El Colegio de la Frontera Sur

Actividad enzimática proteolítica durante el desarrollo larvario de *Anastrepha obliqua* (Diptera: Tephritidae) en mango y dieta artificial

TESIS

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Para usted

Sofia Ciprian García,

con todo mi corazón mamita chula

desde el cielo donde nos mira†

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I. Introducción

Anastrepha obliqua es la segunda especie de importancia económica para el país, después de *A. ludens*. Se encuentra localizada principalmente en las regiones bajas tropicales de México. Se pueden encontrar en hospedantes como: toronja (*Citrus grandis*), carambola (*Averrhoa carambola*), naranja (*Citrus sinensis*), pomarrosa (*Eugenia jombos*), guayaba (*Psidium guajava*), jobo (*Spondias mombin*), almendro tropical (*Terminalia catappa*), siricote (*Cordia dodecandra*), naranja agria (*Citrus aurantium*), jocote (*Spondias purpurea*), arazá (*Eugenia stipitata*), manzana de agua (*Syzygium malaccense*), guayaba (*Psidium guajava*), lúcuma (*Pouteria lúcuma*) (Programa de Sanidad Vegetal-SAGARPA- Guanajuato, Ficha Técnica de *A. obliqua;* Sosa et al 2015; Tigrero 2009), aunque su importancia radica en su preferencia por infestar frutos de *Mangifera indica* (mango), donde puede causar grandes pérdidas económicas para los productores (Aluja 1994).

Dentro de las estrategias que se emplean para su control se encuentra la Técnica del Insecto Estéril (TIE), que consiste en producir y liberar moscas de manera masiva. Las moscas liberadas deben cumplir con estándares de calidad en cuanto al peso, habilidad de vuelo, sobrevivencia y longevidad. La producción de dichas moscas se realiza en biofábricas, donde las larvas son alimentadas con "dietas artificiales" formuladas con ingredientes disponibles en el mercado, en las cuales se les proporciona a los insectos los elementos que requieren para su desarrollo. El objetivo de las dietas artificiales es producir insectos con las cualidades que les permitan copular con las hembras silvestres una vez liberados en el campo.

En general, cuando se requiere establecer la cría la una especie de mosca en condiciones artificiales de laboratorio o producción masiva, no se realizan estudios de sus requerimientos nutrimentales, y ésta se lleva a cabo empleando dietas utilizadas en la producción de insectos de géneros o especies cercanas, realizando únicamente ajustes en las concentraciones para su funcionamiento.

Los trabajos realizados con dietas de insectos se pueden dividir en dos vertientes, los orientados en las disciplinas de la nutrición de insectos y los orientados en la dietética de insectos. En general, la vertiente de la nutrición de insectos ha sido una ciencia destinada a comprender los requisitos y la función de los componentes alimentarios, mientras que la vertiente de la dietética ha sido aplicada de manera tradicional dirigida al desarrollo de dietas de manera práctica, donde el objetivo es que funcionen, sin lograr el entendimiento de cómo lo hacen (Cohen 2015). A la fecha existen muchos trabajos orientados en el desarrollo de dietas larvarias para la cría artificial de *A. obliqua* (Zucoloto et al 1979; Message and Zucoloto 1989; Moreno et al 1997; Saldanha & Silva 1999; Rivera et al 2007, Hernández et al 2016), en donde su funcionamiento se evalúa mediante parámetros como peso, tamaño, y rendimiento, sin poner importancia en las razones del funcionamiento.

Con pocas excepciones como ecología de campo y sistemática, en estudios realizados con insectos son necesarios ejemplares de alta calidad, para asegurar resultados confiables en pruebas, o para lograr algún propósito específico como el de la TIE. Pero para lograr producir éstos insectos saludables, también es necesario contar con dietas de buena calidad (Cohen 2015), lo que es difícil lograr cuando no se tienen conocimientos suficientes sobre el proceso de alimentación de la especie.

Las enzimas digestivas desempeñan un papel importante en el proceso de alimentación de los insectos, ya que éstas actúan sobre los alimentos ingeridos desdoblando las macromoléculas, permitiendo el aprovechamiento de los nutrimentos. De acuerdo a diversos estudios, la actividad catalítica de las serina-proteasas como quimotripsina y tripsina, y de las metaloproteasas como carboxipeptidasa A y carboxipeptidasa B, resultan de gran interés para comprender el proceso digestivo durante el desarrollo de los insectos.

El conocimiento que se tiene sobre el proceso de digestión de moscas de la fruta es limitado, y aunque diversos trabajos realizados con especies del mismo orden que indican que existe variación en la actividad enzimática, de manera particular se desconoce las enzimas que participan en el proceso de digestión de las proteínas en larvas de *A. obliqua,* o si se presentan cambios cuando la alimentación de los insectos se realiza variando su dieta que puedan indicar su mejor aprovechamiento. En el presente trabajo se comparan larvas de *A. obliqua* desarrollada en la dieta artificial utilizada para su cría en condiciones de laboratorio, y desarrolladas en mango, que es un hospedero natural de la especie. Debido a lo anterior el objetivo de esta investigación fue determinar la actividad enzimática de proteasas de larvas de *A. obliqua* criadas en mango y en dieta artificial.

II. Midgut Protease Activity During Larval Development in *Anastrepha obliqua* (Diptera: Tephritidae) fed on Mango and an Artificial Diet

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Midgut Protease Activity During Larval Development in *Anastrepha obliqua* (Diptera: Tephritidae) fed on Mango and an Artificial Diet

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Midgut Protease Activity During Larval Development in Anastrepha obliqua (Diptera: Tephritidae) fed on Mango and an Artificial Diet

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Abstract: Digestive enzymes play important roles in insect feeding because they act on ingested food ingredients to unfold macromolecules, which allows the use of their component nutrients. In nature, larvae feed on the pulp of host fruits, which generally contains less than 1% protein, whereas under artificial conditions, larvae are reared on artificial diet with at least 3.5% of protein content. In this study, we examined the activity of two serine proteases (chymotrypsin and trypsin) and two metalloproteinases (carboxypeptidases A and B) during larval development in *A. obliqua* fed on natural (mango fruit) or artificial (a formulation used in mass-rearing) diet. The results indicated that for both types of diet, the level of carboxypeptidase activity was approximately 100-fold the level of chymotrypsin activity but 10,000-fold that of trypsin. The activity of these serine proteases increased with each instar, and their activity was higher in insects fed mango than in those fed an artificial diet. Carboxypeptidase activity remained constant across larvae of different instars. In general, there was no difference between the two diets.

Key words: Digestive proteases, artificial diet, natural diet, larval development, midgut pH

Introduction

The West Indian fruit fly *Anastrepha obliqua* (Macquart) is one of the main pests of the mango (*Mangifera indica*) and the jobo (*Spondias* spp.) (Hernández-Ortiz & Aluja 1993). This fly causes direct damage by infesting the fruits as well as indirect damage because their presence in orchards requires the implementation of rigorous quarantine measures by fruit-importing countries that are free of this pest (Santiago 2010). One of the most successful control strategies for this pest is the Sterile Insect Technique (SIT), in which massive numbers of these insects are reared, sterilized and subsequently released into infested areas (Knipling 1955, Reyes et al. 2000). These released sterile males must be competitive enough to fulfil their function of reducing the wild fruit fly population. The mass production of insects is achieve by the development of artificial diets that guarantee larval growth (Caceres 2002).

For insects not previously reared on an artificial diet, one approach is to adopt or modify the existing diet of a closely related species (Hernández et al. 2010). Currently, *A. obliqua* larvae can be successfully mass-reared. However, the constant increase in the cost of the ingredients has led to the development or reformulations of artificial diets that support digestive processes (i.e., enzymatic hydrolysis).

In larvae, digestive proteases catalyze the release of peptides and amino acids from diet. Amino acids are essential to maintain the insects' viability and fitness (Chang 2004). However, deficiency of amino acids can affect the insects' progress and abilities by retarding their development and increasing larval and pupal mortality (Cangussu & Zucoloto 1997, Nash &

Chapman 2014). In adults, it can affect emergence, size, oocyte maturation (Chang 2004), and melanisation (Lee et al. 2008).

The composition of the enzymes that are secreted is related to the nature of the meal that an insect can ingest (Agusti & Cohen 2000, Zeng & Cohen 2000, Torres & Boyd 2009).

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 &Ying-Hong 2007, Ping-Ping et al. 2014). Previous studies have determined that the type of host fruit significantly affects enzymatic activity, and this has increased the interest to quantify the effects of artificial diets on the enzymatic activity of sterile insects. As a result, the goal of this study was to evaluate enzymatic activity during larval development in *A. obliqua* fed an artificial or natural diet (mango fruit) (*M. indica*).

Materials and methods

This study was conducted in the Colonization and Rearing Laboratory of the Development Methods Unit of the Moscafrut Program Facilities in Metapa de Domínguez, Chiapas, México and the Department of Ecology of Fruit Flies of 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 150 generations in the Moscafrut facility at Metapa de Domínguez in Chiapas, México (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 baby') or an artificial diet.

The artificial diet was prepared in accordance with the methods described by Artiaga-López et al. (2004) and by Domínguez 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 (Mallinckrodt 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. Larvae of different instars were obtained from multiple sown of newly hatched larvae on this diet. The larvae were separated from the artificial diet on 2, 4, and 7 days old to obtain first, second and third instar larvae, respectively (according to previous analyses, unpublished data).

The natural diet consisted of mango cv. Ataulfo fruits that collected in orchards in Tapachula, Chiapas, Mexico. The fruits were washed and kept until they reached a uniform yellow color before they were exposed to infestation. Second and third instar larvae were obtained by exposed five ripe mangoes, at two hundred mated flies for eight hours in 50 cm x 50 cm x 50 cm wooden frame cages covered with a white mesh. Second (four days old) and third instar (eight days old) larvae were separated from infested mangoes (data obtained in preliminary tests). First instar larvae (two days old) were obtained sowed newly hatched larvae in liquefied mango pulp containing 1% m-p-ben and 1% sodium benzoate (Oster blender), due to difficult to separate first

instar larvae from mangoes. All infested mangoes and diets were stored in laboratory at 25°C±1, 60% humidity and a 12/12 h light : dark photoperiod.

The physiological maturation times for first, second and third instar were previously determined in flies 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 diets

The protein content was determined in non-infested fresh and infested artificial diets at 2, 4, and 7 days after the newly hatched larvae were sown (corresponding to the first, second and third instar, according to preliminary trials), in the same way the protein content of the natural diet was determined in both non-infested and infested fruits after 6, 8, and 12 days after infesting the fruits (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, 0.2 g of a dry sample was digested by adding 2.0 ml of 0.1 N NaOH solution and incubated 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 1N HCl solution. Posteriorly, 1 ml of buffer solution (pH 9.2) was added, and then distilled water was added until the total volume was 10 ml. The Bicinchoninic Acid (BCA) method was used to calculate the amount of protein (Walker 2002, Lowry et al. 1951). For each sample 125 µl of solution was mixed with 125 µl of BCA solution: 25 parts of A reagent (0.18 M Sodium carbonate anhydrous, 0.69 mM Sodium tartrate dihydrate, 0.01 M NaOH, 0.011 M Sodium bicarbonate; pH 11.25), 25 parts of B reagent (4% (w/v) Bicinchoninic acid solution, pH 8.5), 1 part of C reagent (4% (v/w) Cupric sulfate pentahydrate solution). (Sigma-Aldrich, technical

bulletin, Cat. No. QPBCA). The solution was then placed in a water bath (Thermo Precision, 280 Series) for 30 min at 37°C. Five readings were performed for each of the six samples. A calibration curve was constructed using standard bovine serum albumin (BSA, 0.5-30 µg/ml), and the absorbance was then read at 562 nm using a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Finland).

Extraction of gut homogenates

Larvae from each treatment (according to the diet type and sampling day) were washed with distilled water and subjected to chilling pulses to reduce movement of larvae and facilitate their manipulation. The guts of the second and third instar midgut was extracted by dissecting the larvae under stereo microscope, cutting their abdominal region and extracting their midgut in a Petri dish containing a solution of 0.8% NaCl (w/v) at 4°C. The extraction of the gut homogenates of the first instar larvae was performed using decapitated larvae according to methods described in previous studies performed using beetles (Overney et al. 1997, Girard et al. 1998). To keep the guts hydrated and to facilitate their removal, a solution of 0.8% NaCl (w/v) at 4°C was used during the dissections (Broadway & Duffey 1985). Each replicate consisted of a sample of 20 larvae midguts obtained from first and second instar larvae. Tor the third instar larvae, the sample consisted of ten larvae midguts. The sample midguts 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 supernatant was stored at -20°C (Overney et al. 1997, Girard et al. 1998).

Measurement of pH inside the gut

The pH of the larval midgut in each of the three instars was estimated to determine the levels at which the enzymatic activity trials would be conducted. A vital pH indicator stain was mixed at 1% (w/v) with 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), bromocresol green (pH 3.8 - 5.4) and bromothymol blue (pH 5.8 - 7.6) (Ossa-O et al. 2000, Overend et al. 2016).

The mix of the pH indicator and diet were placed in a plastic container of 500 g capacity (i.e., natural or artificial). Each diet was sowed with 0.1 ml (~1500 eggs) of newly hatched larvae. The larvae remained in the diet-pH indicator until the pH was determined. Thirty larvae were separated from diet and were dissected to obtain the midgut. The procedure used for gut extraction is described in a previous section. Photographs were obtained using a BestScope BLM-340 stereoscope (Bestscope International Limited, Beijing, China).

The color pattern midgut of first, second and third instar larvae that were reared on artificial diet was similar. We found that in general, the larval midgut has an acidic pH, and based on the degree of acidity, larval midguts can be divided into the following three regions: the anterior midgut with a pH between 3.0 and 5.0, the mid-midgut has a pH between 3.0 and 3.8, and the posterior midgut has a pH higher than 5.0 but lower than 7.6 (Fig. 1).

It was not possible to add the pH indicators to the closed fruits for the determination of the pH of the midgut of larvae that were reared inside them. It was tried to rearing larvae in liquefied mango pulp, but this presented a rapid increase in its acidity, which generated inconsistent data, reason it was not possible to determine the pH of larvae that were reared in mango

Protein content in midgut homogenates

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

Enzymatic assays

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) in first, second, and third instar midgut homogenates (Wirnt 1974).

The reaction mixture consisted of 1.32 ml Tris-HCl buffer 80 mM (pH 3.0, 4.5 or 6.0), 1.40 ml BTEE 1.18 mM (dissolved in methanol, 63% (v/v)), 0.08 ml $CaCl_2$ 2 M, and 0.2 ml of gut homogenate. In the blank solution, the gut homogenate was replaced with saline solution. Analyses were performed with three repetitions for each instar and pH value. Absorbance was measured at 256 nm (Multiskan GO spectrophotometer, Thermo Fisher Scientific, Finland). Absorbance was readed every 30 seconds for 5 min. One unit of activity (U) is defined as the amount that enzyme activity increased per min at 256 nm (ΔA_{256}/min). The results are expressed as activity units per gram protein and per gut.

Trypsin activity. Benzoyl Arginine p-Nitroanilide (BApNA) was used as the specific substrate to determine the level of trypsin activity (EC 3.4.21.4) in first, second, and third instar midgut homogenates (Erlanger et al. 1961).

The reaction mixture consisted of 2.68 ml Tris-HCl buffer 100 mM / $CaCl_2$ 10 mM (pH 3.0, 4.5 or 6.0), 120 µl BApNA 12.4 mM (dissolved DMSO 12.5 mM), and 200 µl of gut homogenate. In the blank solution, the gut homogenate was replaced with saline solution. Analyses were performed in triplicate for each instar stage and pH value. Absorbance readings were measured at 410 nm (Multiskan GO spectrophotometer, Thermo Fisher Scientific, Finland).

Absorbance was readed every 30 seconds for 5 min. One unit of activity (U) was defined as the amount that enzyme activity increased per min at 410 nm (ΔA_{410}/min). The results are expressed as activity units per gram protein and per gut.

Carboxypeptidase A activity. Hippuryl-L-Phenylalanine was used as a specific substrate to determinate the level of carboxypeptidase A (EC 3.4.17.1) activity in first, second and third instar midgut homogenates (Folk & Schirmer 1963).

The reaction mixture consisted of 2.9 ml of Hippuryl-L-Phenylalanine 1.0 mM in EtOH (diluted in a solution of 25 mM Tris-HCl buffer and 500 mM NaCl, pH 3.0, 4.5 or 6.0) and 0.1 ml of gut homogenate. In the blank solution, the gut homogenate was replaced with saline solution. Analyses were performed in triplicate for each instar and pH value. Absorbance was measured at 254 nm (ΔA_{254}/min) (Multiskan GO spectrophotometer, Thermo Fisher Scientific, Finland). Absorbance readings were obtained every 30 seconds for 5 min. One unit of activity (U) was defined as the increased in enzymatic activity that produced 1 μmol hippuric acid per min per mg of protein. The results are expressed as activity units (U) per gram of protein and per gut.

Carboxypeptidase B activity. Hippuryl-L-Arginine was used as the specific substrate to determinate the level of carboxypeptidase B (EC 3.4.17.2) activity in first, second and third instar midgut homogenate (Hass & 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. In the blank solution, the gut homogenate was replaced with saline solution. Analyses were performed in triplicate for each instar and pH value. Absorbance readings were measured at 254 nm (ΔA_{254}/min) (Multiskan GO, Thermo Scientific). One unit of B-carboxypeptidase hydrolyzes 1.0 μmol of Hippuryl-L-Arginine per min. The results are expressed as activity units (U) per gram of protein and per larvae.

Statistical analysis

The protein contents in the diets were analyzed using Kruskal-Wallis ($\alpha<0.05$) test and the means compared by pairs at the same instar was used the Wilcoxon (U- de Mann-Whitney) test for two

independent samples was used. The analyses were performed using the statistical program InfoStat (version 2016e).

All data obtained from the activity and protein analyses that were performed at different pH values and different larval phases were transformed into arcsine for standardization and then analyzed using analysis of variance (ANOVA) and Tukey's (HSD) multiple comparisons of means test. All analyses were performed using the statistical program JMP (Version 5.0.1, Statistical Discovery Software, SAS Institute, 2003).

Results

Protein content in natural and artificial diets

The natural diet without larvae (mangoes not infested) had the lowest protein content (Fig. 2), while the artificial and natural diets that contained 8-day-old larvae had the highest protein content ($H = 31.370$, $df = 7$, $p = 0.001$). The freshly prepared artificial diet had a higher protein content than was observed in the natural diet without infestation ($W = 57.00$, $p = 0.002$) and that the artificial diet with 2-day-old larvae had a higher protein content than was observed in the natural diet ($W = 57.00$, $p = 0.065$). In the diets with 4-day-old larvae ($W = 48.00$, $p = 0.178$) and 8-day-old larvae ($W = 47.00$, $p = 0.240$), no significant difference was found between the protein content of the natural and artificial diets.

Protein content in midgut homogenates

The highest protein levels were found in the larval midguts of the third instar, and the lowest protein levels were observed in the larval midguts of first instar ($H = 26.350$, $df = 5$, $p = 0.001$) for both diets (Fig. 3).

The protein content was not significantly different between larvae that were reared on an artificial or natural diet in the first ($W = 34.00$, $p = 0.222$), second ($W = 34.00$, $p = 0.222$), and third ($W = 34.00$, $p = 0.421$) instar larvae (Fig. 3).

Proteolytic activity

Trials performed at pH 3.0

Chymotrypsin. The proteolytic activity in the first instar larvae that were reared on natural diet was significantly lower than the activity observed in larvae that were reared on artificial diet ($F = 233.301$, $df = 1, 10$, $p < 0.001$). In the second instar larvae, there was no significant difference in the level of activity between larvae fed the two types of diet ($F = 0.004$, $df = 1, 12$, $p = 0.952$). In third instar, the higher level of activity was observed in larvae reared on natural diet ($F = 315.921$, $df = 1, 13$, $p < 0.001$) (Fig. 4A).

Trypsin. Trypsin proteolytic activity was not registered in first instar larvae reared on natural diet. Comparisons between the natural and artificial diets indicated that the type of food did not influence trypsin activity in second and third instar larvae ($F = 11.312$, $df = 1, 2$, $p = 0.078$; and $F = 3. 263$, $df = 1, 2$, $p = 0.213$, respectively) (Fig. 4B).

Carboxypeptidase A. The specific activity of carboxypeptidase A was significantly higher in larvae reared on natural diet than in larvae reared on artificial diet in all three instars ($F = 16.678$, $df = 1, 4$, $p = 0.011$; $F = 75.659$, $df = 1, 4$, $p = 0.001$; and $F = 16.670$, $df = 1, 4$; $p = 0.012$; for first, second, and third instar larvae, respectively) (Fig. 4C).

Carboxypeptidase B. A comparison of the specific activity of carboxypeptidase B indicated that the type of diet did not affect proteolytic activity in larvae reared on artificial or natural diet, and no difference was found between any pair of instar ($F=6.511$, $df = 1, 4$, $p=0.063$) (Fig. 4D).

Trials performed at pH 4.5

Chymotrypsin. In the first instar, the proteolytic activity observed in larvae reared on natural diet was significantly lower than the activity in the larvae reared on artificial diet ($F = 47.469$, $df = 1, 10$, $p < 0.001$). The second instar larvae reared on natural diet exhibited significantly higher activity than observed in the larvae reared on artificial diet ($F = 39.518$, $df = 1, 13$, $p < 0.001$). A

similar result was observed in third instar larvae, in which a higher level of activity was observed in larvae reared on natural diet ($F = 165.178$, $df = 1, 14$, $p < 0.001$) (Fig. 5A).

Trypsin. The highest level of activity was registered in third instar larvae that were reared on natural diet, while the lowest level of activity was observed in first instar larvae that were reared on artificial diet. Trypsin proteolytic activity was not registered in first instar larvae reared on natural diet. There were no differences in proteolytic activity between second instar larvae reared on natural or artificial diet ($F = 10.038$, $df = 1, 2$, $p = 0.087$). Third instar larvae reared on natural diet showed higher level of activity that larvae reared on artificial diet ($F = 31.893$, $df = 1, 2$, $p = 0.030$) (Fig. 5B).

Carboxypeptidase A. The highest activity level was observed in first instar larvae that were reared on natural diet, while the lowest level of activity was observed in second instar larvae that were reared on artificial diet. In the second instar larvae, carboxypeptidase A specific activity was significantly higher in the larvae reared on natural diet ($F = 11.158$, $df = 1, 4$, $p = 0.029$). There was no difference in proteolytic activity values between the first and third instar larvae ($F = 6.070$, $df = 1, 4$, $p = 0.069$ and $F = 0.434$, $df = 1, 4$, $p = 0.546$ for first and third instar larvae, respectively) (Fig. 5C).

Carboxypeptidase B. Specific carboxypeptidase B activity of first instar larvae reared on natural diet was higher than in larvae reared on artificial diet ($F = 150.382$, $df = 1, 3$, $p = 0.001$). There was no difference in activity between second instar larvae ($F = 3.817$, $df = 1, 4$, $p = 0.123$) and third instar larvae ($F = 0.134$, $df = 1, 4$, $p = 0.733$) reared on either diet (Fig. 5D).

Trials performed at pH 6.0

Chymotrypsin. In first and second instar larvae, there was no difference in proteolytic activity between larvae reared on natural and artificial diet ($F = 0.201$, $df = 1, 10$, $p = 0.664$ and $F = 0.548$, $df = 1, 12$, $p = 0.473$ in first and second instar larvae, respectively). Third instar larvae

reared on natural diet had the highest level of activity ($F = 185.541$, $df = 1, 13$, $p < 0.001$) (Fig. 6A).

Trypsin. The highest activity value was observed in third instar larvae that were reared on natural diet, while the lowest value was observed in first instar larvae that were reared on natural diet. There were no differences in activity values between second instar larvae reared on natural and artificial diet ($F = 1.486$, $df = 1, 2$, $p = 0.347$). In first instar larvae, a higher level of activity was observed in larvae reared on artificial diet ($F = 742.786$, $df = 1, 2$, $p = 0.001$), while in third instar larvae, a higher level of activity was observed in larvae reared on natural diet ($F = 28.134$, $df = 1, 2$, $p = 0.034$) (Fig. 6B).

Carboxypeptidase A. There was no difference in specific activity between larvae reared in artificial or natural diet in any of the three evaluated instars ($F = 3.024$, $df = 1, 4$, $p = 0.157$; $F = 5.104$, $df = 1, 4$, $p = 0.087$; and $F = 5.860$, $df = 1, 4$, $p = 0.073$ for first, second, and third instar, respectively) (Fig. 6C).

Carboxypeptidase B. There was no difference in specific activity between larvae reared on artificial or natural diet in any of the three evaluated instars ($F = 4.075$, $df = 1, 4$, $p = 0.114$; $F = 1.039$, $df = 1, 4$, $p = 0.366$; and $F = 0.641$, $df = 1, 4$, $p = 0.468$) (Fig. 6D).

Discussion

Proteolytic activity was detected in the midgut of larvae of *A. obliqua* that were reared on an artificial or natural diet. The activity of the enzymes chymotrypsin, trypsin, carboxypeptidase A and carboxypeptidase B were detected. Phytophagous insects such as *A. obliqua* possess physiological adaptations that allow them to exploit plant nutrients even in the presence of allelochemicals. These insects also possess regulatory mechanisms that permit them to initiate adaptive enzymatic response to a heterogeneous environment and diverse food sources (Ortego

2012). These mechanisms and adaptations provide the larvae with physiological plasticity and the capacity to develop in a variety of host fruits, including *Mangifera indica* (Anacardiaceae), *Spondia mombin* (Anacardiaceae), *Psidium guajava* (Myrtaceae), and *Averrhoa carambolo* (Oxalidaceae), in which fruit fly larvae find a natural variation in the primary and secondary metabolites.

The highest level of proteolytic activity in *A. obliqua* larvae grown on both natural and artificial diet was observed for the enzymes carboxypeptidase A and carboxypeptidase B, which belong to the metallo-carboxypeptidase group and have a level of activity 80-fold higher than of chymotrypsin and 10,000-fold higher than the activity of trypsin. A similar result was found in *Musca domestica* larvae in which serine protease activity was minimal, whereas the metallo-carboxypeptidases were responsible for a greater proportion of digestive proteolysis (Blahovec et al. 2006). In many insects has been report the higher level of proteolytic activity is due chymotrypsin and trypsin (Johnston et al. 1995, Lee & Anstee, 1995, Lam et al. 1999, Valaitis et al. 1999, Wagner et al. 2002).

In insect the amount of digestive enzyme is determinate by their feeding habits (García et al. 2012, Ortego 2012, Terra & Ferreira 2005), and this can vary even within species depending on food composition (Garcıa et al. 2012).

Studies that have examined the cell structure of the gut in some insects have indicated that species with constant feeding habits (i.e., no limit on food, as in fruit fly larvae) exhibit a continuous release of digestive enzymes, whereas species with discontinuous feeding habits (i.e., those with only occasional access to food) more strictly regulate the release of digestive enzymes (Lehane et al. 1996).

Trypsin activity has been previously reported in several species of the Diptera order, although its proteolytic activity is not always the main one (Billingsley & Hecker 1991, Mahmood &

Borovsky 1993, Muharsini et al. 2000, Silva et al. 2006, Fazito do Vale et al. 2007), and with this study including too *A. obliqua*.

In the current study, we observed that the activity of chymotrypsin and trypsin increased as the larvae matured, which is agree Woodring & Weidlich (2016) that suggesting the basic rate of enzyme secretion is determined simply by the growth and development of the midgut, a larger midgut has more cells that secret more enzymes. But this argument is does no explain what was found with carboxypeptidase A and carboxypeptidase B in this research, where there was no changes in enzymatic activity with increasing the maturity of the larvae, similarly to *Spodoptera frugiperda*, in which there was no change with age (Lwalaba et al. 2010).

In current study, trypsin and chymotrypsin activity increased at pH 6.0 in larvae grown on both natural and artificial diets. This is because an alkaline pH (above 8.0) is optimal for the activity of these enzymes (Terra & Ferreira 1994). The proteolytic activity of chymotrypsin in larvae reared on both diets was approximately 100-fold (at pH 3.0 and 4.5) and 60-fold (at pH 6.0) higher than the level of trypsin activity. Similarly, in *C. capitata* larvae reared on an artificial diet, the highest level of activity was observed for chymotrypsin (Silva et al. 2006). In the current study, chymotrypsin exhibited a lower level of activity in larvae reared on diets with a higher protein content than in larvae reared in diets with a lower protein content, and this might be because of differences in the amino acid profiles of the analyzed diets. Chymotrypsin hydrolyzes the peptide bonds of hydrophobic amino acids, such as tyrosine, phenylalanine, and tryptophan, which may be present in a higher proportion in natural diets even though they have an overall lower protein content.

Trypsin activity was higher in larvae reared on diets with low protein content than in larva reared on diets with high protein content, alike *C. capitata* trypsin activity was higher in larvae reared in low protein content diets, and larvae reared on high protein diets and after transferred to low

protein diet or without protein, displayed a significantly increased in trypsin activity. (Lemos et al. 1992). In *Gryllus bimaculatus*, trypsin activity was higher in insects reared on low protein diets (i.e., sunflower seeds) than in those reared on diets with a protein content higher than 25% (Woodring et al. 2009), this may because when insects are fed a diet with low nutrient content, they increase the production of enzymes to maximize the exploitation of the diet. Trypsin activity in *C. capitata* was relatively lower than that of other enzymes, such as aminopeptidases (Lemos et al. 1992).

The optimal pH of enzyme activity and the acidity of the gut of origin are independent, this was determined by isolating chymotrypsin from midgut of insects, where the optimum pH of activity was between 8 and 9 regardless of the acidity of the intestine of which it was isolated (Terra & Ferreira 1994). In this work the highest enzymatic activity was found at pH 6.0, although it is likely at pH 8.0 the activity would have been higher.

The food may be affect the enzymatic activity because the interactions among components of diet causes changes in the pH of intestine which can affect the synthesis of trypsin molecules.

In an insect population, changes in trypsin activity may be due to changes in the expression of genes originated by a selection pressure for many generations, selecting genotypes with a higher adaptive plasticity that favors the survival of individuals with a most digestive efficient of a particular diet. (Lazarevic & Jankovic-Tomanic 2015). A similar selective process could occur during colonization by mass reared fruit flies, during which the larvae fed an artificial diet with a higher protein content are selected over those reared on the natural fruit host.

Trypsin activity in the midgut increased as larval maturity progressed, indicating that the protein requirements of the larvae increased during physiological maturation. The gut of the larvae of *A. obliqua* displayed the highest level of proteolytic activity by carboxypeptidases and a smaller

contribution by serine proteases that is the opposite that found by Silva et al. (2006) in *C. capitata* larvae, in that digestion involved a greater enzymatic activity of serine proteases.

In the animal kingdom, endopeptidases such as trypsin and chymotrypsin initiate digestion by degrading protein into peptides. This continues the activity of exopeptidases such as carboxypeptidases, which degrade small peptides eventually into amino acids (Blahovec et al. 2006). Carboxypeptidases A and B have similar kinetic structures and mechanisms, suggesting they are derived from the same ancestral polypeptide (Vonk & Western 1984, Puigseiver et al. 1986). Carboxypeptidase A hydrolyzes terminal carbons from aromatic and ramified amino acids but does not hydrolyze arginine, lysine and proline, while carboxypeptidase B hydrolyzes terminal carbons with a positive charge and prefers lysine and arginine. The level of carboxypeptidase A activity was higher than the level of activity of carboxypeptidase B in the midgut of *A. obliqua* in larvae reared on either an artificial or a natural diet. This suggests that both diets contain an abundance of aromatic and ramified amino acids or at least a higher amount of these than lysine and arginine, which are preferentially hydrolyzed by carboxypeptidase B. Carboxypeptidase A activity was higher in third instar larvae reared on an artificial diet than in first and second instar larvae, and this resulted in a gradual change in their diet could be due to microorganisms action. Similarly, there was no difference in the level of carboxypeptidase A activity among different instar larvae reared on a natural diet, and we therefore suggest that natural diet conditions should not be changed during larval development so that enzymatic activity is maintained.

Because there was no change in carboxypeptidase B activity between the different diets or across instars, we suggest that the levels of the amino acids lysine and arginine were the same in both diets and were not affected by changes in the food supplied during larval development.

The multienzymatic complex possessed by *A. obliqua* larvae includes serine proteases, like trypsin and chymotrypsin, and metalloproteases, such as carboxypeptidases A and B. During digestion in *A. obliqua* larvae, carboxypeptidases A and B are primarily responsible for protein degradation.

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References

Agusti, N., and A. C. Cohen. 2000. *Lygus hesperus* and *L. lineolaris* (Hemiptera: Miridae), phytophages, zoophages, or omnivores: Evidence of feeding adaptations suggested by the salivary and midgut digestive enzymes. J. Entomol. Sci. 35: 176–186.

Artiaga-López, T., E. Hernández, J. Domínguez-Gordillo, D. S. Moreno, and D. Orozco-Dávila. 2004. Mass-production of *Anastrepha obliqua* at the Moscafrut Fruit Fly Facility, México, pp. 389-392. In B. N. Brian (ed.), Proceedings of the 6th International Symposium on Fruit Flies of Economic Importance. 6-10, May, 2002. Heriotdale, Johannesburg, South Africa.

Billingsley, P. F., and H. Hecker. 1991. Blood digestion in the mosquito, *Anopheles stephensi* Liston (Diptera: Culicidae): activity and distribution of trypsin, aminopeptidase, and alpha-glucosidase in the midgut. J. Med. Entomol. 28: 865-71.

Blahovec, J., Z. Kostecka, and A. Kocisova. 2006. Peptidolytic enzymes in different larval stadium of house fly *Musca domestica*. Vet. Med. 51: 139–144.

Broadway, R. M., and S. S. Duffey. 1985. The effect of dietary protein on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. J. Insect Physiol. 32: 673–680.

Caceres, C. 2002. Mass rearing of temperature sensitive genetic sexing strains in the Mediterranean fruit fly (*Ceratitis capitata*). Genetica. 116: 107–116.

Cangussu, J. A., and F. S. Zucoloto. 1997. Effect of protein sources on fecundity, food acceptance, and sexual choice by *Ceratitis capitata* (Diptera: Tephritidae). Rev. Bras. Biol. 57: 611–618.

Chang, C. L. 2004. Effect of amino acids on larvae and adult of *Ceratitis capitata* (Díptera: Tephritidae). Ann. Entomol. Soc. Am. 97: 529-535.

Domínguez, J., T. Artiaga-López, E. Solís, and E. Hernández. 2010. Métodos de colonización y cría masiva, pp. 259-276. In: P. Montoya, J. Toledo and E. Hernández (Eds.). Moscas de la Fruta: Fundamentos y Procedimientos para su Manejo. S y G. Editores. México D.F.

Elson-Harris M. M. 1988. Morphology of the immature stages of *Dacus tryoni* (Froggatt) (Diptera: Tephritidae). J. Aust. Ent. Soc. 27: 91-98.

Erlanger, B. F., N. Kokawsky, and W. Cohen. 1961. The preparation and properties of two new chromogenic substances of trypsin. Arch. Biochem. Biophys. 95: 271-278.

Fazito do Vale, V., M. H. Pereira, and N. Figueiredo Gontijo. 2007. Midgut pH profile and protein digestion in the larvae of *Lutzomyia longipalpis* (Diptera: Psychodidae). J. Insect Physiol. 53: 1151–1159.

Folk, J. E., and E. W. Schirmer. 1963. The porcine pancreatic carboxypeptidase A system I. Three forms of the active enzyme. J. Biol. Chem. 238: 3884-3894.

Garcıa, M., G. P. Farinos, P. Castañera, and F. Ortego. 2012. Digestion, growth and reproductive performance of the zoophytophagous rove beetle *Philonthus quisquiliarius*

(Coleoptera: Staphylinidae) fed on animal and plant based diets. J. Insect Physiol. 58: 1334–1342.

Girad, C., M. Le Metayer, M. Bonadé-Botino, M. Pham-Delegue, and L. Jouanin. 1998. High level of resistance to protease inhibitors may be conferred by proteolytic cleavage in beetle larvae. Insect Biochem. Mol. Biol. 28: 229-237.

Hass, G. M., and J .E. Derr. 1979. Distribution of carboxypeptidase isoinhibitors in the potato plant. Plant Physiol. 64: 1029-1031.

Hernández, E., J. P. Rivera, D. Orozco-Dávila, M. Salvador, and J. Toledo. 2010. An artificial diet for rearing of *Anastrepha striata* (Diptera: Tephritidae). Folia Entomol. Mex. 93: 167–174.

Hernández-Ortiz, V., and M. Aluja. 1993. Listado de especies del género neotropical *Anastrepha* (Diptera: Tephritidae) con notas sobre su distribución y plantas hospederas. Folia Entomol. Mex. 88: 89-105.

InfoStat. 2016. InfoStat, Version 2016e. Grupo InfoStat, FCA, Universidad Nacional de Cordoba, Argentina.

Johnston, K. A., M. J. Lee, C. Brough, V. A. Hilder, K. M. R. Gatehouse, and J. A. Gatehouse. 1995. Protease activity in the larval midgut of *Heliotis virescens*: evidence for trypsin and chymotrypsin like enzymes. Insect Biochem. Mol. Biol. 25: 375–384.

Knipling, E. F. 1955. Possibilities of insect control or eradication through the use of sexually sterile males. J. Econ. Entomol. 48: 902-904.

Lam, W., G. M. Coast, and R. C. Rayne. 1999. Isolation and characterization of two chymotrypsins from the midgut of *Locusta migratoria*. Insect Biochem. Mol. Biol. 29: 653–660.

Lazarevic, J., and M. Jankovic-Tomanic. 2015. Minireview. Dietary and phylogenetic correlates of digestive trypsin activity in insect pests. Entomol. Exp. Appl. 57: 123-151.

Lee, K. P., S. J: Simpson, and K. Wilson. 2008. Dietary protein-quality influences melanization and immune function in an insect. Funct. Ecol. 22: 1052–1061.

Lee, M. J., and J. M. Anstee. 1995. Endoproteases from the midgut of larval *Spodoptera littoralis* include a chymotrypsin-like enzyme with an extending binding site. Insect Biochem. Mol. Biol. 25: 49–61.

Lehane, M. J., H. M. Buller, and A. Crisanti. 1996. Mechanisms controlling the synthesis and secretion of digestive enzymes in insects, pp. 195-205. In M. J. Lehane, P. F. Billingsley (Eds.). Biology of the insect midgut. Chapman & Hall. London.

Lemos, F. J. A., F. S. Zucoloto, and W. R. Terra. 1992. Enzymological and excretory adaptations of *Ceratitis capitata* (Diptera: Tephritidae) larvae to high protein and high salt diets. Comp. Biochem. Physiol. 102: 775-719.

Lowry, O. H., N. J. Rosebrough, N. A. Lewis Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lwalaba, D., K. H. Hoffmann, and J. Woodring. 2010. Control of the release of digestive enzymes in the larvae of the fall armyworm, *Spodoptera frugiperda*. Arch. Insect Biochem. Physiol. 73: 14–29.

Mahmood, F., and D. Borovsky. 1993. Biosynthesis of serine proteases in *Lutzomyia anthophora* (Diptera: Pychodidae). J. Med. Entomol. 30: 683–688.

Muharsini, S., Sukarsih, G. Riding, S. Partoutomo, S. Hamilton, P. Willadsen, and G. Wijffels. 2000. Identification and characterization of the excreted/secreted serine proteases of larvae of the old world screwworm fly, *Chrysomya bezziana*. Int. J. Parasitol 30: 705-714.

Nash, W. J., and T. Chapman. 2014. Effect of dietary components on larval life history characteristics in the medfly (*Ceratitis capitata*: Diptera, Tephritidae). PLoS ONE 9 (1): e86029. doi:10.1371/journal.pone.0086029.

Ortego, F. 2012. Physiological adaptations of the insect gut to herbivory, pp. 75–88. In G Smagghe & I. Diaz (eds.), Arthropod-plant interactions: novel insights and approaches for IPM. Springer, Dordrecht, the Netherlands.

Ossa-O., G. A., A. E. Bustillo-Pardey, and A. Valencia-Jiménez. 2000. Determinación del pH en el fluido digestivo de larvas y adultos de *Hypothenemus hampei* (Coleoptera: Scolytidae). Cenicafé. 51: 97-101.

Overend, G., Y. Luo, L. Henderson, A. E. Douglas, S. A. Davies, and J. A. T. Dow. 2016. Molecular mechanism and functional significance of acid generation in the *Drosophila* midgut. Sci. Rep. 6, 27242; doi: 10.1038/srep27242.

Overney, S., A. Fawe, S. Yelle, and D. Michaud. 1997. Diet related plasticity of the digestive system in larvae of the Colorado potato beetle (*Leptinotarsa decemlineata* Say). Arch. Insect. Biochem. Physiol. 36: 241-250.

Ping-Ping B., C. Er-Hu, S. Guang-Mao, W. Dong, W. Dan-Dan, and W. Jin-Jun. 2014. Inhibition of phenoloxidase activity delays development in *Bactrocera dorsalis* (Diptera: Tephritidae). Fla. Entomol. 97: 477-485.

Puigseiver, A., C. Chapus, and B. Kerfelec. 1986. Pancreatic exopeptidases, pp. 235-247. In P. Desnuelle, H. Sjostrom, and O. Noren (Eds.). Molecular and Cellular Basis of Digestion. Elsevier, Amsterdam.

Reyes, J., G. Santiago, and P. Hernández. 2000. The Mexican fruit fly eradication programme, pp. 377-380. In: K. H. Tan (Ed.). Area-wide control of fruit flies and other insect pests. Penerbit Universiti Sains Malaysia. Penang, Malaysia.

SAS Institute, 2003. JMP. Statistical, Discovery Software, Version 5.0.1. SAS Institute Inc., Carg., North Carolina.

Santiago, M. G. 2010. Aplicación del concepto de áreas libres de plagas, pp. 229-242. In P. Montoya, J. Toledo & E. Hernández (Eds.). Moscas de la Fruta: Fundamentos y Procedimientos para su Manejo. S y G. Editores. México D. F.

Sigma – Aldrich. Catalog. Technical Bulletin. Numbers BCA1 and B9643.

Silva, F. C. B. L., A. Alcazar, L. L. P. Macedo, A. S. Oliveira, F. P. Macedo, L. R. D. Abreu, E. A. Santos, and M. P. Sales. 2006. Digestive enzymes during development of *Ceratitis capitata* (Diptera: Tephritidae) and effects of SBTI on its digestive serine proteinase targets. Insect Biochem. Mol. Biol. 36: 561–569.

Terra, W. R., and C. Ferreira. 1994. Review. Insect digestive enzymes: properties, compartmentalization and function. Comp. Biochem. Physiol. 109: 1-62.

Terra, W. R., and C. Ferreira. 2005. Biochemistry of digestion, pp. 171–224. In Li Gilbert, K. Iatrou & S. S Gill (Eds.), Comprehensive molecular insect science, vol. 4. Academic press, London, UK.

Torres, J. B., and D. W. Boyd. 2009. Zoophytophagy in predatory Hemiptera. Braz. Archiv. Biol. Technol. 52: 1199-1120.

Valaitis, A. P., S. Augustin, and K. M. Clancy. 1999. Purification and characterization of the western spruce budworm larval midgut proteases and comparison of gut activities of laboratory–reared and field–collected insects. Insect Biochem. Mol. Biol. 29: 405–415.

Vonk, H. J. and J. R. H. Western. 1984. Comparative Biochemistry and Physiology of Enzymatic Digestion. Academic Press, New York. 501 pp.

Wagner, W., F. Mohrlen, and W. Schnetter. 2002. Characterization of the proteolytic enzymes in the midgut of the European Cockchafer, *Melontha melontha* (Coleoptera: Scarabaeidae). Insect Biochem. Mol. Biol. 32: 803–814.

Walker, J. M. 2002. The bicinchoninic acid (BCA) assay for protein quantitation, pp. 11-14. In: John M. Walker (Ed.). Cap. 3. The protein protocols Handbook. Second edition. Humana Press. Hatfield, UK.

Wirnt, R. 1974. Chymotrypsin: measurements with n-benzoyl-tyrosine ethyl ester as substrate. pp. 1009–1012, in Methods of Enzymatic Analysis. 2nd ed. H. Ulrich Bergmeyer (Ed.). Verlag Chemie, Weinheim, Germany.

Woodring, J., S. Diersch, D. Lwalaba, K. Hoffmann, and M. Meyering-Vos. 2009. Control of the release of digestive enzymes in the caeca of the cricket *Gryllus bimaculatus*. Physiol. Entomol. 34: 144–151.

Woodring, J. and S. Weidlich 2006. The secretion of digestive enzymes and caecal size are determined by dietary protein in the cricket *Gryllus bimaculatus*. Arch. Insect. Biochem. Physiol. 93: 121–128.

Xiao-Zhen L. and Ying-Hong L. 2007. Diet influences the detoxification enzyme activity of *Bactrocera tau* (Walker) (Diptera: Tephritidae). Acta Entomol. Sín. 50: 989-995.

Zeng, F., and A. C. Cohen. 2000. Partial characterization of α-amylase in the salivary glands of *Lygus Hesperus* and *L. lineolaris*. Comp. Biochem. Physiol. B. 126: 9–16.

Figures Captions

Figure 1. Third instar *A. obliqua* larvae midgut grown on artificial diet each with different pH indicator: A) eosin yellowish (orange to red, pH 0-3), B) bromophenol blue (yellow to purple, pH 3-4.6), C) bromocresol green (yellow to green, pH 3.8-5.4), and D) bromothymol blue (yellow to blue, pH 5.8-7.6). Arrows indicating where occur the change in coloration of the midgut due the indicators

Figure 2. The protein content (mg/g of diet) in natural diet (mango), artificial diet without infestation after larval growth was maintained for approximately 2, 4, and 8 days. The means were not significantly different ($p > 0.05$).

Figure 3. The protein content in the midgut of first, second, and third instar *A. obliqua* larvae that were reared on natural and artificial diet (mean ± SE). The means were not significantly different ($p > 0.05$).

Figure 4. Protease-specific activity (mean ± SE) in the midguts of first, second, and third instar *A. obliqua* larvae reared on (■) artificial and (■) natural diet. The results shown were obtained under pH 3.0 conditions. The bars indicate the level of activity in units or milli-units per gut (U/gut or mU/gut), while the lines indicate activity in units or milli-units per mg of protein (U/protein or mU/protein). Lowercase letters indicate differences between diets, while capital letters indicate differences between instar. Different letters indicate significant differences ($p<0.05$).

Figure 5. Protease-specific activity (mean ± SE) in the midguts of first, second, and third instar *A. obliqua* larvae reared on (■) artificial and (■) natural diet. The results shown were obtained under pH 4.5 conditions. The bars indicate the level of activity in units or milli-units per gut (U/gut or mU/gut), while the lines indicate activity in units or milli-units per mg of protein (U/protein or mU/protein). Lowercase letters indicate differences between diets, while capital

letters indicate differences between instar. Different letters indicate significant differences ($p<0.05$).

Figure 6. Protease-specific activity (mean ± SE) in the midguts of first, second, and third instar *A. obliqua* larvae reared on (■) artificial and (■) natural diet. The results shown were obtained under pH 6.0 conditions. The bars indicate the level of activity in units or milli-units per gut (U/gut or mU/gut), while the lines indicate activity in units or milli-units per mg of protein (U/protein or mU/protein). Lowercase letters indicate differences between diets, while capital letters indicate differences between instar. Different letters indicate significant differences ($p<0.05$).

Figure 1

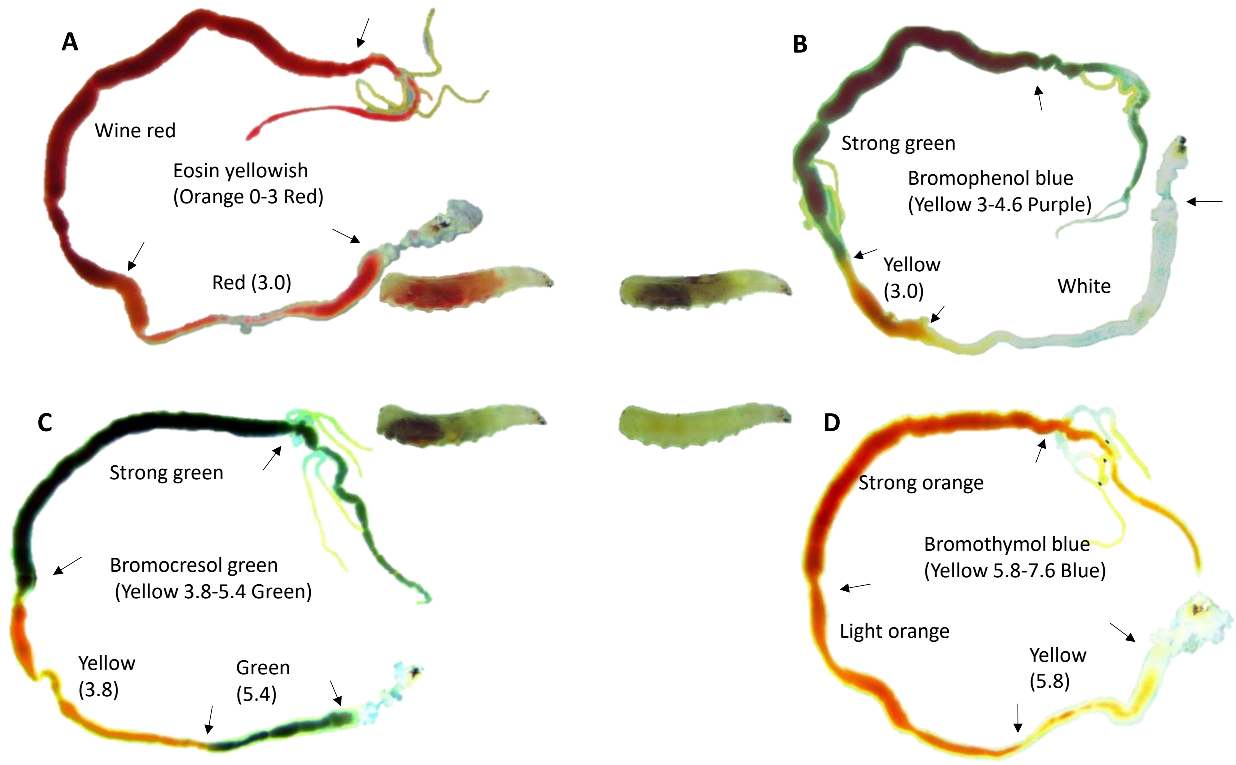


Figure 2

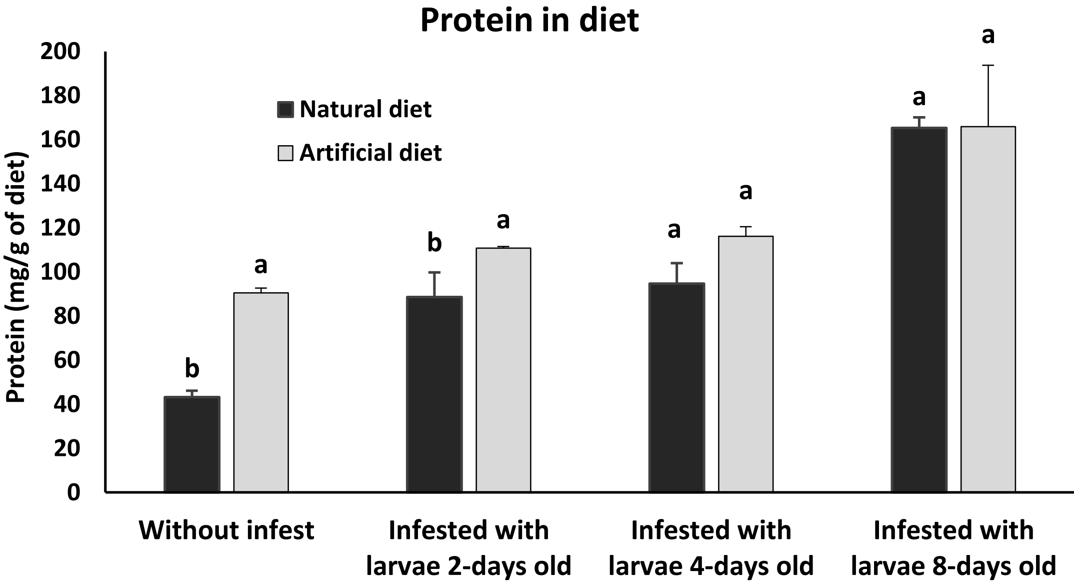


Figure 3

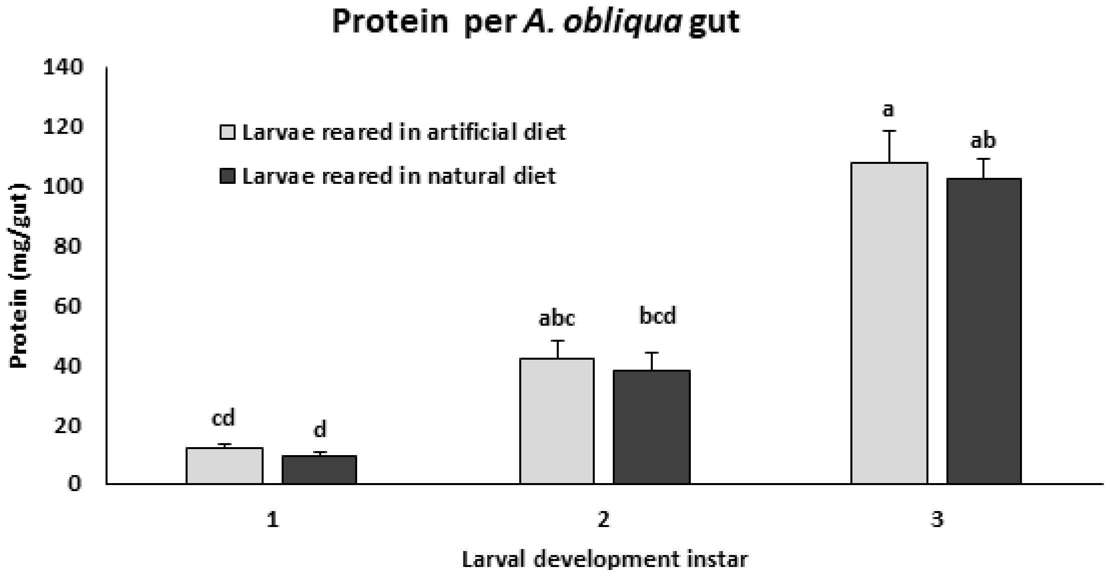


Figure 4

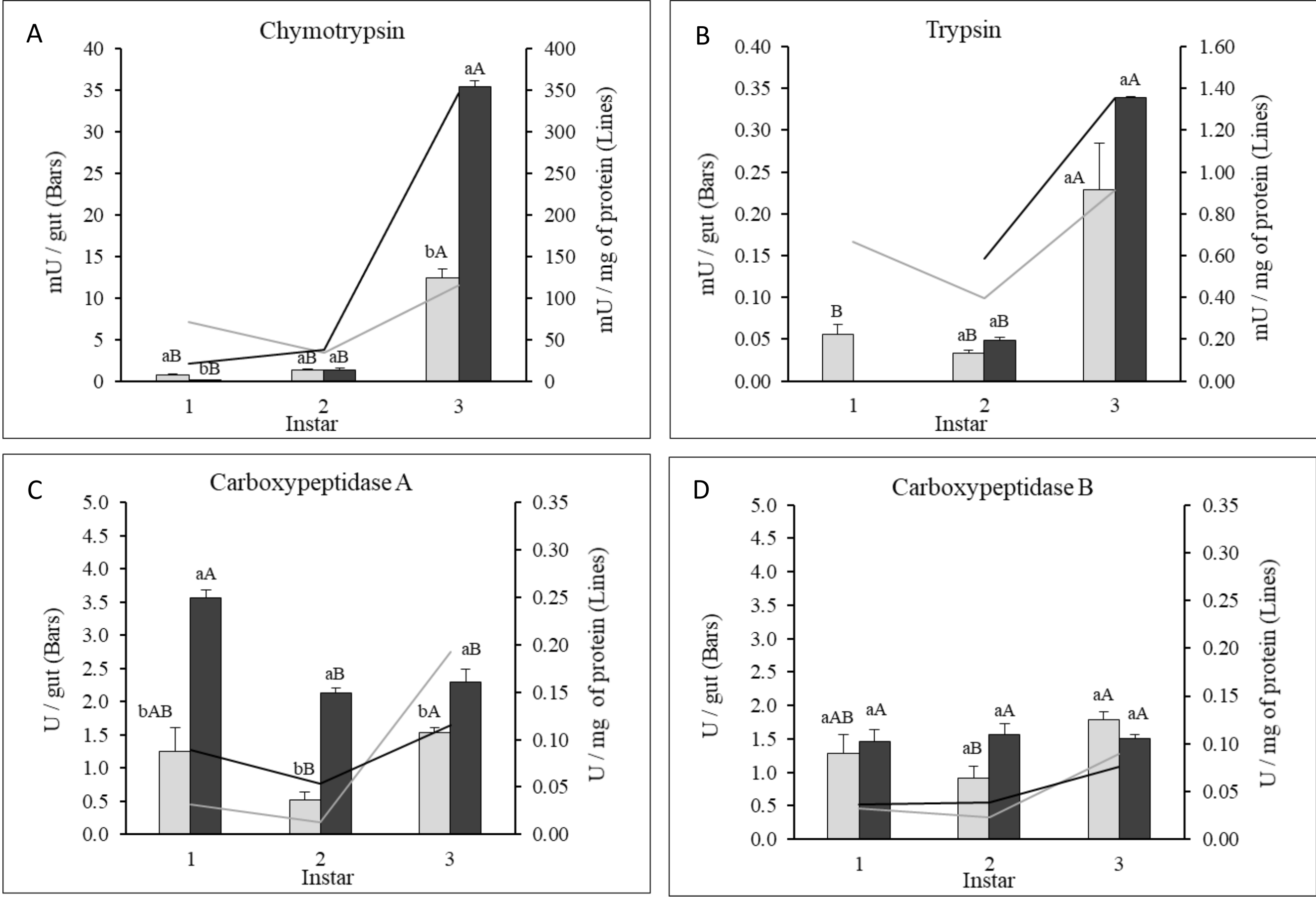


Figure 5

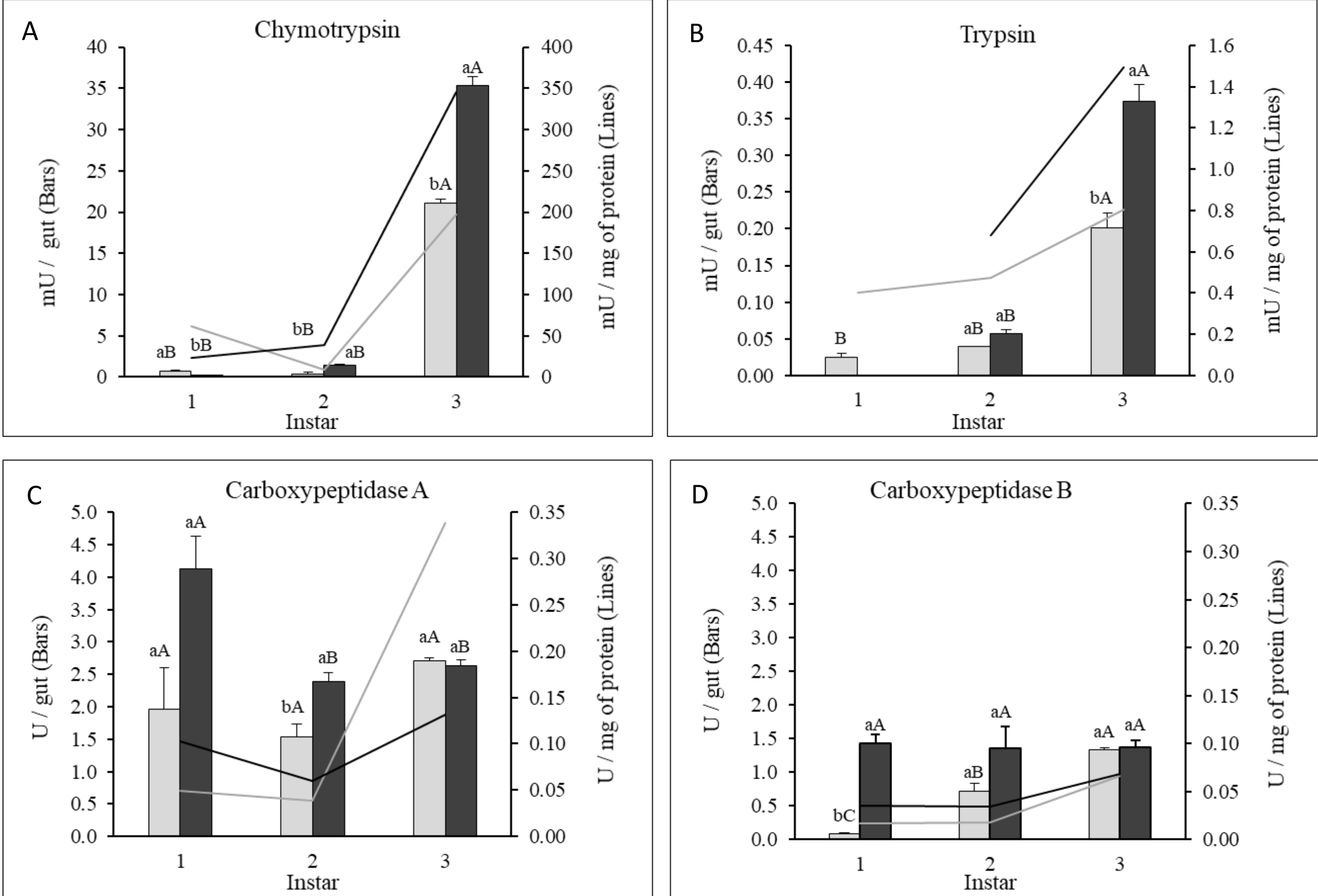
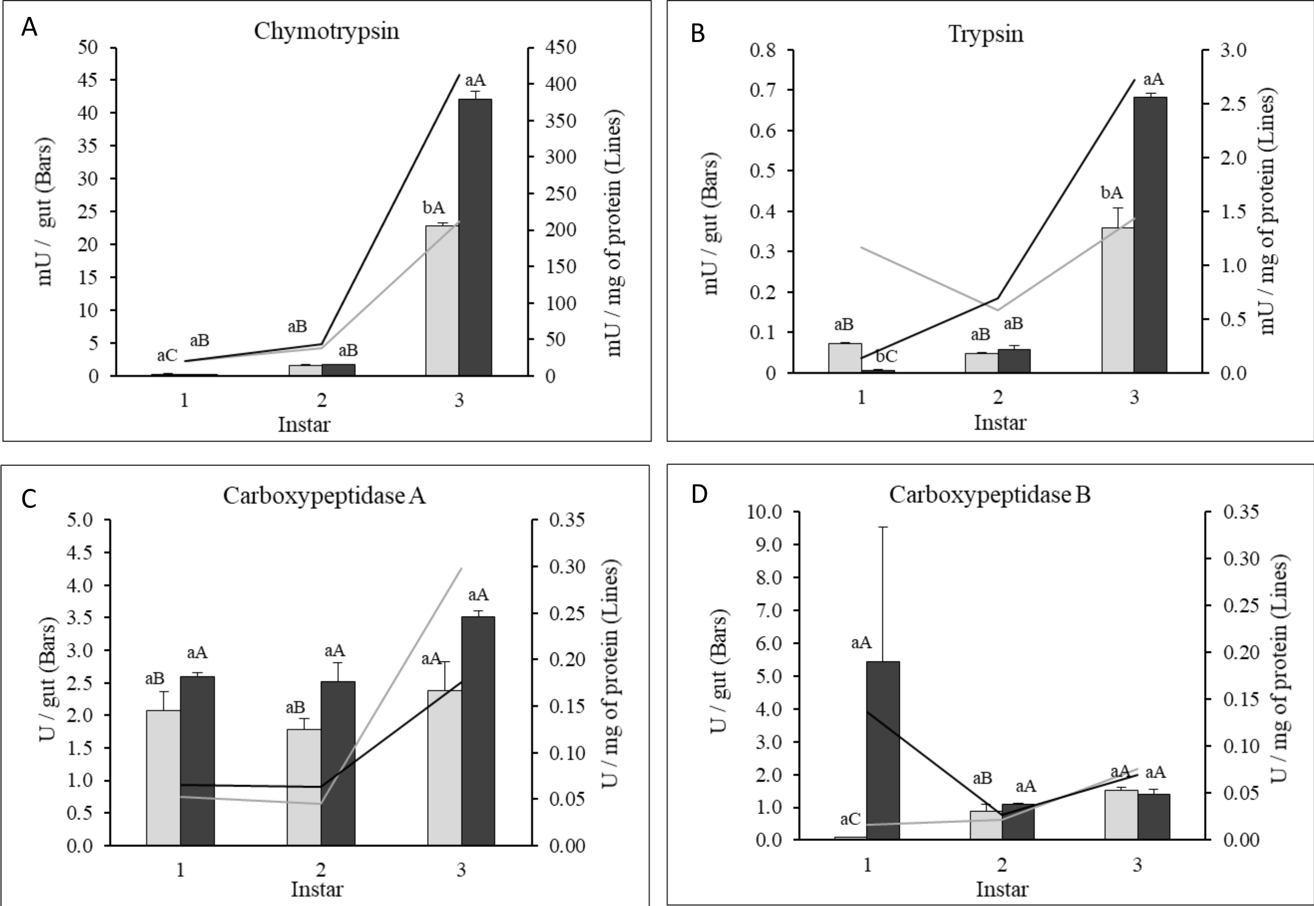


Figure 6



III. Conclusiones

Al determinar el contenido de proteína en dieta artificial natural y artificial se encontró que la proteína en mango sin infestar es menor que en dieta artificial recién elaborada sin infestar, pero después de ocho días, el contenido de proteína de las dos dietas es similar. En la dieta natural el contenido de proteína se incrementa aproximadamente cuatro veces desde que las larvas inician su desarrollo hasta que lo completan, mientras que en la dieta artificial, durante el mismo tiempo el contenido únicamente se duplica, seguramente a causa de la descomposición por microorganismos.

Cuando se determinó el pH en los intestinos de larvas de *A. obliqua* se encontró que en la parte media del intestino medio hay una región con un pH ácido, además en las regiones anterior y posterior del intestino medio el pH es menos ácido. Los valores de pH no se aproximan a la neutralidad o a ser básico como esta reportado para otros dípteros como *Aedes aegypti* con pH >10 (Onken et al 2008), o *Drosophila melanogaster*, donde se hay una región con pH de 9.5 (Overend et al 2016)

De acuerdo a las determinaciones realizadas se puede decir que dentro del complejo multienzimático que poseen las larvas de *A. obliqua*, y que intervienen en el desdoblamiento de las proteínas ingeridas en los alimentos, se encuentran las serina proteasas: quimotripsina y tripsina, y las metaloproteasas: carboxipeptidasa A y carboxipeptidasa B, las cuales se presentan en larvas de primero, segundo y tercer estadio, alimentadas con dieta artificial (empleada en la cría masiva), y con dieta natural (mango cv. 'Ataulfo niño').

En la digestión que ocurre en larvas de *A. obliqua*, las carboxipeptidasas A y B son mayormente responsables del desdoblamiento de las proteínas en comparación a la

tripsina y quimotripsina. Al comparar la actividad entre las enzimas encontramos que la actividad de las carboxipeptidasas A y B es aproximadamente 100 veces mayor que la actividad de la quimotripsina y 10,000 veces mayor que la actividad de la tripsina. La actividad de la quimotripsina es 100 veces mayor que la actividad de la tripsina.

Comparando entre ambas dietas, se encontró que las actividades de la tripsina y quimotripsina fueron mayores en larvas desarrolladas en dieta natural. Las actividades de las carboxipeptidasas de manera general no hubo diferencia en la entre las larvas alimentadas con una u otra dieta, salvo en dieta natural (mango) a pH de 3.0, donde la carboxipeptidasa A presentó una mayor actividad.

Comparando entre diferentes estadios se encontró un incremento en la actividad de la tripsina y la quimotripsina conforme el desarrollo de la larva fue avanzando, a diferencia de la actividad de las carboxipeptidasas A y B en que no se dio tal incremento. Se puede argumentar que el grado de actividad enzimática es independiente al tamaño del intestino, de acuerdo a los resultados encontrados.

IV. Literatura citada

Aluja, M. 1994. Bionomics and management of *Anastrepha*. Annu. Rev. Ent. 39: 155–178.

Cohen AC. 2015. Insect Diets Science and Technology. Boca Raton London New York: CRC Press. Chapter 1. Scope of insect diet science and technology. Second edition. p. 1-20.

Hernández E, Rivera P, Aceituno-Medina M, Aguilar-Laparra R, Quintero-Fong L, Orozco-Dávila D. 2016. Eficiencia de levaduras para la cría masiva de *Anastrepha ludens*, *A. obliqua* y *Ceratitis capitata* (Diptera: Tephritidae). Acta Zool. Mex. 32(3): 240-252.

Message CM, Zucoloto FS. 1989. Effect of some artificial diets on egg production by *Anastrepha obliqua*. Revta. Bras. Biol. 49(3): 699–701.

Moreno DS, Ortega Zaleta DA, Mangan RL. 1997. Development of artificial larval diets for West Indian fruit fly (Diptera: Tephritidae). J. Econ. Entomol. 90(2): 427-434. DOI: https://doi.org/10.1093/jee/90.2.427.

Onken H, Moffett SB, Moffett DF. 2008. Alkalinization in the isolated and perfused anterior midgut of the larval mosquito, *Aedes aegypti*. J. Insect Sci. 8(46): 1-20, available online: insectscience.org/8.46

Overend, GY, Luo, Henderson L, Douglas AE, Davies SA, Dow JAT. 2016. Molecular mechanism and functional significance of acid generation in the *Drosophila* midgut. Sci. Rep. 6, 27242; doi: 10.1038/srep27242.

Programa de Sanidad Vegetal-SAGARPA- Guanajuato. Ficha Técnica, *A. obliqua* Celaya Gto. Noviembre, 2004.

Rivera JP, Hernández E, Toledo J, Salvador M, Silva R. 2007. Dieta texturizada con agar para el desarrollo larvario de tres especies de moscas de la fruta (Diptera: Tephritidae). Fol. Entomol. Mex. 46(1):37–52.

Saldanha LA, Silva NM. 1999. Semiartificial rearing of the larvae of *Anastrepha obliqua* (Diptera: Tephritidae) in Manaus, Amazonas-Brazil. Fla. Entomol. 82: 82-87.

Sosa-Armenta JM, López-Martínez V, Alia-Tejacal I, Jiménez-García D, Guillen-Sánchez D, Delfín-González H. 2015. Hosts of five *Anastrepha* species (Diptera: Tephritidae) in the State of Quintana Roo, México. Fla. Entomol. 98:1000-1002.

Tigrero JO. 2009. Lista anotada de hospederos de moscas de la fruta presentes en Ecuador. Boletín Técnico 8, Serie Zoológica 4-5: 107-116

Zucoloto FS, Puschel S, Message CM. 1979. Valor nutritivo de algumas dietas artificias para *Anastrepha obliqua*. Boletim de Zoologia da Universidade da São Paulo 4:75-80.