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Big particles, best nutrition? Absorption and excretion of protein by *Anastrepha obliqua* larvae (Diptera: Tephritidae)

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Abstract

For purposes of mass-rearing fruit flies, nutrient intake through artificial diets is a challenge, artificial food content and processing should promote sufficient absorption and availability to ensure fly fitness. Bulking agents play an essential role in creating a quality diet, but its physical characteristics, such as particle size, may establish a better microenvironment for feeding and development. Currently, there is a lack of information about protein metabolism in mass-reared fruit flies. Therefore, we evaluated whether the particle size of the bulking agent affects the absorption and excretion of the proteins, as well as their effect on the life-history traits of *Anastrepha obliqua*. We determined the protein content of hemolymph and feces, as well as the presence of nitrogen end-products as indicators of their level of absorption in a diet elaborated with coarse and fine corn cob particles as a bulking agent. The bromatological composition showed that coarse particles increased the bioavailability and content of crude, digestible, and soluble protein for the diet and hemolymph protein of larvae alike. We found an inverse relationship between the protein content of the hemolymph and feces of the larvae. Ammonium was determined to be a product of the catabolism of proteins. Also, *A. obliqua* improved its development (yield and pupal weight) and fitness (adult emergence and flight ability) when larvae were reared on a coarse particle diet. In conclusion, a diet elaborated with a coarse bulking agent features increased protein bioavailability and nutritional quality, which, in turn, increases the life-history traits of *A. obliqua*.

Introduction

The West Indian fruit fly, *Anastrepha obliqua* (Macquart) (Diptera: Tephritidae), is a species widely distributed in America, from the Southern United States to Brazil (Hernández-Ortiz and Aluja, 1993; Aluja, 1994). *Anastrepha obliqua* is considered the second most economically important fruit fly species in Mexico and presents a severe challenge for mango production and export (Aluja *et al.*, 2014). One of the technologies applied to control this fly species is the Sterile Insect Technique (SIT) (Tan, 2000; Wyss, 2000; Hendrichs *et al.*, 2005; Klassen and Curtis, 2005). The SIT implies the massive rearing, sterilization, and release of a large number of sterile male insects that copulate with wild females, which then lay infertile eggs (Knipling, 1979; Cáceres *et al.*, 2007). Mass rearing of fruit flies requires an artificial diet that supports larval growth and development, provides a nutritional advantage, and increases the allocation and bioavailability of larval food resources. These factors improve survival and reproduction as a result of the efficient digestion and assimilation of nutrients. The relationship between absorption and excretion is correlated with the quality of the food consumed, exerting an effect on life-history traits (Klein and Kogan, 1974; Boggs and Freeman, 2005; Carbonell-Capella *et al.*, 2014). Optimized nutrient metabolism could explain the differences in survival and reproduction between natural and mass-rearing conditions.

Conventional diets for *Anastrepha* spp. are created by mixing fine-particle ingredients mainly, a bulking agent, a source of carbohydrates, and a source of protein, along with preservatives and pH regulators (Moreno *et al.*, 1997). The bulking agents are deliberately added as filling agents or carriers of essential molecules (proteins and carbohydrates), although they can be either nutritionally inert or not nutritionally inert (Cohen, 2004). Fine particles of corn cob and coconut fiber (<0.177 mm) are the bulking agents most widely used for the mass rearing of *Anastrepha ludens* and *A. obliqua* (Stevens, 1991; Artiaga-López *et al.*, 2004; Aceituno-Medina *et al.*, 2019). For the last 20 years, these artificial diets have been used to consistently produce 150 and 62 million pupae per week for *A. ludens* and *A. obliqua*, respectively, the Moscafrut facility in Metapa de Domínguez, Chiapas, Mexico (Orozco-Dávila *et al.*, 2017; SENASICA, 2017a, 2017b). The use of coarse bulking agents is desirable because

previous results indicated that their use increases the yield and survival of insects (Artiaga-López *et al*., 2018). Moreover, they promote an increment in nutrient absorption rates when modifying texture, wetness, consistency, hardness, distribution, water availability, biochemical stability, and bioavailability compounds, as well as the palatability of the diet (Bhattacharya and Waldbauer, 1970; Lingappa, 1987; Aguilera, 2005; Parada and Aguilera, 2007; Tomic-Carruthers, 2007; Sun *et al*., 2019). Diet consistency is also relevant because most insects require specific water levels with adequate viscosity. An adequate environment does not asphyxiate eggs and promotes larvae uptake of fluid during feeding, allowing nutrients to pass from the artificial medium toward the mouthparts and, finally, to the interior of the gut (Hendrichs *et al*., 1993). From this perspective, the success or failure of an artificial diet is determined by both the concentrations of diet components and the molecular interactions among them, as well as by the balance (regulation) and dynamics of factors such as humidity, temperature, pH, viscosity, water activity, and particle size, among others, with the bulking agent being a key element in reaching this goal (Cohen, 2004).

In the artificial rearing of fruit flies, the larval diet is not only the food matrix that contains the nutrients. It is 'everything.' The diet not only implies what larvae feed on but also refers to the medium in which larvae perform all of their biological functions: ingestion, absorption, excretion, molting, growth, etc.

Evaluation of nitrogenated metabolites (e.g., proteins, uric acid, ammonium) in larval or adult feces has led to an understanding of the effects of different artificial and natural diets on the amount of metabolized and absorbed protein (Waldbauer, 1968; Parra *et al*., 2012). However, there is still a lack of information about protein metabolism in mass-reared fruit flies. Hence, our study aimed to evaluate the effect of the bulking agent's particle size on the absorption and excretion of protein and, therefore, on the life-history traits of *A. obliqua*. To determine whether particle size could affect the bioavailability of the diet's protein, we determined the protein content in hemolymph and feces, as well as the presence of nitrogen end-products as indicators of the protein-level absorption by the larvae, in a diet elaborated with coarse and fine corn cob particles.

Materials and methods

Biological material

The eggs of *A. obliqua* flies used in this study were provided by the Moscafrut fruit fly facility (SADER-SENASICA) located in Metapa de Domínguez Chiapas, Mexico. *Anastrepha obliqua* has been mass-reared since 2002 following the procedures described by Stevens (1991), Artiaga-López *et al*. (2004), and Orozco-Dávila *et al*. (2017) for at least 174 generations, with a reintroduction of wild material in 2011.

Experimental design

The protein content in hemolymph and feces, as well as the presence of nitrogen end-products, from larvae of *A. obliqua* reared on two diets were compared: (1) a coarse granulometry diet prepared with big particles of corn cob, 0.250–0.420 mm, and (2) a fine granulometry diet prepared with fine particles of corn cob, 0.177–0.210 mm. Their effect on life-history traits was tested. The experimental unit consisted of a tray of 20 × 15 × 4 cm with 500 g of diet. Nine replicates were performed for each type of diet, considering each experimental unit as a replicate. The eggs used for each replicate corresponded to egg-laying by a different cohort of adults ($n = 9$ cohorts).

Preparation of the larval diet

Fine and coarse granulometry bulking agent

The particle sizes were obtained by passing samples of milled corn cob through a series of sieves (US Standard sieve), stacked with progressively smaller openings from top to bottom (40, 50, 60, 70, and 80). All the material retained on and above sieves 50 and 60 corresponded to coarse corn cob particles (0.250–0.420 mm size) and all the content retained on and above sieves 70 and 80 corresponded to fine corn cob particles (0.177–0.210 mm size). Both ingredients were the products of milled corn cob and were supplied by Mafornu Company (Mafornu, Cd. Guzmán, Jalisco, México).

Larval diet preparation

The proportion (w/w) of the nutrients in the matrix was the same in both diets, with coarse and fine granulometry. The larval diet was prepared by mixing all of the solid ingredients, 18% corn cob particles (coarse or fine) (Mafornu), 8.33% cornflour (Maíz Industrializado del Sureste, Arriaga, Chiapas, México), 6.33% Torula yeast (Lake States, Div. Rhinelander Paper, Rhinelander, WI, USA), 9% saccharose (Ingenio Huixtla, Chiapas, México), 0.33% sodium benzoate (Cia. Universal de Industrias, S.A. de C.V., México), 0.18% nipagin (Mallinckrodt Specialty, Chemicals Co., St. Louis, MO, USA), and 0.43% citric acid (Anhidro Acidulantes FNEUM, Mexana S. A. de C.V., Morelos, México) (Orozco-Dávila *et al*., 2017).

A volume of 325 ml of water was then added per 213 g of dry ingredients and mixed for 3 min using an industrial food mixer (CRT Model CPM-30: 127 V, 60 Hz, 1100 W, CRT Global S.A. de C.V., Santa Catarina, N.L., México). The wet diet was distributed in containers, each with 500 g, and they were sown with 0.25 ml of *A. obliqua* eggs (~4150 eggs) suspended in 2.5 ml of a 0.4% gum agar solution. The egg suspension was poured carefully and homogeneously over each diet. For the first 2 days of incubation, the containers were kept at 29 ± 1°C and R.H. of 90% until the eggs hatched. Afterward, they were held at 27 ± 1°C and RH 85–90% for seven more days until larval development was complete. The diet was then diluted with water and the larvae were recovered with a sieve (mesh size 14). Afterward, the larvae were collocated on fine coconut fiber (50% powdered/50% short fiber: CF Coirtech, Colima, México) to promote pupation (Aceituno-Medina *et al*., 2017) and kept at 21 ± 1°C and R.H. 70%. After 24 h, the pupae were covered with fine coconut fiber and then transferred to a second room at 25 ± 1°C and R.H. 80% for 14 days. Two days before adult emergence, the pupae were separated from the coconut fiber with a sieve (mesh size 14) and weighed.

Bromatological composition

Eighteen diets were prepared, each was considered a replicate, nine diets for coarse and nine diets for fine granulometry, respectively. From each type of granulometry, three diets were randomly selected to carry out the bromatological assays. Thirty-gram samples were taken from each selected diet. The bromatological assays of fresh diets were realized 2 h after their preparation and before the seeding of eggs.

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Crude fiber

Crude fiber was determined based on the protocol described in NMX-Y-094-SCFI (2012) with some modifications. Two grams were digested with 200 ml of a solution 0.128 M of H_2SO_4. This mixture was boiled for 30 min. Then the samples were filtered and washed with acetone. Afterward, alkaline digestion was performed with 200 ml of a solution 0.313 M of NaOH and boiled for 30 min. This was followed by a final filtering and acetone washing steps. Residues were calcined at 600°C for 30 min. The crude fiber was estimated as the difference between the initial and residual weight samples. The tests were carried out in triplicate for each replicate ($n = 3$).

Gross energy

The gross energy was determined based on the protocol described by the American Society for Testing and Materials (ASTM, 1974). One gram of sample was analyzed in an adiabatic bomb calorimeter (IKA C7000, Staufen, Germany) through the measuring of the temperature inside the oxygen bomb calorimeter before and after the sample was burned with pure oxygen. Benzoic acid was used, as a standard, for calibration of the calorimeter system with a known caloric value (26.4 MJ kg^{-1}). The tests were carried out in triplicate for each replicate ($n = 3$).

Ammoniacal and non-protein nitrogen, and crude protein

The determination of ammoniacal and non-protein nitrogen and crude protein was determined using a sample of 1 g of diet according to the standard Kjeldahl procedure (method 984.13) (AOAC, 1990; NMX-Y-346-SCFI, 2007). The tests were carried out in triplicate for each replicate ($n = 3$).

Digestible protein

Digestible protein was measured according to the protocol described in NMX-Y-085-SCFI (2006) and AOAC method 971.09 (1999) with some modifications. One gram of diet was digested with 150 ml of a solution of 0.0002% pepsin (in 0.075 M HCl). This mixture was placed at 150 rpm in a rotary shaker (IKA KS 4000, Wilmington, NC, USA) for 16 h at 45°C. Samples were then filtered and washed with acetone. Residues were used to determine protein with the standard Kjeldahl (method 984.13) procedure of the AOAC (1990). The tests were carried out in triplicate for each replicate ($n = 3$).

Soluble protein

Soluble protein was determined according to the protocols described in NMX-Y-320-SCFI (2004) and AOAC method 984.13 (1990). Two grams of diet were placed in a flask and added to 100 ml of 0.2% KOH (w/v), then placed in agitation for 20 min. Subsequently, they were centrifuged at 700 *g* for 20 min. The supernatant was recovered and a volume of 20 ml was taken for the determination of protein with the standard Kjeldahl procedure. The tests were carried out in triplicate for each replicate ($n = 3$).

Total protein

The total protein content was measured using 5 g of the sample according to the protocol described by Smith *et al.* (1985). Five grams of diet were placed in a drying oven (drying oven DGH9070A) at 105°C for 24 h. Then, the samples were digested with 5 ml of a solution 0.1 N of NaOH in a water bath for 1 h at 60°C. Next, the solution was centrifuged at 4000 *g* (Eppendorf 5424 Centrifuge, Hamburg, Germany) for 15 min. The supernatant was neutralized with 2.5N HCl, and 1 ml of borate buffer (6.21 g l^{-1} H_3BO_3, 4 g l^{-1} NaOH, 3.7 g l^{-1} KCl, pH 9.25) was added. A volume of 10 ml was completed with distilled water. The total protein was determined with the bicinchoninic acid (BCA) method. The absorbance was measured at 562 nm using a spectrophotometer (Multiskan GO Thermo Fisher Scientific, Finland). Total protein was calculated with a calibration curve of bovine serum albumin of 0.5–30 µg ml^{-1} (QuantiPro™ BCA Assay Kit, Sigma Aldrich® Catalog No. QPBCA). The tests were carried out in triplicate for each replicate ($n = 3$).

Bioavailability of the protein

Absorption

Protein bioavailability was measured as the amount of protein absorbed, and that is present in the hemolymph of the larva. Total protein in the hemolymph was determined in larvae fed and grown on three independent diets, randomly selected from the nine prepared for coarse and fine granulometry diets, respectively, and which were selected for bromatological assays. Samples of 20 larvae were taken from each chosen diet. One microliter of hemolymph was collected by puncturing each 8-day-old larvae and transferred to Eppendorf tubes. Proteins were precipitated with 1 ml of trichloroacetic acid (10% w/v). Then the samples were centrifuged for 10 min at 3000 g. The supernatant was removed, and the pellet was dissolved in 1 ml of 1 M NaOH. The samples were stored at 5°C until use. We estimated the total protein according to the BCA method, described previously. The tests were carried out in triplicate for each replicate ($n = 3$).

Excretion

Protein excretion was determined indirectly by the content of protein in the feces, as well as the presence of nitrogen end-products. To do so, larvae feces were collected from six groups of 100 8-day-old larvae (third stage). The larvae were starved for 24 h, later, the larvae were separated from the diet in a container without food, to empty their digestive tracts, then larvae were removed, and the feces were collected washing with 5 ml of distilled water. Immediately, the solution with feces was processed to determine the protein, uric acid, and ammonium content. Feces were dried at 105°C for 24 h and samples of 0.3–0.5 mg were then weighed. Next, 5 ml of 0.3N NaOH was added. We estimated the total protein according to the BCA method, described previously.

To determine uric acid and nitrogen end-products, we added 6 ml of 0.6% Li_2CO_3 (w/v) to larval feces, then left the samples for 1 h for the extraction of uric acid (Bhattacharya and Waldbauer, 1969, 1972). The samples were then centrifuged at 4000 *g* for 15 min, and the supernatant recovered. Uric acid was determined using the Uricostat enzymatic AA™ Assay Kit (Wiener Lab® Catalog No. 1840107). Absorbance was measured at 505 nm and the concentration was calculated using a calibration curve generated with a previously prepared standard solution of uric acid (10 mg dl^{-1}). The presence/absence of ammonium in the larval feces was obtained using the QUANTOFIX® Ammonium Test Kit (Sigma Aldrich Catalog No. 37212).

The test to determine protein, uric acid, and ammonium content was performed independently three times on each replicate and there were three replicates in total ($n = 3$).

Life-history traits

The life-history traits were assessed following the procedures described by the international product quality manual for sterile

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fruit flies (Hernández *et al.*, 2009; FAO/IAEA/USDA, 2014). Nine replicates ($n = 9$ cohorts) were performed for each type of diet, with each experimental unit (EU) considered as a replicate. Yield (EU = 500 g diet sown with ~4150 eggs), larval and pupal weight (EU = 3 g larvae or pupae), emerged flies (EU = 100 pupae), and flight ability (EU = 100 pupae) were the variables measured to compare the effect of the diets, and they were estimated for each replicate (cohort). The mean of life-history traits was estimated by each replicate using the total summation of the three samples instead of the mean of the means.

The yield was expressed as the mean number of larvae in 1 g, which was estimated using triplicate counts for each replicate, and used to determine the total recovery larvae in the total grams of diet. This value was expressed as the number of larvae per gram of diet. The yield was defined as the number of larvae recovered in 1 g of diet.

The mean weights of larvae and pupae were estimated by counting the total number of individuals in three samples of 3 g of larvae or pupae, with a total of 9000 mg of larvae for each replicate.

$$\text{Larval weight} = \frac{9000\ \text{mg}}{\text{Number of larvae in 9000 mg diet}}$$

Adult emergence was assessed from three samples of 100 pupae of 13 days old for each replicate. Each sample group was kept in cylinders (140 mm high and 80 mm in diameter); the numbers of emerged flies were quantified 5 days later (estimated emergence time for the 95%).

$$\text{Emerged flies (\%)} = \frac{300\ \text{pupae} - \text{non emerged} - \text{not complete emerged}}{300\ \text{pupae}} \times 100$$

Flight ability was estimated from three samples of 100 pupae of 13 days old for each replicate in the same cylinders that were used for adult emergence. Five days later, we counted the flies that were able to fly and escape from the cylinder and these values were expressed as a percentage.

$$\text{Flight ability (\%)} = \frac{300\ \text{pupae} - \text{non emerged} - \text{not complete emerged} - \text{non fliers}}{300\ \text{pupae}} \times 100$$

Statistical analyses

Data with homogeneity variances according to Bartlett's test (Box, 1953; Glass, 1966) were compared by the Student's *t*-test for equal variances, yield (larvae g^{-1} of diet), pupal weight (mg), emergence (%), fliers (%), protein in the diet (mg g^{-1} of diet), protein in hemolymph ($\mu g\,\mu l^{-1}$ hemolymph), protein in feces (μg larva^{-1}), crude protein (mg g^{-1} of diet), digestible protein (mg g^{-1} of diet), and gross energy (kcal g^{-1}). Variables with non-homogeneous variance, larval weight (mg), and total protein (mg g^{-1} of diet) were analyzed by the Student's *t*-test for unequal variances. The analyses were performed using the R Statistical Software (R Development Core Team, 2014).

Results

Bromatological composition

Diets elaborated with a coarse particle size had higher crude fiber ($t = 59.42$; d.f. = 4; $P < 0.0001$), ammoniacal nitrogen ($t = 673.00$; d.f. = 4; $P < 0.0001$), non-protein nitrogen ($t = 50.83$; d.f. = 4; $P < 0.0001$), crude protein ($t = 16.76$; d.f. = 4; $P < 0.0001$), digestible protein ($t = 13.21$; d.f. = 4; $P = 0.0002$), and soluble protein ($t = 64.48$; d.f. = 4; $P < 0.0001$). In contrast, gross energy ($t = 4.24$; d.f. = 4; $P = 0.0132$) and total protein ($t = 0.09$; d.f. = 4; $P = 0.9320$) did not show a non-significant effect (table 1).

Bioavailability of the protein

Larvae developed on coarse granulometry diets showed a higher protein content in their hemolymph ($t = 6.07$; d.f. = 4; $P = 0.0037$) in comparison to the protein content in larvae developed on a fine granulometry diet (fig. 1a). In contrast, the effect of particle size on the protein content in the feces was inverse; larvae developed on coarse granulometry diets showed lower protein content in their feces ($t = 6.29$; d.f. = 4; $P = 0.0033$) in comparison to the protein content in larvae developed on a fine granulometry diet (fig. 1b).

Uric acid and ammonium in larval feces

Uric acid was not detected in any of the treatments evaluated. Ammonium was detected in both coarse granulometry diet and fine granulometry diet treatments.

Life-history traits

Larvae developed on coarse granulometry diets showed higher values for life-history traits, larval yield ($t = 2.95$; d.f. = 16; $P = 0.0095$), pupal weight ($t = 2.14$; d.f. = 16; $P = 0.0481$), percentage of emergence ($t = 4.12$; d.f. = 16; $P = 0.0008$), and flight ability ($t = 3.36$; d.f. = 16; $P = 0.0040$). The larval weight did not show a significant difference as a result of the effect of the bulking agent's particle size in the diet ($t = 0.8060$; d.f. = 16; $P = 0.4370$) (table 2).

Discussion

Our study showed that a coarse granulometry diet coming from a big-size particle bulking agent increased the bioavailability and improved nutrition, thereby improving the content of the digestible and soluble protein in the diet and hemolymph of the larvae alike. In addition, it promoted the fitness of the mass-reared insect.

This study provides the first insight into establishing a relationship between the physicochemical properties of the larval diet and the nutritional and life-history traits of mass-reared fruit flies. Assuring the nutritional quality of the larval diet is the first step in the mass-rearing production process. Nevertheless, the evaluation of ingredients and acceptance currently established at Moscafrut Facility includes only pH, moisture, and microbiological quality (Hernández-Ibarra *et al.*, 2015). These measurements do not include tests for protein content and its bioavailability. Our results indicated that while both diets contained the same quantity of total protein, a diet elaborated with a coarse particle bulking agent showed higher crude, digestible, and soluble protein content, as well as a higher content of crude fiber, non-protein nitrogen, and ammoniacal nitrogen.

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Table 1. Crude fiber, energy, and ammoniacal nitrogen in larval diets formulated with different granulometries for *Anastrepha obliqua*

Particle size of the bulking agent	Fine (0.177–0.210 mm size)	Coarse (0.250–0.420 mm size)
Crude Fiber (%)	3.89 ± 0.01 b	4.75 ± 0.01 a
Gross energy (kcal g^{-1})	4.30 ± 0.01 a	4.28 ± 0.01 a
Ammoniacal nitrogen (%)	4.64 ± 0.01 b	6.88 ± 0.01 a
Non-protein nitrogen (%)	40.15 ± 0.08 b	44.45 ± 0.01 a
Crude protein (mg g^{-1} diet)	36.63 ± 0.09 b	38.40 ± 0.06 a
Digestible protein	16.45 ± 0.03 b	16.96 ± 0.04 a
Soluble protein (mg g^{-1} diet)	10.58 ± 0.03 b	12.98 ± 0.02 a
Total protein (mg g^{-1} diet)	18.49 ± 0.04 a	18.43 ± 0.77 a

Different lowercase letters in the rows indicates that the means (±SE) between treatments (columns) are significantly different ($P > 0.05$).

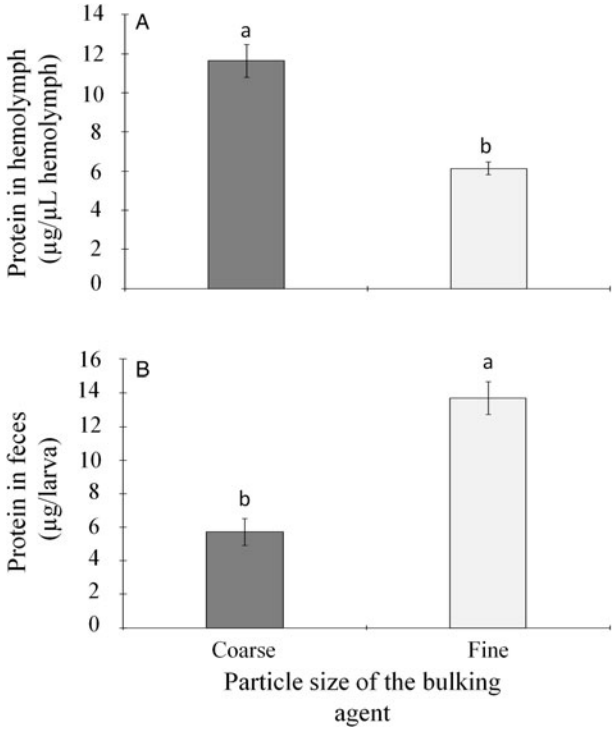

Figure 1. Protein in hemolymph (a) and feces (b) larvae of *Anastrepha obliqua* developed in diets prepared with fine and coarse bulking agent. Different lower case letters on the bars (means ± SE) indicate that the means are significantly different ($P > 0.05$).

The above indicated that a diet elaborated with a coarse bulking agent increased the protein bioavailability, as was reflected by high protein in the hemolymph and lower content in the feces of *A. obliqua*. A coarse granulometry diet might make the larvae capable of selecting a more favorable balance of nutrients as compared to a diet with smaller particle sizes (Bhattacharya and Waldbauer, 1970, 1972). According to Parada and Aguilera (2007) and Palma *et al.* (2019), increasing particle size has a positive effect on a diet's porosity and viscosity. It also influences the mixture, dispersion, and bioavailability of nutrients.

Table 2. Life history traits of *Anastrepha obliqua* developed on diets formulated with different granulometries

Life history traits	Particle size of the bulking agent: Fine (0.177–0.210 mm size)	Particle size of the bulking agent: Coarse (0.250–0.420 mm size)
Yield (larvae g^{-1} diet)	4.11 ± 0.52 b	6.34 ± 0.56 a
Larval weight (mg)	16.85 ± 0.32 a	17.50 ± 0.73 a
Pupal weight (mg)	11.80 ± 0.31 a	13.14 ± 0.55 b
Emergence (%)	72.44 ± 0.70 b	90.00 ± 2.4 a
Flight ability (%)	54.98 ± 3.82 b	73.78 ± 4.08 a

Different lowercase letters in the rows indicates that the means (±SE) between treatments (columns) are significantly different ($P > 0.05$).

Our results indicate that the crude fiber content has a direct relationship to the crude protein content, perhaps due to the minor amounts of nitrogen compounds that fiber contains. Additionally, it influences the carbon:nitrogen ratio in the medium and affects food intake, though this must still be proved in the context of mass-rearing diets for fruit flies. We observed that *A. obliqua* had improved development (yield and pupal weight) and fitness (adult emergence and flight ability) when the larvae were fed a coarse granulometry diet with high nitrogen content. Similarly, *Bombyx mori* optimized its development, preferring the consumption of foods containing 3.14% nitrogen compared to foods with 2.67% nitrogen (Fukuda *et al.*, 1961).

The objective of our study was to determine whether particle size affects protein absorption. We discovered that the particle size modifies the media and improve the life-history traits of *A. obliqua*. Then, we aimed to determine whether grain size in the diet changed life-history traits and protein absorption. As previously stated, though the diets with fine and coarse granulometry contained similar concentrations of total protein, the soluble and digestible protein content was higher in a diet with coarse granulometry. This can be explained by the fact that the milling or grinding process shears and opens the cellular structures. The protein matrix is then exposed to the environmental hydrolytic enzymes, which enhance protein digestibility (Joye, 2019). Although all nutritional content is potentially bioaccessible, the reality shows that almost no nutrient is totally converted during digestion into a potentially absorbable form. It is also known that a protein in a soluble state has good dispersibility, which increases its bioaccessibility and bioavailability through the physical properties of the food matrix. This affects the efficiency of physical, enzymatic, and chemical digestion processes, thus modulating the bioavailability of food components through its structure and composition (Boyer and Liu, 2004; Capuano and Pellegrini, 2019). This argument could explain the high protein content in the hemolymph of the larvae reared on a diet with coarse granulometry. The digestible protein content could be used as a specific parameter to characterize the nutritional quality of artificial diets. However, it would be interesting to evaluate this variable with some specific enzymes of significant concentration from the larvae gut of *A. obliqua* (Rivera-Ciprian *et al.*, 2017) rather than the pepsin used in this study. We found an inverse

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ratio between protein content in larvae's hemolymph and feces. Hence, both tests are suitable as indicators of a nutrient's bioavailability and nutrition quality.

The evaluation of nitrogenated end-products in feces is essential to understanding the general use of the ingested nutrients (Waldbauer, 1968). In insects, larvae, and adults, uric acid has been described as one of the main products of excretion and could be an indicator of nitrogen metabolism (Bursell, 1967; O'Donnell, 2008). In this study, uric acid and ammonium were measured as products of the catabolism of proteins. Surprisingly, our results showed that uric acid was not detected in larval feces. Ammonium was detected as the main nitrogen end-product; consequently, an assay was conducted in the feces of newly emerged adults, in which uric acid determination was positive (O. Rincón-Betancurt, personal communication, January 2020). This finding suggests that this compound is synthesized in the larval stage and stored at low concentrations in the fatty tissue and Malpighi tubules. When the insect reaches the adult stage, it is excreted (O'Donnell, 2008; O'Donnell and Donini, 2017). During the processes of excretion in insects, uric acid synthesis implies a high energetic cost. However, under humid conditions such as those found in fruits and the larval diet (approximately 60% water content), the synthesis and excretion of ammonium could be favored (Moloo, 1973). We observed that the size of the particle of the bulking agent modifies the larval media. This finding, according to Shukla *et al.* (2016) and Voirol *et al.* (2018), influences the gut microbiome and, consequently, the assimilation of the nutrients, which can even change from one developmental stage to another. In this sense, we can hypothesize that a proportion of the protein and its end-products in the larval diet of fruit flies could be the result of microbial activity.

Also, the increment of protein content in the hemolymph of insects reared on the coarse particle diet may be related to a higher concentration of ammoniacal nitrogen and non-protein nitrogen in the diet. These compounds could become a source of nitrogen for the formation of non-essential amino acids and the synthesis of hemolymph proteins in the fatty body through the reabsorption of the insect itself or by the action of microorganisms (Hirayama *et al.*, 1996; Gold *et al.*, 2018).

Our diets elaborated with fine and coarse bulking agents contained the same quantity of total protein in their composition. Therefore, it could be proposed that they had the same nutritional quality. However, a diet prepared with a coarse bulking agent produced insects with a higher hemolymph protein pool. This result is similar to that of the study of Lee *et al.* (2008). They observed that a high-quality-protein diet produced *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae with high hemolymph protein content.

This is the first step in developing a whole, high-quality diet using a holistic approach. It includes not only the effect of the diet's microstructure and components in the food matrix but also the medium for the larvae to perform all of their biological functions through appropriate nutrient absorption.

In summary, this study evidenced that the particle size of the texturizing agent has a significant effect on the absorption and excretion of protein by *A. obliqua* larvae. Therefore, its categorization as 'inert' should be reconsidered. The digestible protein content could be a specific parameter for characterizing the nutritional quality of artificial diets. In conclusion, a diet elaborated with a coarse bulking agent increases the bioavailability of the protein and nutritional quality of the diet and the life-history traits of *A. obliqua*.

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