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Research Article



# Effects of Larval Density and Support Substrate in Liquid Diet on Productivity and Quality of Artificially Reared *Anastrepha ludens* (Diptera: Tephritidae)

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## Abstract

The larval developmental environment can affect the quality of artificially reared insects used in pest control programs that apply the sterile insect technique. We used a liquid larval diet lacking corncob fractions (an ingredient which may be contaminated by toxins that inhibit larval development and drastically reduce insect production in mass-rearing facilities) to examine quality indicators of *Anastrepha ludens* (Loew) (Diptera: Tephritidae) as a function of larval density (1–5 larvae per ml of diet) and on two types of supporting materials (synthetic sponge and carpet felt) compared with a standard solid diet with corncob fractions inoculated with a fixed quantity of *A. ludens* eggs. Pupal weight and adult emergence of *A. ludens* reared on a liquid artificial diet were negatively and positively density dependent, respectively. One tray of solid diet produced an average (95% CI) of 689.5 (594.4, 794.3) pupae, whereas one tray of liquid diet with synthetic sponge and carpet felt produced an average of 266.0 (208.7, 332.9) and 43.7 (23.2, 73.9) pupae, respectively. Pupal weight and adult emergence of flies reared on the solid diet were atypically low probably due to the type of rearing tray used, whereas pupal weight and adult emergence on liquid diet were equal to, or exceeded 20 mg and a proportion of 0.93, respectively. The highest proportion of adults capable of flight was observed in the liquid diet with carpet felt. The potential causes by which larval density and support substrate affected productivity and quality of *A. ludens* reared on liquid diet are discussed.

**Key words:** artificial diet, mass rearing, sterile insect technique, Tephritidae, quality control parameter

The Mexican fruit fly, *Anastrepha ludens* (Loew) (Diptera: Tephritidae), is a major fruit fly pest distributed in southern United States, Mexico, and Central America (Aluja 1993, Hernández-Ortíz and Aluja 1993). Larvae of *A. ludens* feed on the fruit of more than 38 wild and cultivated plant species including white sapote (*Casimiroa edulis* [La Llave & Lex] [Sapindales: Rutaceae]), citrus (*Citrus* spp. [Sapindales: Rutaceae]), and mango (*Mangifera indica* L. [Sapindales: Anacardiaceae]), among others (Aluja et al. 2000, Birke et al. 2013). Novel host fruit, including apples (*Malus* × *domestica* Borkh [Rosales: Rosaceae]), are also starting to be attacked under current climate change conditions (Birke et al. 2013, Aluja et al. 2014). Direct losses (i.e., infested fruit) caused by *A. ludens* infestation can be above 30% of annual yields in the case of citrus (Orozco-Dávila et al. 2017).

*A. ludens* is mass reared as part of ongoing sterile insect technique (SIT)–based programs (Gutiérrez et al. 2013). Mass production of sterile flies in the Moscafrut facility of the National Campaign against Fruit Flies SENASICA-SAGARPA Mexico, involves the use of artificial diets which commonly contain corncob fractions as a readily available texturizing and bulking agent (Domínguez et al. 2010, Orozco-Dávila et al. 2017). However, corncob, an agricultural byproduct, is often contaminated by mycotoxins that inhibit larval development and cause a high prevalence of larval mortality (Aceituno-Medina et al. 2016). Therefore, the development of a diet that does not require corncob texturizing and bulking agents could avoid the serious insect production issues associated with this diet component.

Liquid larval diets are an alternative that has been explored for species in the genera *Ceratitis*, *Bactrocera*, *Dacus*, and *Anastrepha* (Chang 2009), but with one exception (Hernández et al. 2010), they have not been studied for the production of *A. ludens*. The liquid diet–rearing system requires an inert solid substrate to act as a support medium for developing larvae (Chang et al. 2004, Chang 2009). Supporting materials for liquid diets should be lightweight, highly water absorbent, easy to clean, and reusable (Chang et al. 2004). The use of locally available low-cost supporting materials is highly desirable for rearing fruit flies on liquid diet (Cáceres et al 2014, Resilva et al. 2014, Vera et al. 2014). Supporting materials that have been tested include sponge cloth, cotton fabrics, and all-purpose rag, among others (Chang et al. 2004, Resilva et al. 2014).

The larval developmental environment can strongly affect the principal phenotypic traits considered as quality indicators of insects used in SIT programs (Calkins and Parker 2005). Hence, the determination of the conditions required for the production of high-quality insects for SIT requires an evaluation of key variables of the rearing system, including larval densities in artificial diet (Cohen 2018), and different types of supporting materials for larvae in the case of liquid diets (Vera et al. 2014, Resilva et al. 2014). Previous studies on alternative diets for *A. ludens* (Hernández et al. 2010) had not considered the role of larval density and rearing substrate on insect production. Therefore, in the present study, we examined established quality indicators of *A. ludens* reared on a liquid artificial diet based on a standard formulation lacking corncob fractions as a function of larval density. We further examined the influence of two support materials on larvae feeding on a liquid diet in comparison with insects reared on a standard solid diet inoculated with the same number of *A. ludens* eggs.

## Materials and Methods

### *A. ludens* Colony

Flies were obtained from a laboratory colony of *A. ludens* kept at the Red de Manejo Biorracional de Plagas y Vectores (RMBPV) of the Instituto de Ecología, A.C., in Veracruz, Mexico. This colony had been maintained on artificial diet for over 120 generations with occasional introductions of wild flies from citrus fruit to maintain genetic variation (Pascacio-Villafán et al. 2016). Details on the rearing process of *A. ludens* at the RMBPV can be found in Aluja et al. (2009).

### Artificial Diets

Diets were based on a standard formulation for artificial rearing of *A. ludens* (Domínguez et al. 2010, Pascacio-Villafán et al. 2015). The solid diet and the liquid diet differed in that the solid diet had corncob fractions and the liquid diet lacked this ingredient and had 19% (wt/wt) more water than the solid diet (Table 1). Our liquid diet excluded corncob fractions from the solid formulation (Table 1) because this ingredient may contain mycotoxins that inhibit the development of *A. ludens* larvae (Aceituno-Medina et al. 2016). Apart from corncob fractions and water, we did not remove or modify the quantities of any other ingredients in the diet because we did not want to alter further a formulation that is effective for rearing of *A. ludens* (Pascacio-Villafán et al 2015). Our liquid diet differed from one previously tested by Hernández et al. (2010) that comprised 4.2% more water and lacked guar gum and corn flour. To prepare 150 ml of the liquid diet, all solid ingredients were weighed on a digital scale (Sartorius CP64). All ingredients were placed in a glass jar with a screw cap, shaken briefly until no lumps were observed and the resulting suspension was stirred for 35 s in a domestic blender (Magic Bullet).

**Table 1.** The artificial diet formulations tested, costs of diet ingredients, and support materials used in liquid diet

| Ingredient | Solid diet (% by weight) | Liquid diet (% by weight)[a] |
|---|---|---|
| Yeast[b] | 6.0 | 6.0 |
| Sugar[c] | 8.2 | 8.2 |
| Corn flour[d] | 5.3 | 5.3 |
| Corncob fractions[e] | 19.0 | – |
| Guar gum[f] | 0.1 | 0.1 |
| Citric acid[g] | 0.44 | 0.44 |
| Sodium benzoate[b] | 0.4 | 0.4 |
| Nipagin[i] | 0.1 | 0.1 |
| Water | 60.46 | 79.46 |

[a]Liquid diet used either synthetic sponge Scotch Brite (a package with three 20- × 18-cm sponges cost US$1.7), or carpet felt (1 $m^2$ cost US$3.5) as support material.
[b]Lallemand Mexico SA de CV (1 kg cost US$5.1).
[c]Ingenio de Huixtla SA de CV Mexico (1 kg cost US$0.78).
[d]Maíz Industrializado del Sureste, Arriaga, Chiapas, Mexico (1 kg cost US$0.46).
[e]Mt. Corncob fractions 100 Pulaski, Chicago, (1 kg cost US$0.82).
[f]Tic Gums, Belcamp, MD, USA (1 kg cost US$5.9).
[g]Cava Nutrielimentos SA de CV, Mexico (1 kg cost US$1.4).
[h]Joaquín Lambaren Valencia, Mexico (1 kg cost US$3.2).
[i]Joaquín Lambaren Valencia, Mexico (1 kg cost US$9.4).
Exchange rate at the time of writing was US$1 = $18.8 Mexican pesos.

To prepare 150 g of the solid diet (Table 1), all diet ingredients except water, sodium benzoate, nipagin, and citric acid were mixed by hand for 2 min in a plastic tray; then, water, and preservatives and citric acid diluted in 25% of the total volume of water were added to the tray and mixed with the other ingredients for additional 3 min.

### Supporting Materials

As support materials for larvae in liquid diet, we tested a synthetic sponge (Scotch Brite) made from regenerated cellulose (70%) and cotton (30%) with an absorption capacity of 10 times its own weight as informed by the supplier (Experiments 1 and 2), or locally available carpet felt, a textile material made from wool and synthetic fibers (Experiment 2). The synthetic sponge is a material previously used in liquid diets and carpet felt was tested as an alternative support medium for larvae because it is a locally available low-cost absorbent material that is used in rearing facilities to provide water to adult flies. The synthetic sponge and carpet felt were washed thoroughly with tap water and left to dry for 48 h prior to use.

### Experiment 1—Influence of Larval Density in Liquid Diet on Fly Quality

The influence of larval density (1–5 larvae per ml of diet) on established quality control parameters for tephritid rearing was examined using the liquid diet. The highest larval density (5 larvae per ml of diet) was higher than the density of 3.8–4.8 larvae per g of diet used for mass rearing of *A. ludens* at the Moscafrut facilities (DGSV-DMF 2009). Densities 1 and 5 larvae per ml of diet (i.e., extreme points) were replicated eight times, densities 2 and 4 larvae per ml of diet were replicated two times, and density 3 (i.e., center point) was replicated five times. The experimental unit was a plastic tray (22-cm long by 12-cm wide by 3.5-cm tall with a dome lid) containing 150 ml of diet and a rectangular piece of synthetic sponge (20-cm long by 7-cm wide) soaked in the diet. Depending on the larval density evaluated (1–5 larvae per ml of diet), between 150 and 750 neonate *A. ludens* larvae were placed on the ridged side of a rectangular piece (6 × 4.5 cm) of moistened synthetic sponge, which was then placed into the sponges soaked with diet in each tray, in such a way that the ridges of the sponges were at 90° to one another (Supplementary Table S1). These pieces of sponge remained for 5 d in the diet and then were removed. The lids of the plastic trays were placed onto trays to reduce evaporation but were not sealed tightly. The entire experiment was performed in one block and consisted of 25 experimental units (Supplementary Table S1).

Following standard procedures established for *A. ludens* rearing (Aluja et al. 2009), plastic trays with diet and larvae were incubated for 10 d in a dark room at 30 ± 1°C and 70 ± 5% relative humidity (RH). Then, all larvae were recovered by gently washing the trays

and sponges with tap water through a plastic strainer (18 cm in diameter) with nylon mesh (1 mm). Recovered larvae were counted and placed in a plastic cup (7 cm in diameter by 6 cm in height) with vermiculite. The container was closed with a lid that had a 5-cm-diameter hole covered with organdy cloth, and placed in a laboratory at 22 ± 1°C, 70 ± 5% RH, and a photoperiod of 12:12 (L:D) h to promote pupation. After pupation, the pupae were recovered daily over a 3-d period. Recovered pupae were moved to a laboratory at 26 ± 1°C, 60 ± 5% RH, and a photoperiod of 12:12 (L:D) h. A randomly selected sample of thirty 3-d old pupae were weighed individually on an analytical balance (Sartorious CP64). Samples of pupae (range of 7 to 100 pupae) from each diet were placed inside black PVC cylinders (9-cm diameter by 10 cm in height) coated with unscented talcum powder on the interior to prevent adult flies from walking up the sides of the cylinder. A cardboard ring (6-cm diameter) was placed on the bottom of the cylinder surrounding the pupae. Cylinders with pupae were placed in a 90-cm-long- by 100-cm-wide- by 90-cm-tall-mesh cage that had four sticky traps and two plastic bottle traps baited with 300 ml of grape soft drink (Sangría Casera) hanging from the ceiling of the cage. Adults were allowed to emerge and fly out of the tubes. Flies that escaped from the tubes and were not captured in traps were removed manually twice a day. At the end of emergence, the remaining contents of the cylinders were counted and categorized as follows: empty puparia (i.e., emerged individuals), dead adults (i.e., flightless individuals), and partially emerged adults.

Another group of 30 pupae from each diet was used to obtain adults. Four single male and female pairs of newly emerged adults from each diet were placed inside plastic cages (16-cm long by 7-cm wide by 11.5-cm tall) and provided ad libitum access to water and food (a 3:1 mixture of sugar:hydrolyzed protein). Eight days after emergence, when flies had reached sexual maturity (Aluja et al. 2009), a spherical oviposition device (4-cm diameter by 5-cm height, 50-ml capacity) made of green linen cloth with a silicon cover and a screw cap was hung from the ceiling of the cage (Supplementary Fig. S1). The oviposition device was filled with 40 ml of a 0.2% (wt/vol) sodium benzoate solution. The oviposition devices were exposed for 7 d to pairs of flies from each diet. On a daily basis, the content of individual spheres was poured over a piece of black cloth on top of moistened cotton inside an 8.5-cm-diameter plastic Petri dish. Eggs were counted and incubated in a dark room at 30 ± 1°C and 70 ± 5% RH. After 5 d of incubation, egg hatch was recorded.

### Response Variables

The response variables examined were based on quality control parameters established for tephritid flies (FAO/IAEA/USDA 2014): 1) pupation, expressed as the proportion of larvae that pupated in relation to the total number of larvae placed in each diet; 2) mean pupal weight (mg), measured from thirty 3-d old pupae; 3) adult emergence, expressed as the proportion of adults that emerged from samples of pupae (range of 7 to 100 pupae per replicate) placed in black PVC tubes; 4) flight ability, expressed as the proportion of emerged adults that flew out of PVC tubes; 5) eggs laid, expressed as the mean number of eggs a female produced during a 7-d period; and 6) egg hatch, expressed as the proportion of eggs hatched from the total number of eggs produced by each female during the 7-d period.

## Experiment 2—Comparison of a Solid Diet and a Liquid Diet With Two Types of Support Substrate

The explanatory variable was artificial diet with three levels: 1) liquid with synthetic sponge, 2) liquid with carpet felt, and 3) solid diet. The synthetic sponge was the same as that used in Experiment 1. The experimental units consisted of plastic trays as described in Experiment 1, with either 150 g of solid diet or 150 ml of liquid diet with a piece of synthetic sponge or carpet felt as support substrate (Table 1). As in the case of the trays with the solid diet, the lid had a rectangular opening (6 by 7 cm) covered with organdy cloth for ventilation. All diets were inoculated with an average (±SE) of 1,408 ± 44 eggs (equivalent to 100 µl of eggs), mean hatch = 72.1% (average of 12 samples analyzed). Four days prior to diet preparation, eggs were placed into pieces of moistened sponge (6 × 4.5 cm) inside Petri dishes and incubated in a dark room at 30 ± 1°C and 70 ± 5% RH. On the day of diet preparation, these pieces of sponge were placed in contact with the sponge soaked with diet as described previously. The lid of the plastic trays was not sealed. Plastic trays with diet and larvae were incubated for 10 d as described in Experiment 1. After this period, larvae from each diet were recovered by washing the trays and sponges through a plastic strainer. Insects that were observed to have pupated prior to separation from the diet were counted and considered in the analysis of number of pupae recovered (Supplementary Table S2). However, because we did not know the exact day of pupation and because the pupation environment was different from the rest of the larvae that pupated later, these pupae were ignored in the other analyses. Larvae recovered from diets were placed in plastic containers with vermiculite at 22 ± 1°C, 70 ± 5% RH, and a photoperiod of 12:12 (L:D) h to promote pupation. Pupae were recovered 24 h later, following standard rearing procedures and moved to a laboratory at 26 ± 1°C, 60 ± 5% RH, and a photoperiod of 12:12 (L:D) h. A sample of 10 randomly selected 3-d-old pupae from each diet were weighed individually and samples of pupae (range of 3 to 100 pupae per replicate) from each diet were placed inside black PVC cylinders and treated in the same way as mentioned in Experiment 1. Each diet was replicated eight times for a total of 24 experimental units. Diet treatments were randomized, and the experiment was performed in one block.

### Response Variables

The following response variables were measured: 1) number of pupae recovered, 2) mean pupal weight (mg), 3) adult emergence, and 4) flight ability, as described in the previous experiment.

### Statistical Analyses

The nature of the explanatory (continuous in Experiment 1 and categorical in Experiment 2) and response variables (proportions, counts, and continuous variables) make Generalized Linear Models (GLMs) appropriate for the analyses.

In Experiment 1, pupation, adult emergence, flight ability, and egg hatch (i.e., proportional response variables) were modeled with a quasi-binomial error distribution and identity link function to account for overdispersion (Buckley 2015). Pupal weight was modeled with a Gaussian error distribution and identity link function. The mean number of eggs/female/day was modeled using a gamma error distribution and inverse link function.

In Experiment 2, the number of pupae recovered was modeled with a quasi-Poisson error distribution and log link function to account for overdispersion; pupal weight was modeled with a Gaussian error distribution and identity link function; and adult emergence and proportion of flight-capable insects were modeled with a quasi-binomial error distribution and identity link function to account for overdispersion.

The significance of changes in model deviance following fit to response variables was determined by a likelihood ratio test based

on a $\chi^2$ distribution for Gaussian and gamma models, and an $F$ distribution for quasi-binomial models (Buckley 2015). In Experiment 2, when significant effects were detected, pairwise comparisons based on the linear predictor of the model were used to test for differences among diet levels. The suitability of statistical models was checked graphically by examination of the distribution of model residuals.

All analyses were performed in R (R Core Team 2017) using the packages gtools (Warnes et al. 2015a) and gmodels (Warnes et al. 2015b). Details on data modeling, the R scripts to fit GLMs and create the figures (Supplementary Text S1), and the databases of Experiment 1 (Supplementary Table S1) and Experiment 2 (Supplementary Table S2) are presented in Supplementary Material.

## Results

### Experiment 1—Influence of Larval Density in Liquid Diet on Fly Quality

Pupation was not significantly affected by larval density ($F$ = 2.52; df = 1, 23; $P$ = 0.126) and averaged a proportion of 0.50 (95% CI: 0.44, 0.57) across all larval densities (Fig. 1A). Pupal weight decreased significantly with increasing larval density ($\chi^2$ = 16.32; df = 1; $P$ = 0.0013; Fig. 1B). Adult emergence increased significantly as the larval density increased ($F$ = 40.89; df = 1, 23; $P$ < 0.001; Fig. 1C). Flight ability was not significantly influenced by larval density ($F$ = 2.75; df = 1, 23; $P$ = 0.1109), and an overall mean proportion of 0.89 (95% CI: 0.87, 0.92) provided the best description of adult flight capacity across all larval density treatments (Fig. 1D). Larval density had no significant effect on the number of eggs laid by females ($\chi^2$ = 0.0013; df = 1; $P$ = 0.8988), which averaged 45.56 eggs per female per day (95% CI: 40.84, 51.04) across all larval densities (Fig. 1E). Egg hatch was not significantly affected by larval density ($F$ = 0.135; df = 1, 23; $P$ = 0.7167), and an overall mean proportion of 0.86 (95% CI: 0.83, 0.89) provided the best description of the data across all larval densities (Fig. 1F).

### Experiment 2—Comparison of a Solid Diet and a Liquid Diet With Two Types of Support Substrate

The number of pupae recovered differed significantly among diets ($F$ = 93.72; df = 2, 21; $P$ < 0.0001). More pupae were recovered from the solid diet than from liquid diet, and the number of pupae recovered from the liquid diet with a synthetic sponge was significantly higher than from the liquid diet with carpet felt (Fig. 2A). Pupal weight differed significantly among diets ($\chi^2$ = 230.11; df= 2; $P$ < 0.0001). Pupae from liquid diet with synthetic sponge or carpet felt were significantly heavier than pupae from the solid diet, by approximately 33–35% (Fig. 2B). A higher proportion of adults emerged from liquid diet with either type of substrate than from the solid diet ($F$ = 166.19; df = 2, 19; $P$ < 0.0001; Fig. 2C). Flight ability differed significantly between liquid diet with synthetic sponge and carpet felt ($F$ = 3.9; df = 2, 19; $P$ = 0.0376). A higher proportion of flies reared on liquid diet with carpet felt flew out of PVC cylinders compared with flies reared on liquid diet with sponge, but neither of the liquid diet + substrate treatments differed significantly from the solid diet (Fig. 2D).

## Discussion

This study extends previous research on the suitability of liquid diet for mass-rearing of *A. ludens* (Hernández et al. 2010), by examining the influence of larval density and support substrate as two

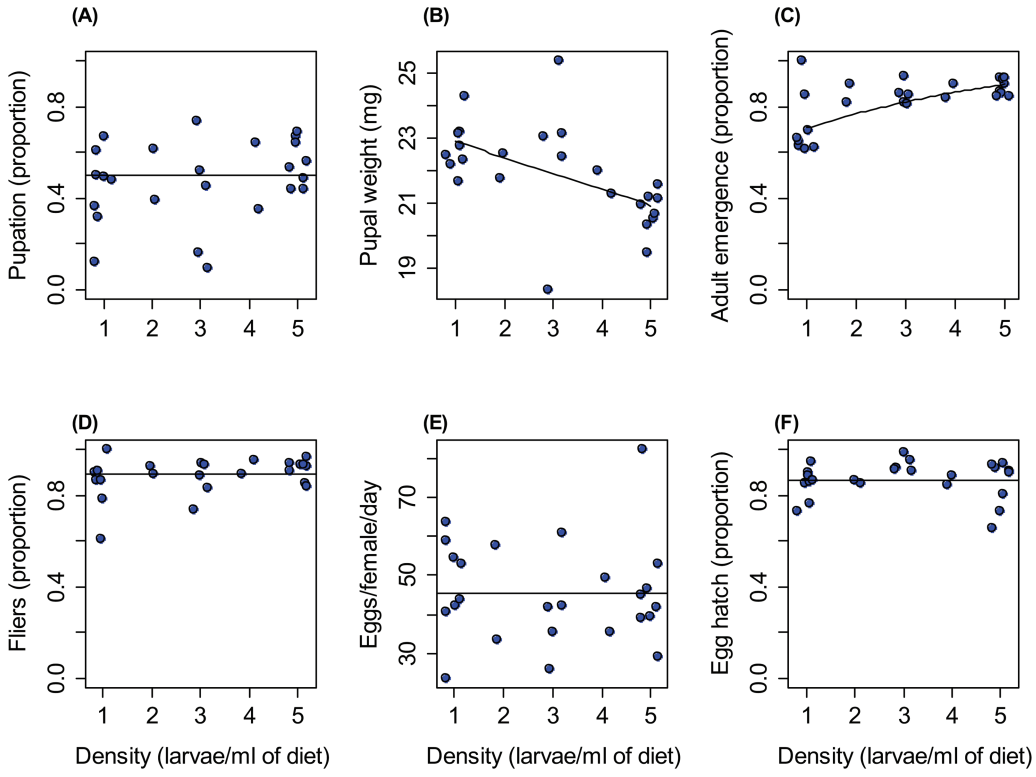


**Fig. 1.** Quality indicators of *A. ludens* reared on a liquid artificial diet with synthetic sponge as support material as a function of larval density (larvae per ml of diet). (A) Pupation (proportion), (B) pupal weight (mg), (C) adult emergence (proportion), (D) fliers (proportion), (E) eggs per female per day, and (F) egg hatch (proportion). The data points were jittered for graphical clarity. The line represents the fitted model. See Results and Supplementary Material for statistical details. Color figure online.

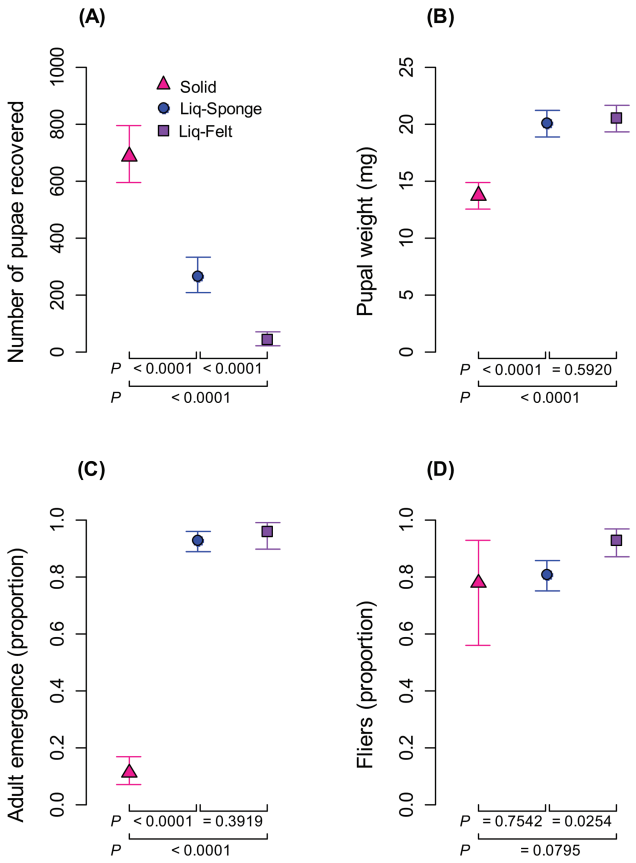


**Fig. 2.** Mean of (A) number of pupae recovered, (B) pupal weight (mg), (C) adult emergence (proportion), and (D) adult flight capacity (proportion) of *A. ludens* reared on a solid diet (Solid) or a liquid diet with synthetic sponge (Liq-Sponge) or carpet felt (Liq-Felt) as support material for feeding larvae. The *P* values of *t*-test contrasts among diets are shown at the bottom of each figure. Error bars indicate 95% CI. See Results and Supplementary Material for statistical details. Color figure online.

critical variables that can affect the outcome of rearing this fruit fly on liquid diet. One difference of our liquid diet was that it contained corn flour, which has amino acids and vitamins that could contribute to the development and reproduction of *A. ludens* (Rivera et al. 2007), although it remains to be determined whether corn flour is necessary in the liquid diet formulation for continuous rearing of *A. ludens*. Overall, we obtained encouraging results in the liquid diet we tested indicating that, compared with the solid diet, the mean weight of pupae and prevalence of adult emergence were significantly higher following rearing in the liquid diet. The latter, despite the fact that our insect colony had never been exposed to a liquid rearing medium. This represents an opportunity for the removal of corncob fractions as a bulking agent, since despite their availability and long-standing use in *A. ludens* diet, these fractions are frequently contaminated with mycotoxins (Aceituno-Medina et al. 2016). The results of this study are similar to those of previous studies in that the liquid diet does not equal or exceed the yields of pupae or adult insects obtained with the solid diet (Fay and Wornoayporn 2002, Chang et al. 2004, Chang et al. 2006), indicating that additional refinements of the liquid diet and the rearing system are required to improve overall yields of insects.

The results from Experiment 1 showed that pupal weight and adult emergence of *A. ludens* reared on liquid diet were significantly affected by larval rearing density. Indeed, variation in the larval rearing density could affect growth and development of dipterans through several mechanisms including: 1) direct competition among larvae for food, 2) increased energetic expenditure owing to a greater frequency of conspecific interactions, 3) by exposure to potentially toxic waste products from nearby larvae, or 4) by variation in the generation of metabolic heat (Calkins and Parker 2005, Wu et al. 2011, Jannat and Roitberg 2013). In our study, reduced pupal weights at higher larval densities were likely the result of a limited availability of nutrients due to food competition among larvae (Fig. 1B). In fact, when we recovered larvae from trays with diet, we observed that, in general, there were fewer traces of uneaten diet in the trays that had high larval densities compared with the trays with low larval densities. If high larval densities had affected larval nutrition in our study, we would have expected a reduction in the proportion of emerged adults with increased larval density because larval nutrition is positively correlated with adult emergence (Calkins and Parker 2005, Orozco-Dávila et al. 2017). However, this was not the case, and instead, the proportion of emerged adults increased as a function of larval density in the liquid diet (Fig. 1C). The cause of this unexpected finding is not clear, but it is possible that larvae in the low densities consumed an excess of energetic resources and accumulated large amounts of fat, which reduced their ability to emerge as adults (Warbrick-Smith et al. 2006). These results suggest a potential trade-off between pupal weight and adult emergence mediated by larval density in the diet. This is important to consider in a mass rearing context where both pupal weight and adult emergence are robust quality indicators of artificially reared tephritid flies (Fanson et al. 2014, Orozco-Dávila et al. 2017).

Our results contrast with those of a study on the Oriental fruit fly, *Bactrocera dorsalis* Hendel, which indicated that pupal weight and adult emergence were not influenced by larval density in a liquid artificial diet (Chang et al. 2006). These divergent results may reflect different strategies for the acquisition and allocation of resources across the immature and adult stages of these species (Liendo et al. 2016; Nestel et al. 2016). It may also be that pupal weight and adult emergence are the parameters that are most sensitive to variations in developmental conditions in *A. ludens*. Testing higher larval densities than those considered in this study, and measuring nutrient allocation in immature and adult stages, should help us to clarify this.

In Experiment 2, although we did not quantify the number of larvae recovered at the moment of larval separation from diets, we noted that liquid diets had fewer larvae than the solid diet. Accordingly, the number of pupae recovered from the solid diet was about threefold and tenfold higher than the number of pupae recovered from the liquid diet with synthetic sponge and carpet felt, respectively (Fig. 2A). A possible explanation for the low productivity in the liquid diets is that a high proportion of eggs and larvae died immediately after eclosion by suffocation. A reduction in the volume of diet in rearing trays (in this study 150 ml of diet was about 40% of the capacity of the rearing trays) might improve immature survival and pupal recovery. Determination of the optimal relationship between diet volume and larval density would improve survival and result in complete exploitation of dietary resources (Chang 2009), making it easier to implement the liquid diet rearing system for mass production of *A. ludens*. Other methods of introducing eggs or neonate larvae to the diet such as suspending them in gels may also improve survival. In fact, a previous study in which *A. ludens* eggs and larvae were placed in a guar gum solution that was poured directly on to the sponge in a liquid diet reported an average larval recovery above 70% and over 90% pupation of larvae (Hernández et al. 2010).

The low values obtained with the solid diet for pupal weight, adult emergence, and the prevalence of flight-capable adults in Experiment 2 are not typical of the values usually obtained with this type of diet (Figs. 2B–D) (Pascacio-Villafán et al. 2015). We noticed that even when the lids of the trays with solid diet had an opening for ventilation, most of the trays had moisture condensation on the top and sides of the lids. This indicates that insects in the trays with solid diet were exposed to excessive heat and humidity, which may have caused cellular damage (Caro-Corrales et al. 2015) that resulted in reduced survival to adulthood and a reduction in the proportion of flight-capable adults.

The liquid diet with a synthetic sponge substrate produced ca. sixfold more insects than the liquid diet with carpet felt (Fig. 2A), whereas the mean proportion of flying adults reared from larvae on the liquid diet with synthetic sponge was 0.12 lower than in the liquid diet with carpet felt (Fig. 2D). These results may reflect differences in the water absorption capacity, structure, and texture of the synthetic sponge and carpet felt which may have affected larval movement through the diet and have limited their access to certain nutrients (Fay and Wornoayporn 2002). We consider that the synthetic sponge is a better material than carpet felt as a support substrate in liquid diet for rearing of *A. ludens*, and that the slight reduction in the proportion of fliers reared from the synthetic sponge in liquid diet might be overcome by the addition of ingredients that promote flight ability, such as certain vitamins or fatty acids (Chang and Vargas 2007).

A piece of sponge for one tray of liquid diet had a cost of US$0.28, whereas the amount of corncob fractions for one tray of solid diet (28.5 g) had a cost of US$0.02. As such, one tray of liquid diet for rearing of *A. ludens* was US$0.26 more expensive than a tray with the standard solid diet. It would be necessary to use a single sponge more than 10 times so that the cost of the liquid diet we used could be comparable to the cost of the standard solid diet. Nevertheless, because the liquid diet could avoid the insect production issues associated with contaminants in corncob fractions, the potential benefits of using a liquid diet for rearing of *A. ludens* could outweigh its cost. To reduce the costs, however, future research should examine how many times a single sponge or another support substrate can be reused (Vera et al. 2014). Reducing the size of the sponge could also help to reduce costs.

Liquid diets have advantages over solid diets for rearing of fruit flies including savings in labor and storage space, waste reduction, and the elimination of contaminants in bulking agents which can inhibit larval development (Chang et al. 2004, 2006; Cáceres et al. 2014), and the liquid diet with synthetic sponge we tested could have potential applications in the mass rearing of *A. ludens*. Liquid diets also have disadvantages that could limit their use for large-scale rearing including yeast fermentation, additional management for water recovery, and the separation of diet components that could limit the access of early stage larvae to particular nutrients (Moadeli et al. 2017). To overcome these complications and eliminate the risk of high levels of larval mortality probably due to the reduced oxygen conditions of liquid diets, and to reduce the cost associated with the use of a solid substrate, the addition of gelling agents to the liquid diet could also be explored as gel-based diets are increasingly attracting the attention of researchers for the mass production of tephritids with promising initial results (Rivera et al. 2007, 2012; Moadeli et al. 2017, 2018).

This study contributes to the continuous improvement of protocols for artificial rearing of *A. ludens* on liquid diet. We conclude that larval density and support substrate affect the quantity and quality of *A. ludens* reared on liquid diet probably by regulating larval nutrition and larval movement trough the diet, although further research will be required to determine the mechanism by which density and substrate affect pupal weight, adult emergence, flight ability, and the overall yields of insects. Further research will also be required to determine the optimal larval density of *A. ludens* reared on a liquid artificial diet that maximizes pupal weight, which is positively correlated with sexual performance of males, without compromising adult emergence, which should be as high as possible to maximize the number of sterile males that can be released in area-wide management programs.

## Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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