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# Pupal size distribution and sexual dimorphism in wild and laboratory populations of two species of *Anastrepha* (Diptera: Tephritidae) fruit flies

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## Abstract

Body size is one of the most determining traits in the fitness of insects. For fruit fly (Diptera: Tephritidae) control programs using sterile insect technique, size is a valuable indicator of the quality of the mass-reared insects. However, laboratory colonization and mass-rearing conditions can contribute to the disparity in phenotypic traits between laboratory and wild populations, reducing the performance of sterile males and the effectiveness of the sterile insect technique. Hence the relevance of evaluating the possible variations in body size (size and shape) in 2 economically important species: *Anastrepha ludens* (Loew) and *Anastrepha obliqua* (Macquart) (both Diptera: Tephritidae). In this study, we compared pupal size distribution of wild and laboratory populations, using 3 parameters: pupal length, width, and weight. Additionally, we recorded the sex of the emerged adults to determine the sexual dimorphism related to pupae size. In *A. ludens*, male and female wild pupae were longer than pupae of their laboratory congeners, while laboratory pupae were wider and heavier than the wild pupae. In *A. obliqua*, male and female wild pupae were significantly larger than pupae of their laboratory congeners in all size parameters. We confirmed the sexual dimorphism in pupal size in both species and both populations. Females were bigger than males in all pupal size parameters. This study provides useful information about size distributions and dimorphism from pupal size, providing baseline data with potential implications and applications in mass rearing of *A. ludens* and *A. obliqua* for the application of the sterile insect technique.

Key Words: *Anastrepha ludens*; *Anastrepha obliqua*; mass-rearing; phenotypic changes; sterile insect technique

## Resumen

El tamaño corporal es uno de los rasgos más determinantes en la aptitud de los insectos. Para los programas de control de moscas de la fruta (Diptera: Tephritidae) que utilizan la técnica del insecto estéril, el tamaño es un indicador valioso de la calidad de los insectos de cría masiva. Sin embargo, la colonización en laboratorio y las condiciones de cría masiva pueden contribuir a la disparidad en los rasgos fenotípicos entre las poblaciones silvestres y de laboratorio, reduciendo el desempeño de los machos estériles y la efectividad de la técnica del insecto estéril. De aquí la relevancia de evaluar las posibles variaciones en el tamaño corporal (tamaño y forma) en dos especies económicamente importantes: *Anastrepha ludens* (Loew) y *Anastrepha obliqua* (Macquart). En este estudio, comparamos la distribución del tamaño de pupas de una población silvestre y de laboratorio, utilizando 3 parámetros: longitud, ancho y peso de pupas. Además, registramos el sexo de los adultos emergidos para determinar el dimorfismo sexual relacionado con el tamaño de la pupa. En *A. ludens*, las pupas de machos y hembras silvestres fueron más largas que las pupas de sus congéneres de laboratorio, mientras que las pupas de laboratorio fueron más anchas y pesadas que las pupas silvestres. En *A. obliqua*, las pupas de machos y hembras silvestres fueron significativamente más grandes que las pupas de sus congéneres de laboratorio en todos los parámetros de tamaño. Confirmamos el dimorfismo sexual en el tamaño de las pupas en ambas especies y en ambas poblaciones. Las hembras fueron más grandes que los machos en todos los parámetros de tamaño de pupa. Este estudio proporciona información útil acerca de las distribuciones del tamaño y el dimorfismo del tamaño de la pupa proporcionando datos de referencia con potenciales implicaciones y aplicaciones en la cría masiva de *A. ludens* y *A. obliqua* para la aplicación de la técnica del insecto estéril.

Palabras Clave: *Anastrepha ludens*; *Anastrepha obliqua*; cría masiva; cambios fenotípicos; técnica del insecto estéril

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Body size is one of the most determining features in the fitness of insects due to its close relationship with various physiological and ecological characteristics (Kalinkat et al. 2015). For programs that use the sterile insect technique to control pest species of fruit flies (Diptera: Tephritidae), body size is one of the main indicators of the quality of mass-reared insects because a large male and large female are

strongly correlated with greater mating success and fecundity in some species, respectively (Churchill-Stanland et al. 1986; Liedo et al. 1992; Fernandes-da-Silva & Zucoloto 1997; Tejeda et al. 2020). However, the processes of domestication and mass-rearing might lead to inadvertent behavioral, life history, and morphometric changes. These changes can result in positive attributes for mass-rearing, but in adverse aspects

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for field performance of the laboratory reared individuals (Cayol 2000; Weldon 2005; Sørensen et al. 2012; Hoffmann et al. 2005; Hoffmann & Ross 2018).

Because the effectiveness of sterile insect technique depends on the ability of sterile males to compete against their wild congeners for matings with wild females (Knippling 1955), it is crucial to understand how laboratory colonization and mass-rearing conditions can contribute to differences between laboratory and wild populations, especially in relevant traits such as body size or morphometric features related to male competitiveness (Clarke & McKenzie 1992; Gómez Cendra et al. 2014; Meza et al. 2014; Parker et al. 2021).

The assessment of mass-reared sterile insect quality allows the identification of possible causes of low field performance of the released flies (Chambers 1977). In tephritid fruit fly sterile insect technique programs, quality control protocols use pupal weight and diam, an indicator of pupal size, as the first indicators of sterile insect quality. Pupal size is an indicator of adult size, as well as an indirect measure of nutritional status, such as energy reserves, which are essential for the dispersal of insects and desiccation resistance that affects longevity (Weldon et al. 2013, 2021; FAO 2019). However, the relevance of producing large males is based on evidence that male size influences female choice (Aquino & Joachim-Bravo 2014; Benelli et al. 2015).

Because the adult size is determined by pupa size, and this in turn by the larva size, the larval diet (quality and amount) is considered one of the main factors of size variation in the mass-rearing of fruit flies. Nevertheless, this phenotype also reacts to different environmental conditions, such as adaptation to artificial rearing conditions or stressful environments (Clarke & McKenzie 1992; Nijhout et al. 2014). For example, in *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae), it was found that laboratory flies were larger than wild flies, laboratory males had greater head width and eye length, although both laboratory males and females had narrower wings than their wild congeners (Gómez Cendra et al. 2014). Recently, morphometric differences between laboratory and wild males from similar pupal size were determined in *A. ludens* and *A. obliqua* (Sánchez-Rosario et al. 2021). These differences could be due to environmental or genetic factors, or as a response to adaptation to artificial conditions.

Ecological and evolutionary processes act differently in each sex, thus the relevance of studying size variation in the context of sexual size dimorphism (Fairbairn 1997; Stillwell et al. 2010; Testa et al. 2013). In *Anastrepha suspensa* (Loew) and other species of tephritids of economic importance, such as: *A. ludens* (Loew), *A. obliqua* (Macquart), *Bactrocera dorsalis* (Hendel), *Bactrocera cucurbitae* (Coquillett), *Bactrocera oleae* (Rossi), *Ceratitidis capitata* (Wiedemann), *Rhagoletis pomonella* (Walsh) (all Diptera: Tephritidae), females are larger than males (Sivinsky & Dodson 1992). Sex-specific allometry was reported in *B. dorsalis*, particularly in weight relative to tibial length. Females gained disproportionately more weight than males with the increase of hind-tibial length (Zhou et al. 2016). In *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae), sexual dimorphism in body size and wing shape also has been reported, but with similar symmetrical growth. Females had a larger body size but shorter and wider wings than males, which may be associated with their reproductive biology or locomotion (Sivinsky & Dodson 1992; Zhou et al. 2020).

Given the evidence of morphometric differences (size and shape) between wild and laboratory populations, improved understanding is needed on how mass-rearing conditions affect intraspecific size-frequency distributions in tephritid fruit flies. Therefore, the objective of our research was to compare the pupal size distributions and dimorphism of 2 populations, wild and laboratory, of 2 economically important species, *A. ludens* and *A. obliqua*. In this study we used a pupal sorter machine, and we used the same calibration for studying the

size distribution of 2 populations. We measured the length, width, and weight of male and female pupae of wild and laboratory populations of *A. ludens* and *A. obliqua*, 2 species used in the sterile insect technique.

## Materials and Methods

### ANASTREPHA LUDENS AND ANASTREPHA OBLIQUA PUPAE

This study was carried out in 2 species of fruit flies: *A. ludens* and *A. obliqua*. For each species, we evaluated a laboratory and a wild population. Laboratory flies were obtained as pupae from a batch of the mass-reared colony (bisexual strain) at the Moscafrut facility in Metapa de Domínguez, Chiapas, Mexico. Wild flies were obtained as larvae from naturally infested hosts. *Anastrepha ludens* was obtained from sour oranges (*Citrus aurantium* L.; Rutaceae), whereas *A. obliqua* was obtained from mangoes (*Mangifera indica* L. cv. 'coche'; Anacardiaceae). The hosts were collected in different localities of Chiapas, Mexico. Mangoes were collected in Mapastepec (15.4069028°N, 92.8226778°W; 15.3914361°N, 92.7982944°W) and sour oranges in the Soconusco region (14.8975111°N, 92.1831444°W; 14.9192194°N, 92.1861056°W; 14.9220500°N, 92.1812556°W; 14.9569778°N, 92.6271667°W; 14.9785917°N, 92.2639250°W).

Infested fruits were maintained under laboratory conditions (25 ± 2 °C, 65 ± 5% RH, and a 12:12 h [L:D] photoperiod) in trays with vermiculite until the larvae reached the third instar. Later, the larvae were extracted and placed in vermiculite at 60% humidity to promote pupation (Orozco-Dávila et al. 2017). Assessments of length, width, and weight, as well as sorting was made when pupae were 11 and 10 d old for *A. ludens* and *A. obliqua*, respectively.

### SIZE DISTRIBUTIONS

Size distribution in wild and laboratory populations was determined using the pupae sorting machine (FAO 2019) that consists of 2 horizontally placed steel tubes that rotate in opposite directions. The tubes are separated from each other by an adjustable gap, through which the pupae pass and are separated into 10 size categories based on pupal diam.

Before sorting the pupae by size, we standardized a calibration for both populations in each species. The machine was calibrated so that the size distribution of a laboratory batch of pupae from each species (< 5,000 pupae) approximated to a normal distribution in such a way that the most pupae fell in the sixth size category, with the least number in the first and tenth categories. Since the machine is adjustable at both ends, the gap was measured with a 25 sheet (mm) feeler gauge set (Powerbuilt model 648394, Briggs & Stratton Corporation, Wauwatosa, Wisconsin, USA). The calibration used for *A. ludens* was 1.84 to 2.98 mm, and for *A. obliqua* was 1.72 to 2.76 mm.

### MEASUREMENTS OF PUPAL SIZE

The average mass was determined for each species by weighing 50 pupae per category individually (about 500 pupae for each species) using an analytical balance (Sartorius basic, model BA160P, Sartorius AG Lab Instruments GmbH & Co, Goettingen, Germany). Pupae were maintained for identification of the sex of the emerged adult (male or female) in individual 3.5 mL tissue culture cells (Falcon® 24-Well Clear Multiwell Plate, Corning Inc., Corning, New York, USA). Subsequently, each pupa was photographed with a Zeiss AxioCam (ERc 5s) microscope (Stemi 2000-C, Carl Zeiss AG, Oberkochen, Germany) to record its length and width using AxioVision 3.6 software (Carl Zeiss Vision GmbH, Aalen, Germany). The sex of each fly was determined

by identifying the external genital morphological structures (male and female terminalia). Females have an ovipositor, a female reproductive tract, whereas males do not (Norrbon et al. 2000).

## STATISTICAL ANALYSIS

The length, width, and weight of pupae of each population and species were compared with a Pearson's correlation coefficient. The size distribution of laboratory and wild male and female pupae were analyzed by multivariate techniques. A multivariate analysis of variance (MANOVA) with a Pillai test for each species was performed to determine the differences between populations, sexes, and their interactions. Subsequently, a canonical analysis was performed to determine relationships between groups of variables: population (laboratory and wild), and sexes (male and female), and measured variables of pupal size (length, width, and weight). This multivariate technique transforms the original variables into a canonical space of maximal differences, displaying the data in a canonical dimension (can 1), where the groups populations and sexes are represented by boxplots, and the pupal size variables are represented by vectors of means. Differences between groups can be interpreted from the differences in the positions of the boxplots, and it is not indicative of the mean value, but of the influence of the measured variable (vector). The length of the vector shows the relative importance in the distribution of the groups, and direction of the vector is proportional to their contribution to explain the variability between the groups. Statistical analyses were carried out with R software v.3.6.1 (R Core Team 2020).

## Results

Pupal size distribution data for *A. ludens* are based on the emergence record of 184 wild pupae (103 males and 81 females), and 316 laboratory pupae (123 males and 193 females). For *A. obliqua*, the pupal size distribution data is based on 224 wild pupae (123 males

and 101 females) and 243 from laboratory pupae (133 males and 110 females).

The highest proportion of males and females of both populations and species was distributed in categories 5 to 7, although this distribution tended to be smoother in the wild population. In *A. ludens*, the peak of distribution in wild females was skewed more towards the large size relative to the laboratory flies. Regarding males, both distributions were quite similar. In both the laboratory and the wild population, a higher proportion of males and females was observed in *A. ludens* compared to *A. obliqua*. In *A. obliqua*, male and female pupae from the laboratory population were distributed principally in the middle categories (5 to 7), whereas the wild population showed a wider distribution. In this species, unlike *A. ludens*, the distribution was skewed more to the right (Fig. 1).

According to Pearson's correlation, all variables (width, weight, and length) were positively and significantly correlated with each other in laboratory and wild pupae of both species ( $P < 0.0001$ ). In *A. ludens*, the association between width and weight was stronger in the laboratory population than in the wild population (0.9651 and 0.8339, respectively), in such a way that the weight was more correlated with the width in laboratory pupae, whereas in the wild pupae the correlation between the parameters was more uniform. In *A. obliqua*, the correlation values were quite similar in both populations.

The canonical analysis performed for males and females of each species was significant (Figs. 2 & 3). In *A. ludens*, there were significant differences between the populations (Pillai test,  $F_{3,494} = 54.73$ ;  $P < 0.0001$ ) and between the sexes ( $F_{3,494} = 46.31$ ;  $P < 0.0001$ ), but there was no significant interaction between populations and sexes ( $F_{3,494} = 2.44$ ;  $P = 0.063$ ). According to the canonical analysis, wild males and females were longer than laboratory pupae, whereas laboratory males and females were wider and heavier in comparison to wild congeners (Fig. 2). For *A. obliqua*, there also were significant differences between populations (Pillai test,  $F_{3,461} = 33.52$ ;  $P < 0.0001$ ) and between sexes ( $F_{3,461} = 26.18$ ;  $P < 0.0001$ ); the interaction between population and sex

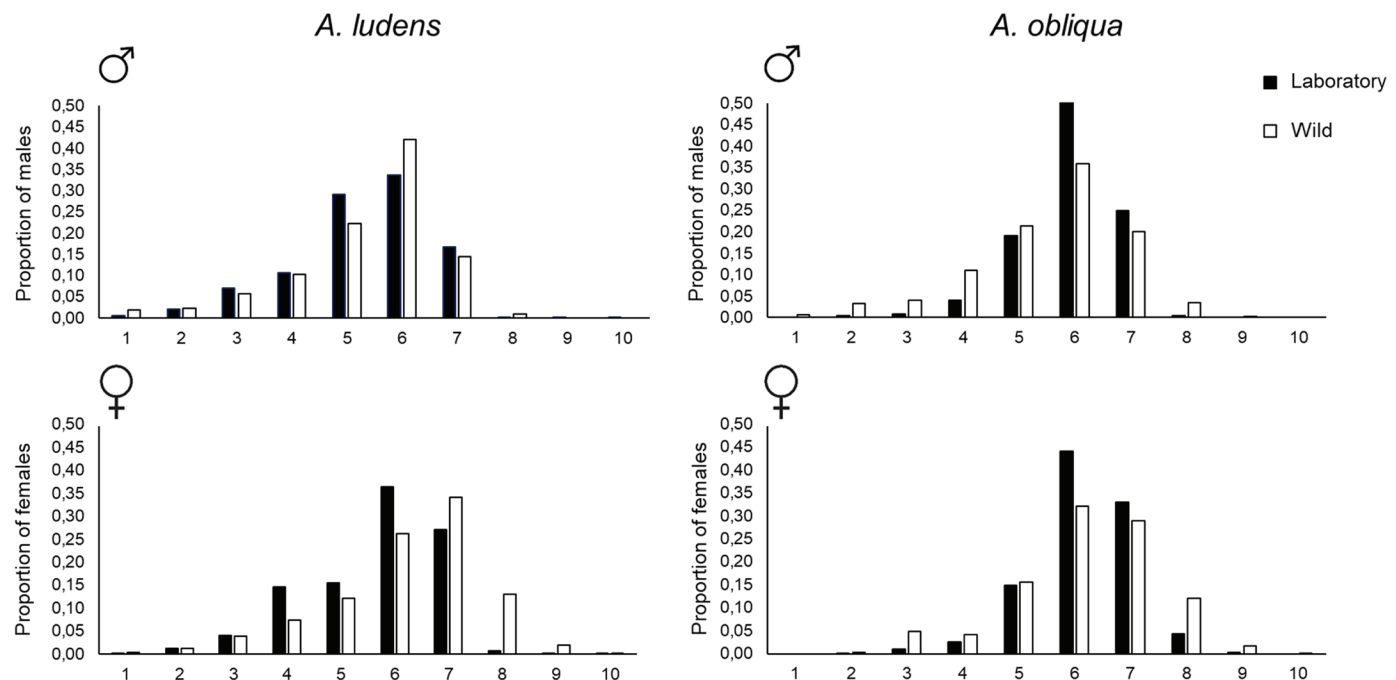
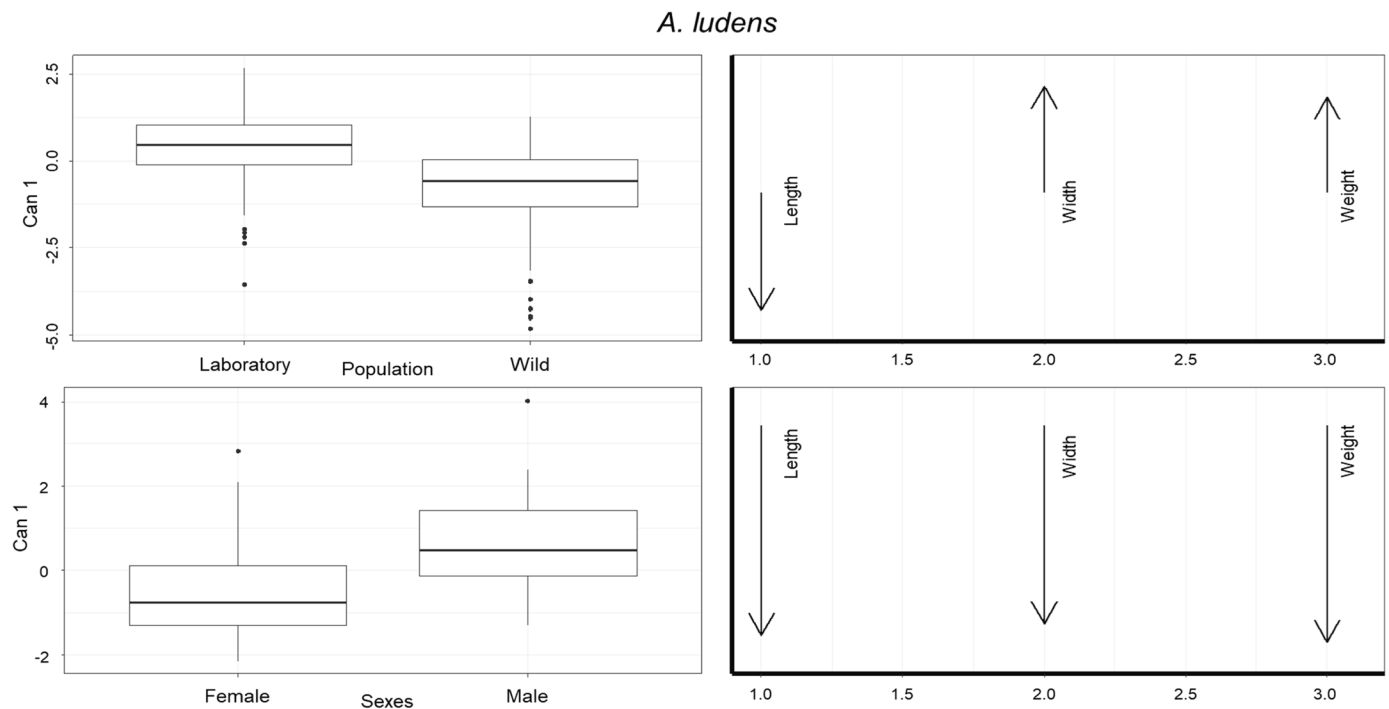


Fig. 1. Size distribution of laboratory and wild *Anastrepha ludens* and *Anastrepha obliqua* male and female pupae. The proportion of male and female pupae is shown in 10 pupal size classes (pupal diam mm) on the x-axis.



**Fig. 2.** Canonical analysis for pupae size parameters of males and females of laboratory and wild populations in *Anastrepha ludens*. The canonical analysis is represented on the first canonical axis (can 1), where the boxplots indicate the populations (laboratory and wild) and sexes (males and females) (left). The variables of pupae size: length (mm), width (mm), and weight (mg) are indicated by vectors (right).

also was statistically significant ( $F_{3,461} = 3.90$ ;  $P = 0.008$ ) (Fig. 3). Wild males and females were larger in pupal weight, width, and length than their laboratory congeners.

## Discussion

In this study we compared the pupal size distributions of 2 populations, laboratory and wild, of 2 economically important species: *A. ludens* and *A. obliqua*. We evaluated 3 parameters associated with pupal size: length, width, and weight. Given the correlation with the adult size (male or female), we inherently considered the sexual size dimorphism in the analysis of pupal size distribution.

Our findings showed differences in size distribution between laboratory and wild populations within each species, as well as between males and females. We confirmed sexual size dimorphism in pupae of both species. Although sexual dimorphism has been demonstrated in some species of tephritids, this study constitutes the first reference regarding sexual size dimorphism in pupae (Sivinski & Dodson 1992; Tejeda et al. 2014; Zhou et al. 2016, 2020). In some experiments where body size was evaluated, size selection was performed through manipulation of larval densities in the diet. However, this leads to different nutritional status of the adult, and therefore an impact on size and fitness components (Aluja et al. 2001; Kaspi et al. 2002; Nash & Chapman 2014). Here, all laboratory-reared males were obtained at controlled larval densities (Orozco-Dávila et al. 2017; FAO 2019) and wild flies were obtained from infested fruits and collected randomly. This approach allowed us to reduce the effects of other causes of variation in male size.

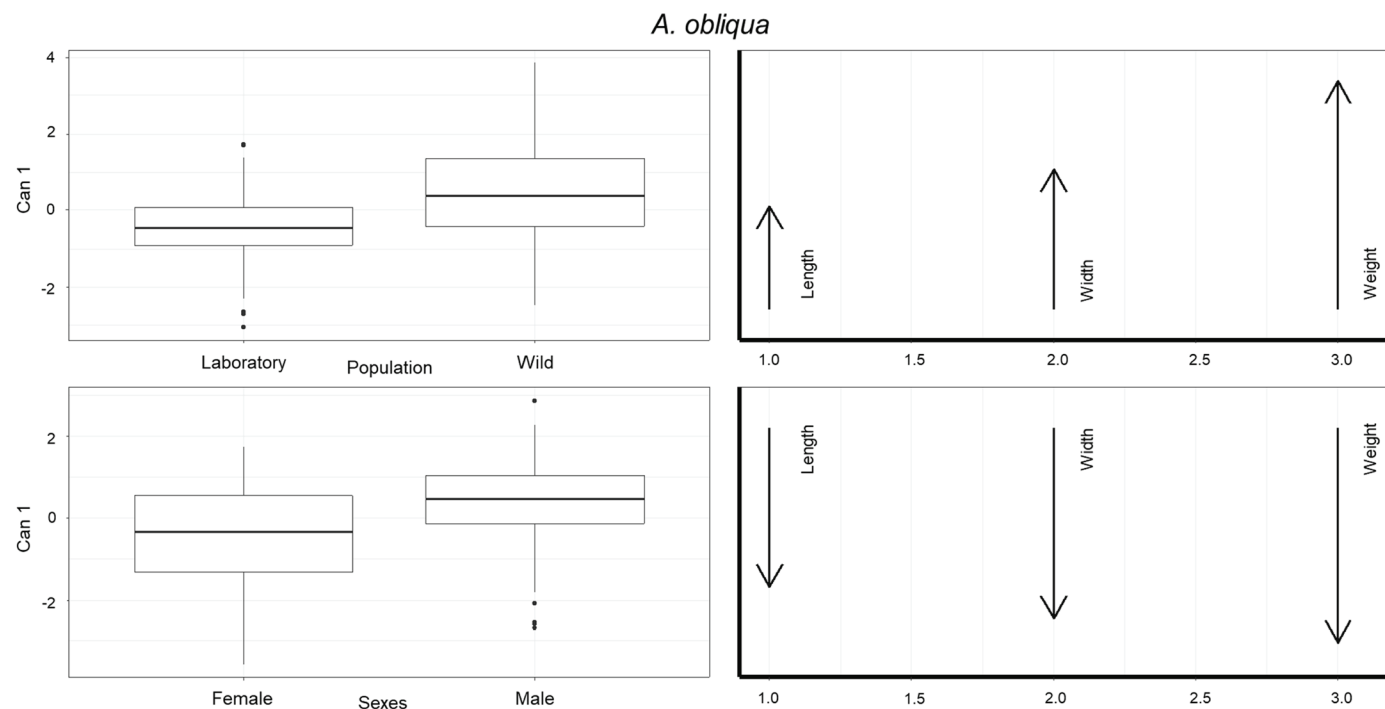
The size distribution showed some differences between the 2 populations. In general, in laboratory *A. obliqua*, a lower proportion of males and females was recorded in the first categories; this was

due to low emergence in the first and second category. According to Tejeda et al. (2014), resistance to desiccation increases with size, so it is likely that small pupae lose more water, which in turn is due to a lower amount of lipids. This could explain the lower emergence observed in small size categories. Regarding the differences in the size distribution between populations, wild males and females showed a smoother normal distribution than laboratory congeners. Wild females of both species showed a higher frequency in the large size categories. The standardized environmental variables used in mass-rearing to increase production and quality of insects, such as quantity and quality of the diet, probably explains a higher frequency in the size distribution of the middle categories (5–7) (Orozco-Dávila et al. 2017). In *C. capitata*, analysis of body mass frequency distributions determined that this distribution tends to be normal to slightly skewed to the right (Gouws et al. 2011). Although we separated the pupae based on their diam, in both species we observed that the highest frequency of individuals was distributed in size categories 5 to 7, so they show a similar trend.

Sexual dimorphism in pupal size was evident in both species and populations. Females were bigger than males in all pupal size parameters, although these differences were more evident in *A. ludens* than in *A. obliqua*. These results on pupal size dimorphism were as expected due to body size dimorphism in adults of several tephritid species (Sivinski & Dodson 1992). The explanation for this marked dimorphism is closely related to the reproductive roles. In *B. tryoni*, the larger body size of the female is related to the increase in the number of ovarioles, whereas in *A. ludens*, *Anastrepha serpentina* (Wiedemann) (Diptera: Tephritidae), and *A. obliqua*, large flies presented greater fecundity (Fitt 1990; Liedo et al. 1992).

Pupal size parameters differed markedly according to sex between populations. However, in *A. ludens* we observed differences in the pupae shape. Wild pupae were longer, whereas laboratory pupae were wider and heavier. These differences in the pupae shape also explain





**Fig. 3.** Canonical analysis for pupae size parameters of males and females of laboratory and wild populations in *Anastrepha obliqua*. The canonical analysis is represented on the first canonical axis (can 1), where the boxplots indicate the populations (laboratory and wild) and sexes (males and females) (left). The variables of pupae size: length (mm), width (mm), and weight (mg) are indicated by vectors (right).

that classification criteria discriminated more strongly in the laboratory than in the wild population. In *A. obliqua*, wild pupae were bigger than laboratory pupae in all size parameters.

In wild populations, body size differences may be influenced by the host fruit and the season of the yr, whereas in laboratory populations, size differences probably were due to larval density in artificial diets, diet formula, texture, and rearing temperatures (Navarro-Campos et al. 2011; Shelly 2018). Although the factors that affect body size were not studied here, it is important to consider that differences among individuals contribute to disparities in body size, whereas differences in populations may be related to genetic or environmental factors, and their interactions (Chown & Gaston 2010; Nijhout et al. 2014). Environmental conditions can strongly affect the degree but not the direction of sexual size dimorphism within species. Female size appears to be more sensitive to environmental conditions than male size (Teder & Tammaru 2005). Although much of the body size variations are due to body size plasticity, it is recognized that many of the variations are adaptive, and therefore inherently linked to biological functions and behavior (Stillwell et al. 2010).

All measured variables were correlated highly with the pupae classification criteria (diam), thus verifying the feasibility of using the pupae sorting machine for size characterization, quality control, and selection of males as a colony management strategy to improve the performance of sterile males (McInnis 1987). Furthermore, we found evidence of the increase in size in mass-reared populations and reduction in the variation of pupal size.

Morphological differences between laboratory and wild populations have been documented in adult fruit flies (Gómez Cendra et al. 2014; Sánchez-Rosario et al. 2021). Although body size is a trait with genetic plasticity, it is also known that mass-rearing conditions represent selection agents that can lead to evolutionary changes in traits related to size.

This research constitutes a reference in the attempts to explore and characterize the potential differences between laboratory and wild populations in an immature stage, and that could be related to the differences described in adults. Therefore, our results can be used as a baseline in the quality control protocols in mass-rearing programs for these species of fruit flies. More research is needed to analyze the effects of mass-rearing at the morphological level, as well as its potential effects on the performance of mass-reared sterile insects.

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