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Apilactobacillus intestinapis: A bacterium with potential benefits in mass-reared Anastrepha obliqua (Diptera: Tephritidae) for SIT purposes

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Abstract

Provisioning mass-reared *Anastrepha obliqua* with gut bacteria from wild flies may be a strategy to reduce the difference in sexual competitiveness between wild and mass-reared and irradiated flies under a Sterile Insect Technique (SIT) scenario. However, in the case of *A. obliqua*, as in other tephritid fruit flies, the bacteria that have been identified in the digestive tract of wild specimens are mainly enterobacteria with potential pathogenic effects in humans. Thus, given that the family *Lactobacillae* is considered safe to humans, we aimed to determine the effect of consuming *Apilactobacillus intestinapis* on the biological attributes of *A. obliqua* adults. We performed tests of sexual competitiveness and survival under starvation with mass-reared flies fed with a sugar diet, standard diet (3:1 sugar: protein), sugar + *A. intestinapis*, or standard diet + *A. intestinapis.* Consuming *A. intestinapis* improved the sexual competitiveness of *A. obliqua* males fed with sugar. We did not find statistical differences between the treatments of standard diet, standard diet + *A. intestinapis*, and sugar diet + *A. intestinapis* in the sexual competitiveness tests, while males fed only with sugar were the least favored.

Females and males of *A. obliqua* fed with the sugar diets showed increased survival compared to flies fed with the standard diet. The addition of *A. intestinapis* resulted in an increased life expectancy of females and males compared to the treatments without the bacterium. Our results suggest that *A. intestinapis* may be a bacterium with potential benefits for sterile males of *A. obliqua*.

Key Message

- Wild *Anastrepha obliqua* gut bacteria, identified as beneficial for these flies, are potentially pathogenic to humans.
- *Apilactobacillus intestinapis* is a safe alternative to enterobacteria in the gut microbiota of *A. obliqua*.
- We observed that *A. intestinapis* improves the sexual competitiveness of *A. obliqua* males fed with sugar.
- Diets containing *A. intestinapis* improve the survival of *A. obliqua* under fasting conditions.
- *A. intestinapis* has the potential to optimize mass rearing of *A. obliqua* in SIT control programs.

Introduction

The study of the interactions between microorganisms and tephritid flies is currently of great importance, especially for the mass-rearing of flies for the application of the sterile insect technique (SIT) (Deutscher et al. 2019; Cáceres et al. 2019). At present, there is evidence that the gut microbiota of wild *Anastrepha obliqua* differs from that of mass-reared *A. obliqua* (Ventura et al. 2018; Roque-Romero et al. 2022). Given the importance of gut bacteria for health, performance, and sexual competitiveness (Hamden et al. 2013; Roque-Romero et al. 2020; Kyritsis et al. 2019; Deutscher et al. 2019), provisioning mass-reared males with gut bacteria from wild males may be a strategy to reduce the difference in sexual competitiveness between wild and mass-reared flies (Rull et al. 2012; Gallardo-Ortiz et al. 2018a).

However, in the case of *A. obliqua*, the bacteria that have been detected in its digestive tract are mainly enterobacteria that are potentially pathogenic to humans (Ventura et al. 2018; Gallo-Franco and Toro-Perea 2020; Muhondwa 2020; Roque-Romero et al. 2022). This limits the use of such bacteria to enrich diets in a mass-rearing setting as personnel in charge of preparing and providing the diet as well as handling the insects could be at risk of being infected (Rock and Donnenberg 2014). The implementation of required additional biosafety measures may increase the costs of production and release of sterile flies.

Nevertheless, the potential of microorganism to improve the cost-quality ratio of insect diets and enhance the sexual competitiveness and performance of sterile insects remains largely unexplored. A wide range of bacteria that are not pathogenic to humans, although not directly associated with fruit flies, could be explored as potential performance enhancers for mass-reared flies. In the present study, we investigated the effects of *Apilactobacillus intestinapis* (formerly classified as *Lactobacillus kunkeei*), a lactic acid bacterium isolated from the gut of *Apis mellifera*. Previous studies of *Apilactobacillus* species associated with honeybees have reported tolerance to conditions encountered in the bee gut, high cell-surface hydrophobicity, co-aggregation with pathogenic bacteria, biofilm-forming capacity, and inhibition of pathogen adhesion. These characteristics are among the criteria commonly used to identify lactic acid bacteria with potential probiotic properties (Endo et al. 2012; Pachla et al. 2021; Rodriguez-Diaz et al. 2021). In addition, *Apilactobacillus kunkeei* has been investigated for its potential probiotic applications in humans (Asama et al. 2015; Asama et al. 2016). However, these findings cannot be directly extrapolated to *A. intestinapis*. Considering its origin from the gut of *A. mellifera* and the reported beneficial properties of closely related *Apilactobacillus* species, we aimed to determine whether consumption of *A. intestinapis* affects the biological attributes of *A. obliqua* adults.

Materials and Methods

Insects origin

We obtained wild *A. obliqua* in the larval stage from infested creole mango (*Mangifera indica* L.) fruits collected in orchards in Tapachula, Chiapas, Mexico (14°54'35.2"N, 92°12'29.3"W). The collected fruits were transported to the laboratory in El Colegio de la Frontera Sur (ECOSUR), where they were kept under environmental conditions of 26 ± 2°C, 70% ± 10 RH, and a 12:12 light:dark photoperiod for eight days to allow for larval full development. We then removed the larvae from the fruits and transferred them to containers with moist (60%) vermiculite to promote pupation. After 13 days, we separated the pupae from the vermiculite with a sieve and transferred them to Plexiglass cages (30 × 30 × 30 cm) for adult emergence.

Mass-reared flies in the pupal stage were provided by the Moscafrut facility in Metapa de Domínguez (Chiapas, Mexico), where they are produced following the methodology described by Orozco-Dávila et al. (2017). Two days before adult emergence, the pupae were subjected to gamma irradiation with cobalt^{60}

at a dose of 80 Gy using a Gamma Beam 127 irradiator (FAO/IAE/USDA 2019). Two days after adult emergence the wild and mass-reared sterile adults, we sorted by sex to prevent them from mating.

Wild flies and sterile males were kept in Plexiglass cages (30 × 30 × 30 cm) at a density of 20 ± 5 flies per cage under the environmental conditions described above. Water and food were provided to the wild females and to the males according to the corresponding treatment (see below).

Bacterial strain origin

The bacterial strain used in this study was isolated from the gut of *Apis mellifera* by Rodas-Hilerio (2021) and was initially identified as *Lactobacillus sp*. based on biochemical characterization.

Genomic DNA isolation and molecular identification of the bacterial strain

Genomic DNA was extracted from six 10-ml bacterial cultures using the AquaPure Genomic DNA Isolation Kit (Bio-Rad, CA, USA; Cat. No. 732–6340). DNA was resuspended in 50 µl of DNA Hydration Solution, and its integrity was assessed by electrophoresis on a 1% agarose gel.

The DNA samples were diluted 1:20 in DNA Hydration Solution, and 1 µl of each dilution was used as template for PCR amplification of the 16S rRNA gene with primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3'). These primers amplify an approximately 1,500-bp fragment encompassing nine hypervariable regions of the 16S rRNA gene according to the *Escherichia coli* numbering system (Chakravorty et al. 2007).

PCR reactions were performed in a final volume of 20 µl using Promega PCR Master Mix 2X (Cat. No. M7502, Wisconsin, USA) and 0.2 µl of each primer. The cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 90 s, followed by a final extension at 72°C for 5 min. PCR products were verified by electrophoresis on 1% agarose gels, purified using the GenElute PCR Clean-up Kit (Cat. No. NA1020) and subsequently checked by agarose gel electrophoresis.

The six purified PCR products were submitted to Macrogen Inc. (Seoul, South Korea) for bidirectional Sanger sequencing. Macrogen provided a consensus sequence for each sample. The sequences were evaluated by inspecting the corresponding electropherograms to assess signal quality and sequence reliability and were subsequently compared with sequences in the NCBI nucleotide database using BLAST.

Bacterial growth kinetics

To determine the logarithmic growth phase and establish the incubation time for harvesting viable bacterial cells, a growth curve was constructed. A single colony (approximately 1 mm in diameter) was inoculated into 10 ml of sterile MRS broth (Conda, Cat. No. 1215) adjusted to pH 7. The culture was incubated at 35 ± 2°C with constant shaking at 120 rpm for 48 h. Optical density at 600 nm (OD_{600}) was recorded every 3 h using a spectrophotometer (Thermo Scientific Multiskan GO, User Manual Rev. 1.2, Cat. No. N10588, Finland). At each sampling time, bacterial concentration was estimated by serial dilution of 100 µl of culture. Aliquots from the 10^{-4}, 10^{-5}, and 10^{-6} dilutions were plated in triplicate on MRS agar using the pour-plate method. After 48 h of incubation at 35 ± 2°C, colony-forming units (CFU) were counted. The resulting data were used to construct the growth curve and estimate bacterial concentration throughout the incubation period. The cultures reached the logarithmic growth phase after 24 h of incubation, with an estimated concentration of 4×10^8 CFU ml^{-1} at this time point.

Provisioning flies with the bacterial consortium

Each of the six bacterial isolates was cultured separately in 10 ml of sterile MRS broth under the conditions established from the growth curve (35 ± 2°C, 120 rpm, 24 h). After incubation, each culture was harvested separately by centrifugation at 4,500 rpm for 10 min. The resulting pellets were individually resuspended in 10 ml of sterile 0.8% saline solution. The six suspensions were then pooled to obtain the bacterial consortium (60 ml total). No further adjustment of the bacterial concentration was performed after pooling. The consortium was used to moisten previously autoclaved sterile cotton balls, with 10 ml of the suspension applied to each cotton ball. Each cotton ball was placed in a sterile lidless Petri dish, which served as a feeding station. One feeding station was provided in each cage containing flies assigned to the bacterial treatments, together with water and the diet corresponding to each experimental treatment.

Viability of the bacterial consortium in cotton balls

To verify that *A. obliqua* adults were exposed to viable bacterial cells throughout the feeding period, we evaluated the viability of the bacterial consortium in the cotton balls. Cotton balls moistened with 10 ml of the bacterial suspension were placed in sterile Petri dishes and maintained at room temperature (30 ± 2°C) for 0, 1, 2, 3, and 4 days. At each time point, a cotton ball was aseptically transferred onto MRS agar using sterile forceps, and the plates were incubated at 30 ± 2°C. Bacterial growth was evaluated after 24–48 h. The bacterial growth observed on the plates was examined by optical microscopy to verify that the cells exhibited morphological characteristics consistent with those of the bacterial isolates comprising the consortium. Each evaluation was performed in duplicate and compared with a negative control consisting of cotton balls moistened with 10 ml of sterile saline solution.

Fly diets

Wild flies were provided with a standard adult diet (D) consisting of sugar and hydrolyzed yeast (3:1, w/w) and were used as the wild control (W). Mass-reared sterile males and females received one of four dietary treatments: standard diet (D), sugar only (S), standard diet supplemented with the bacterial consortium (DL), or sugar supplemented with the bacterial consortium (SL). All diets and bacterial treatments were provided from the day of adult emergence. Cotton balls moistened with bacterial suspension were not replaced during the experimental period.

The treatment codes DL and SL were established according to the preliminary biochemical identification of the bacterial isolates as *Lactobacillus sp*.

Tests of survival under starvation

Two days after adult emergence, groups of 25 males and 25 females from the same treatment (D, S, DL, or SL) were placed in Plexiglas cages (30 × 30 × 30 cm) supplied with water and the corresponding diet. Four replicate cages were established for each treatment. An additional cage containing 25 males and 25 females per treatment was maintained to replace individuals that died during the first five days of feeding, before the starvation assays began.

Flies received their respective diets for five days after adult emergence, following the feeding protocol used at the packing and release facility (FAO/IAEA/USDA 2019). Diets were then removed to initiate the starvation assay, while water remained available throughout the experiment. Cages were inspected every 24 h, and dead flies were removed and recorded according to treatment.

Sexual competitiveness

The sexual competitiveness tests were conducted using wild flies 13–15 days after adult emergence and sterile males 8–10 days after emergence, corresponding to sexually mature adults (Aluja 1993). Sexual maturity was confirmed by the presence of the characteristic behavioral traits described for this developmental stage (Tellez-Romero et al 2011; Gomez et al 2024).

Two days before the tests, males were marked on the dorsal thorax with vinyl paint (Vinci de México, S. A. de C. V., Mexico City, Mexico), using a different color for each treatment (Vazquez et al 2024). The assays were conducted in a mango orchard using amber-colored fiberglass mesh cages (3 m in diameter × 2 m in height) supported by a metal frame. A mango tree was placed at the center of each cage to simulate the natural environment in which *A. obliqua* mating behavior occurs.

Each cage received 30 males from each of the 5 treatment (D, DL, S, SL, and W), and 30 wild females. The experiment was performed in triplicate. Males were allowed to acclimate for 30 min and establish calling behavior and lek positions before 30 wild females were introduced. Each cage therefore contained 180 flies (150 males and 30 females), corresponding to a 5:1 male to female ratio. Mating pairs were collected as they formed using 16 × 100 mm glass tubes, and the treatment of each

successful male was recorded. Observations were conducted from 06:30 to 12:00 h, corresponding to the period of highest mating activity in *A. obliqua* (Aluja et al. 2009).

Statistical analysis

Data from the sexual competitiveness tests were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test for pairwise comparisons. Before the analysis, we verified that the data met the assumptions of normality and homogeneity of variances. Survival under starvation was analyzed using Cox proportional hazards regression, followed by Tukey-adjusted multiple comparisons. All statistical analyses were performed in R version 4.1.2 (R Core Team 2022).

Results

Molecular identification of the bacterial strain

All six independently sequenced DNA preparations showed 99.73–99.93% sequence identity and 100% query coverage to *Apilactobacillus intestinapis* strain 1-1-2 (GenBank accession no. NR_201648.1) in BLASTn searches against the NCBI nucleotide database.

Viability of the bacterial strain

Abundant bacterial growth was observed on MRS agar from cotton balls sampled between days 0 and 3, indicating that *A. intestinapis* remained viable throughout this period. On day 4, bacterial growth was still detected but at a lower density. No bacterial growth was observed on plates inoculated with cotton balls moistened only with sterile saline solution (negative control).

Survival under starvation

Sterile males and females fed with sugar (S) or sugar supplemented with *A. intestinapis* (SL) survived significantly longer under starvation than flies fed with the standard diet (D) or the standard diet supplemented with *A. intestinapis* (DL) (Cox proportional hazards model, $P < 0.0001$; Fig. 1A, B). No significant differences in survival were detected between the S and SL treatments or between the D and DL treatments for either sex (Table 1; Fig. 1A, B).

Table 1
Life expectancy (e_x, days) of sterile adults of *A. obliqua* fed with one of the following diets from the day of emergence until day 5: sugar (S), sugar + *A. intestinapis* (SL), standard diet (D), and standard diet + *A. intestinapis* (DL).

Treatment	Male	Female
S	9.38a	8.83a
SL	9.35a	9.13a
D	7.64b	7.66b
DL	8.03b	7.8b

*Values followed by different lowercase letters differ significantly according to Tukey-adjusted pairwise comparisons following the Cox proportional hazards analysis ($P < 0.0001$).

Sexual competitiveness

Wild males achieved significantly more matings than sterile males from all dietary treatments ($P < 0.0001$; Fig. 2). Among sterile males, those fed only with sugar (S) obtained the fewest matings, whereas males fed with the standard diet (D), standard diet supplemented with *A. intestinapis* (DL), and sugar supplemented with *A. intestinapis* (SL) showed intermediate mating success. No significant differences were detected between D and DL or between S and SL. However, males receiving sugar supplemented with *A. intestinapis* exhibited an intermediate level of sexual competitiveness, overlapping statistically with both the S and D treatments (Fig. 2).

Discussion

Females and males fed sugar-based diets survived longer under starvation than flies fed the standard diet, regardless of *A. intestinapis* supplementation. One possible explanation is that the standard diet used in this study consisted of sugar and hydrolyzed yeast (3:1, sugar:protein). Protein from hydrolyzed yeast is required for sexual maturation in tephritid flies, including egg production, gonadal development, pheromone synthesis, replenishment of sperm reserves, and production of male accessory gland secretions (Drew and Yuval 2000). Previous studies have also shown that amino acid intake enhances male mating performance (Pérez-Staples et al. 2007; Liedo et al. 2013). Consequently, flies that completed sexual maturation under the standard diet likely allocated a greater proportion of their energy reserves to reproductive processes, reducing their ability to withstand starvation. In contrast, flies maintained on sugar-only diets, which lacked the protein required for sexual maturation, probably allocated more energy to survival (Kaspi and Yuval 2000; Yuval et al. 2007). This interpretation agrees with the lower mating success observed in sugar-fed males.

Although survival differences between treatments with and without *A. intestinapis* were not statistically significant, both males and females supplemented with the bacterium showed a consistent tendency toward increased survival under starvation. This pattern could result from probiotic activity following gut

colonization or from the nutritional contribution of bacterial metabolites and cell components released during digestion (Hernández-García et al. 2020; Kyritsis et al. 2019). Distinguishing between these mechanisms will require experiments comparing live and inactivated bacterial cultures.

Prolonged survival of sterile males could increase the period during which they remain available to encounter and mate with wild females, potentially improving the efficiency of the sterile insect technique (SIT). Because mass production and release of sterile insects represent a substantial operational cost, extending male longevity could improve the cost-effectiveness of SIT. However, increased survival is advantageous only if older males retain their ability to compete successfully for mates and inseminate females (Yuval et al. 2007). It will also be important to determine whether the survival trend observed under laboratory conditions is maintained under field conditions, where sterile males are exposed to predators, parasitoids (Hendrichs et al. 2007; Dor et al. 2014, 2019), and environmental stressors (Facon et al. 2021). In addition, increased longevity could be advantageous in integrated pest management programs that combine SIT with entomopathogenic fungi. In such strategies, sterile males may serve as vectors for fungal dissemination, and a longer lifespan could increase the likelihood of contacting and contaminating wild females, even when mating attempts are unsuccessful (Flores et al. 2013; Montoya et al. 2020). Although this possibility was not evaluated in the present study, it represents an interesting direction for future research.

Wild females consistently preferred wild males over mass-reared sterile males, a pattern widely reported in validation studies of tephritid SIT programs (Meza-Hernández and Díaz-Fleischer 2006; Telles-Romero et al. 2011; Rull et al. 2012; Liedo et al. 2013; Gallardo-Ortiz et al. 2018b). Among sterile males, those fed only sugar exhibited the lowest mating success, supporting previous reports that protein-deficient diets compromise sexual performance (Shelly et al. 2002; Yuval et al. 2007; Hernández et al. 2010; Ben-Yosef et al. 2014). Although the difference between the S and SL treatments was not statistically significant, males supplemented with *A. intestinapis* achieved a numerically higher mating success than those fed only sugar. This trend suggests a potential beneficial effect of *A. intestinapis* that warrants further investigation. A plausible explanation is that *A. intestinapis* either transiently colonizes the digestive tract and exerts probiotic effects or provides amino acids and other nutrients that partially compensate for the nutritional limitations of a sugar-only diet (Drew and Yuval 2000; Leitão-Gonçalves et al. 2017; Kyritsis et al. 2019).

Apilactobacillus intestinapis was selected because it was originally isolated from the gut of *Apis mellifera* and belongs to the genus *Apilactobacillus*, which includes lactic acid bacteria associated with honeybees. Several *Apilactobacillus* species, particularly *A. kunkeei*, have been investigated for their beneficial effects in honeybees and for their potential probiotic properties (Endo et al. 2012; Pachla et al. 2021; Rodríguez-Díaz et al. 2021; Asama et al. 2015, 2016). However, these properties have not been established for *A. intestinapis*. Therefore, we evaluated whether consumption of *A. intestinapis* could affect the survival and sexual competitiveness of *A. obliqua* adults. Importantly, supplementation with *A. intestinapis* did not impair either survival or sexual competitiveness under any dietary treatment. Moreover, supplementation was associated with a numerical increase in mating success in sugar-fed

males and a consistent tendency toward longer survival under starvation. Although these effects were not always statistically significant, they identify *A. intestinapis* as a promising probiotic candidate for improving the biological quality of sterile *A. obliqua* males. Previous studies have shown that *Lactobacillus* species can improve the performance of *Anastrepha* flies despite the absence of a shared evolutionary history between the bacteria and their hosts (Caravantes-Villatoro et al. 2022). Future studies should compare live and inactivated *A. intestinapis* cultures to determine whether the observed responses result from probiotic activity or simply from the nutritional value of bacterial biomass.

In conclusion, supplementation with *Apilactobacillus intestinapis* did not negatively affect the survival or sexual competitiveness of sterile *Anastrepha obliqua* adults. Although supplementation did not produce statistically significant improvements in either trait, sugar-fed males receiving *A. intestinapis* showed a numerical increase in mating success, and both males and females exhibited a consistent tendency toward longer survival under starvation. Together, these findings identify *A. intestinapis* as a promising probiotic candidate for improving the biological quality of sterile *A. obliqua* used in sterile insect technique (SIT) programs. Further studies are needed to determine the mechanisms underlying these responses and to evaluate their relevance under field conditions.

Declarations

Author Contributions

LR-R and PL conceived and designed the research. LR-R performed experiments. LR-R, PL, PM, EZ, KG-N analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data Availability

The 16S rRNA gene sequences generated in this study have been deposited in GenBank under accession numbers PZ834073–PZ834078.

Ethical approval

This study required no ethical approval.

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Figures

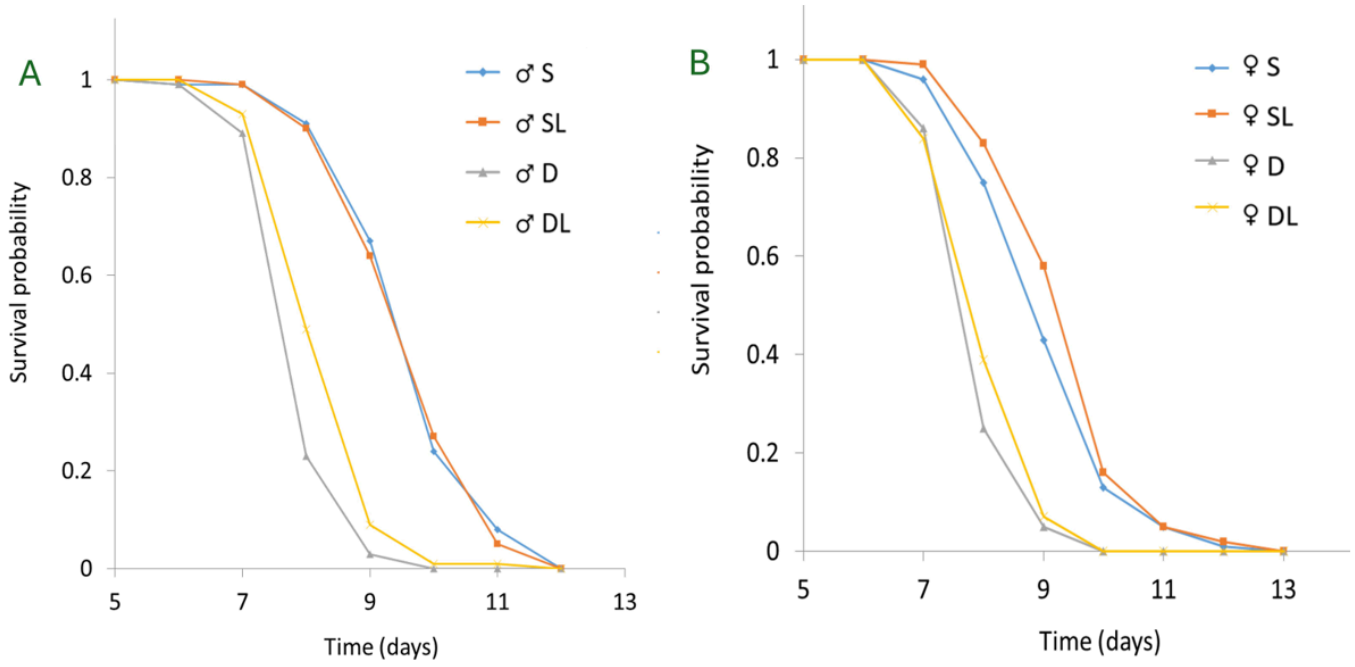


Figure 1

Survival of mass-reared males (A) and females (B): S: sugar diet, SL: sugar diet + *A. intestinapis*, D: standard diet, DL: standard diet + *A. intestinapis*. Food was removed after five days of feeding, and survival under starvation was monitored thereafter.

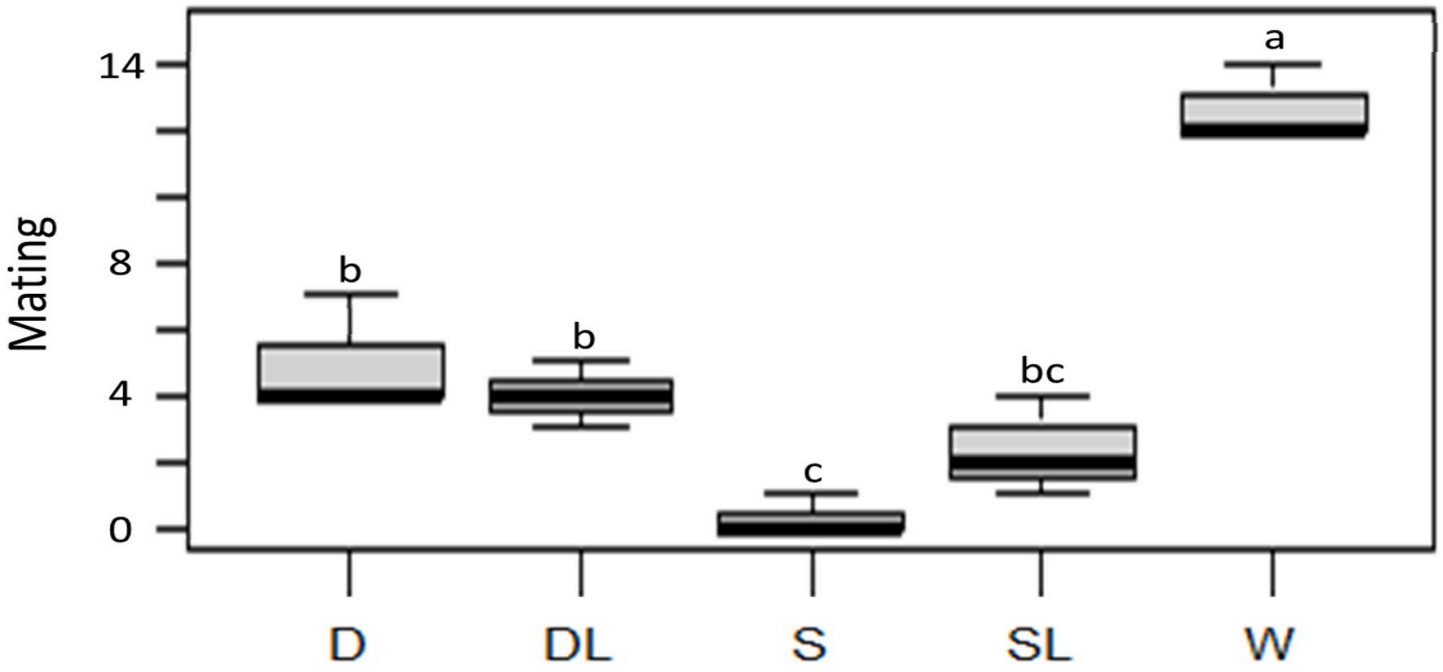


Figure 2

Number of matings obtained by *Anastrepha obliqua* males in sexual competitiveness tests conducted in field cages. D: mass-reared males fed with standard diet, DL: mass-reared males fed with standard diet + *A. intestinapis,* S: mass-reared males fed with sugar,SL: mass-reared males fed with sugar + *A. intestinapis*, and W: wild males fed with standard diet.