5; P < 0.001) (Fig. 3).

Enzymatic Assays
The interaction between the pH, diet type, and instar was significant for the chymotrypsin activity (F = 11.95; df = 4, 107; P < 0.001), carboxypeptidase A (F = 3.80; df = 4, 36; P = 0.011), and trypsin (F = 5.89; df = 4, 18; P = 0.003). The carboxypeptidase B, no significant differences were observed between the pH, diet type, and instar, but the interaction between the diet type and instar was significant (F = 6.79; df = 2, 34; P = 0.003).

Chymotrypsin
The proteolytic activity at pH 3.0 (F = 233.30; df = 1, 10; P < 0.001) and pH 4.5 (F = 47.47; df = 1, 10; P < 0.001) in the first instar reared on the natural diet was significantly lower than the activity observed in larvae reared on the artificial diet. In the first instar at pH 6.0, there was no difference in proteolytic activity between larvae reared on natural and artificial diets (F = 0.21; df = 1, 10; P = 0.664). In the second instar, there was no significant difference at pH 3.0 (F = 0.01; df = 1, 12; P = 0.952) and 6.0 (F = 0.55; df = 1, 12; P = 0.473) in the activity between larvae fed the two types of diet. The second instar reared on the natural diet at pH 4.5 showed a significantly higher activity than observed in the larvae reared on the artificial diet (F = 39.52; df = 1, 13; P < 0.001). In the third instar, the highest activity at pH 3.0 (F = 315.92; df = 1, 13; P < 0.001), 4.5 (F = 165.18; df = 1, 14; P < 0.001), and 6.0 (F = 185.54; df = 1, 13; P < 0.001) was observed in larvae reared on the natural diet (Fig. 4A–C).

Trypsin
Trypsin proteolytic activity was not detected at pH 3.0 and 4.5 in the first instar reared on the natural diet, while at pH 6.0 a higher activity was observed in larvae reared on the artificial diet (F = 742.79; df = 1, 2; P < 0.001). Trypsin proteolytic activity for the second instar was not different between the diets at pH 3.0 (F = 11.31; df = 1, 2; P = 0.078), 4.5 (F = 10.04; df = 1, 2; P = 0.087), and 6.0 (F = 1.47; df = 1, 2; P = 0.347). For the third instar at pH 3.0, the type of diet was not a significant predictor (F = 3.26; df = 1, 2; P = 0.213). At pH 4.5 (F = 31.89; df = 1, 2; P = 0.030) and pH 6 (F = 28.13; df = 1, 2; P = 0.034), the activity of the trypsin was higher in the larvae reared on the natural diet than in larvae reared on the artificial diet (Fig. 4D–F).

Carboxypeptidase A
The activity of carboxypeptidase A at pH 3.0 (F = 16.67; df = 1, 4; P = 0.011) and 6.0 (F = 16.67; df = 1, 4; P = 0.012) was significantly higher in larvae reared on the natural than on the artificial diet for the first and third instar. At pH 4.5, there was no observed difference between the first and third instar (F = 6.07; df = 1, 4; P = 0.069 and

Fig. 1. Different pH of the larval midgut third instar of Anastrepha obliqua grown on artificial diet, as shown by the following biological indicators: A) eosin yellowish (orange to red, pH 0–3.0), B) bromophenol blue (yellow to purple, pH 3.0–4.6), C) bromocresol green (yellow to green, pH 3.8–5.4), and D) bromothymol blue (yellow to blue, pH 5.8–7.6). The arrows indicate the limits sections of different color.
$F = 0.43; \text{df} = 1, 4; P = 0.546$, respectively), and similar results were observed at pH 6.0 ($F = 3.02; \text{df} = 1, 4; P = 0.157$ and $F = 5.86; \text{df} = 1, 4; P = 0.073$, respectively). The enzymatic activity in the second instar at pH 3.0 ($F = 75.66; \text{df} = 1, 4; P = 0.001$) and 4.5 ($F = 11.16; \text{df} = 1, 4; P = 0.029$) was significantly higher in the larvae reared on the natural diet. There was no difference in enzymatic activity at pH 6.0 between larvae reared on the natural and artificial diet in the three analyzed instars ($F = 5.10; \text{df} = 1, 4; P = 0.087$) (Fig. 4G–I).

Carboxypeptidase B

A comparison of the activity of the first instar at pH 3.0 ($F = 6.51; \text{df} = 1, 4; P = 0.063$) and 6.0 ($F = 4.07; \text{df} = 1, 4; P = 0.114$) of carboxypeptidase B indicated that the type of diet did not affect the proteolytic activity in larvae reared on the artificial or natural diet. Carboxypeptidase B activity at pH 4.5 of the first instar larvae reared on the natural diet was higher than that of the larvae reared on the artificial diet ($F = 150.38; \text{df} = 1, 3; P = 0.001$). There was no difference in activity between the natural and artificial diet for the second ($F = 0.13; \text{df} = 1, 4; P = 0.733$) and third instar ($F = 3.82, \text{df} = 1, 4, P = 0.123$) (Fig. 4J–L).

Discussion

Assuming that the synthesis of digestive enzymes could be modulated by protein intake (Alarcòn et al. 2002), we expected a direct relationship between the protein content in the midgut and the enzymatic activity. Consequently, we also expected a direct relationship between the protein content in the midgut and the protein content in the diet. The results of this experiment for the second and third instar indicated that this postulate was not supported, since the protein content in both the natural and artificial diets was similar and the same trend was observed with the protein content in the larvae midgut. On the other hand, for the first instar, the protein content in the midgut of the larvae fed with the natural and artificial diets was similar, even when the natural diet had lower protein content. Therefore, the enzymatic activity was expected to be lower but this did not happen.

Another finding of our research were that the proteolytic activity of chymotrypsin, trypsin, carboxypeptidase A, and carboxypeptidase B was detected in the midgut of different instars of *A. obliqua*, and it is strongly affected by the pH and diet type.

In general, carboxypeptidases are usually a minor component of the protease complement in the gut of insects (Novillo et al. 1999, Pauchet et al. 2008). While, high proteolytic activity for chymotrypsin and trypsin has been reported in some insect species including Diptera (Billingsley and Hecker 1991, Johnston et al. 1995, Lee and Anstee 1995, Lam et al. 1999, Valaitis et al. 1999, Muharsini et al. 2000, Wagner et al. 2002, Silva et al. 2006, Fazito do Vale et al. 2007). Unexpectedly, we found that the highest proteolytic activity in *A. obliqua* larvae was observed for the enzymes carboxypeptidase A and carboxypeptidase B, which belong to the metalloproteases group and have a level of activity 80-fold higher than that of serine proteases and chymotrypsin and 10,000-fold higher than the activity of trypsin. A similar result was found in *Musca domestica* L. (Diptera: Muscidae) larvae, in which serine protease activity was minimal, but metalloproteases were responsible for a greater proportion of digestive proteolysis (Blahovec et al. 2006).

The carboxypeptidase A activity was higher than the level of activity of carboxypeptidase B in the midgut of *A. obliqua* larvae reared on either an artificial or a natural diet. Carboxypeptidase
Fig. 4. Protease-specific activity (mean ± SE) in the midguts of first, second, and third instar *Anastrepha obliqua* larvae reared on (black shade) artificial and (gray shade) natural diet. The bars indicate the level of activity in units per gut (U/gut) or (mU/gut). Lowercase letters indicate differences between diets, while capital letters indicate differences between instars. Differences between means (±SE) for type of diet for each infested diet with the same letter are not significantly different (P > 0.05).
A hydrolyzes C-terminal amino acids except arginine, lysine, and proline, while carboxypeptidase B preferentially releases C-terminal lysine and arginine (Terra and Ferreira 1994). The activity of the A type has been found to be predominant in the cases studied (Bown et al. 1998).

In this work, we find that the pH of the midgut of A. obliqua varies from acid to neutral. As expected, the activity of the endopeptidases (chymotrypsin and trypsin) was lower than those of the exopeptidases (carboxypeptidases A and B). This can be explained by the pH conditions, as the endopeptidases have higher proteolytic activity at basic pH values (>8), while the exopeptidases have higher proteolytic activity at acid pH values (range ~3–5) (Fazito do Vale et al. 2007). The lower activity of the endopeptidases suggest that the larvae has extra-oral digestion during which solid materials can be broken down and nutrients can be liquefied and ingested similar to in larvae of Cyclorrhapha. Additionally, live bacteria may predigest food, change its nutritional content, and/or improve digestion inside the gut (Lemaître and Miguel-Aliaga 2013). In general, enzymatic activity was higher in the midgut of the larvae fed on the natural diet than in larvae fed on the artificial diet, which suggests that the protein from the natural diet was more bioaccessible (i.e. type and sequence of amino acids and protein to carbohydrate ratio) (Le Gall and Behmer 2014). In addition, the type and amount of some secondary metabolites produced by bacteria, i.e., the hydroxycarboxylic acids (e.g., lactic acid) present in greater concentrations in the natural diet than in artificial diet. Hydroxycarboxylic acids serve as substrates for some enzymes, including carboxypeptidase A (Auld 2013). On another hand, hydrolysis in the artificial diet was less effective because the interactions with other macronutrients such as fiber and polysaccharides such that when they come in contact in the mouth or digestive tract, various interactions may occur affecting bioaccessibility (Carbonell-Capella et al. 2014). Consequently, controlled studies are necessary to determine the bioaccessibility and bioavailability of macromolecules of interest.

In summary, of the four studied enzymes, carboxypeptidase A and B are the main proteases involved in the digestion of the proteins in the larvae of the fruit fly, A. obliqua, and their activity is strongly affected by the pH and the diet type. The natural diet showed high bioaccessibility expressed as a high enzyme activity. Trypsin and chymotrypsin activities showed a progressive increase from first to third instar. The activity of carboxypeptidase A at an acidic pH (3.0 and 4.5) on the natural diet showed a clear tendency to decrease as the instars develop. In contrast, the activity of carboxypeptidase A on the artificial diet showed a slightly tendency to increase as the instars developed.

The information our research contributes for planning and developing novel bioaccessibility assays to understand the insect digestion is especially important for improving the diet for mass-rearing fruit fly larvae for SIT application. We suggest that this information is also suitable for strengthening the parameters of quality control of ingredients for the mass-rearing of fruit flies in the future.

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