Gut bacterial diversity and physiological traits of *Anastrepha fraterculus* Brazilian-1 morphotype males are affected by antibiotic treatment

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Abstract

Background: The interaction between gut bacterial symbionts and Tephritidae became the focus of several studies that showed that bacteria contributed to the nutritional status and the reproductive potential of its fruit fly hosts. *Anastrepha fraterculus* is an economically important fruit pest in South America. This pest is currently controlled by insecticides, which prompt the development of environmentally friendly methods such as the sterile insect technique (SIT). For SIT to be effective, a deep understanding of the biology and sexual behavior of the target species is needed. Although many studies have contributed in this direction, little is known about the composition and role of *A. fraterculus* symbiotic bacteria. In this study we tested the hypothesis that gut bacteria contribute to nutritional status and reproductive success of *A. fraterculus* males.

Methods: Wild and laboratory-reared males were treated with antibiotics (AB) and provided sugar (S) or sugar plus protein (S+P) as food sources. The effect of AB on the gut bacteria diversity was assessed through DGGE and sequencing of the V6-V9 variable region of the bacterial 16S *rRNA* gene.

Results: AB affected the bacterial community of the digestive tract of *A. fraterculus*, in particular bacteria belonging to the Enterobacteriaceae family, which was the dominant bacterial group in the control flies (i.e., non-treated with AB). AB negatively affected parameters directly related to the mating success of laboratory males and their nutritional status. AB also affected males' survival under starvation conditions. The effect of AB on the behaviour and nutritional status of the males depended on two additional factors: the origin of the males and the presence of a proteinaceous source in the diet.

Conclusions: our results suggest that *A. fraterculus* males gut contain symbiotic organisms that are able to exert a positive contribution on *A. fraterculus* males' fitness, although the physiological mechanisms still need further studies.

Key words: South American fruit fly, symbiont, antibiotics, nutritional reserves, survival, Sterile Insect Technique

Background

Insects maintain a close and complex association with microbial communities, ranging from parasitic relationships to commensalism and obligate mutualism [1, 2]. The contributions of gut bacteria to their insect hosts are diverse [see 3 for a review], but probably the most important is associated to its nutrition. Insects use the metabolic pathways of bacteria to obtain nutritional resources otherwise unavailable and thus are able to survive on suboptimal or nutrient-poor diets [3-6]. Bacterial symbionts have also been shown to have a protective function of their insect hosts, to the point that are considered to act as an additional immune system [4, 7, 8]. Although the way that this occurs is still unknown in most cases [3], Brownlie and Johnson [8] describe the production of toxins or antibiotics by gut bacteria that would protect the host against pathogens. Other benefits include improving digestion efficiency, the acquisition of digestive enzymes, some of them associated with detoxification, and the provision of vitamins, nitrogen, specific amino acids and carbon [4]. Bacterial symbionts have also been shown to contribute with chemical compounds that participate in the communication between the hosts and other individuals, present either in the volatiles emitted or retained in the insect cuticle [3, 4, 9]. Moreover, the presence of gut bacteria has been associated to the improvement of developmental and reproductive parameters, such as mating behavior [3, 10].

The study of the interactions that bacteria and their hosts establish has followed different experimental approaches [6]. One of these approaches is to phenotypically characterize the bacterial community present in the gut by culture-dependent techniques or to determine its function inferred from their genome sequence by culture-independent molecular methods [11-18]. Another indirect way to assess the effect of gut bacteria is to evaluate the effect of adding antibiotics (AB) into the insect diets and compare parameters associated to the fitness of AB-

95 treated and non-treated insects [5, 19-23]. Alternatively, other studies have taken a more direct
96 approach in which insects were fed specific bacterial species to determine potential benefits
97 associated with the increase of bacteria titers in their gut [10, 24-31].

98 The sterile insect technique (SIT) is an environmentally friendly and species-specific
99 control method commonly used against tephritid fruit fly pests. The SIT consists of mass
100 production, sterilization, and release of males to mate with wild females [32, 33]. For an effective
101 implementation of the SIT, a deep understanding of the biology of the targeted species is needed,
102 particularly its sexual behavior [33]. Thus, the interaction between gut bacteria and fruit flies has
103 become the focus of several studies in recent years. Combining traditional microbiological
104 methods and molecular techniques, the composition of the bacterial community associated to
105 Tephritidae fruit flies has been characterized for some species. Studies on *Ceratitis capitata*
106 Wiedemann, the Mediterranean fruit fly, showed that gut bacterial community is comprised
107 mainly by members of the family Enterobacteriaceae [10, 12, 34, 35]. However, the
108 monophagous olive fruit fly *Bactrocera oleae* Gmelin is characterized by the presence of the
109 obligatory symbiont *Candidatus* Erwinia dacicola that colonize a specialized evagination of the
110 digestive tract while in the gut a limited number of the bacterial species have been reported such
111 as *Acetobacter tropicalis* [36-38]. Through indirect (AB treatment) or direct (feeding larvae or
112 adults) approaches, gut bacteria were shown to contribute to several biological parameters of
113 their hosts, such as longevity [20, 22, 27], fecundity [5, 21, 29], development, productivity and
114 mating success [10, 19, 25, 27, 30, 31, 39]. The South American fruit fly, *Anastrepha fraterculus*
115 Wiedemann (Diptera: Tephritidae), is a major pest causing considerable damage to a wide
116 spectrum of host fruit species, many of them of economic importance [40, 41]. Currently, the
117 only control method for this species is through the use of insecticides which prompt the
118 development of alternative control methods such as the SIT. The efficacy of the technique

depends on the mating success of males released in the field. Many studies so far have provided valuable information in this regard [42-49]. However, despite the important role that gut bacteria have on the development, productivity and the reproductive success of other Tephritidae flies, no study addressed the significance of these interactions for *A. fraterculus* so far. Because understanding how bacterial symbionts affect the overall fitness of sterile males may contribute to the efficacy of the SIT, in the present study, and as an initial approach, we tested the hypothesis that gut bacteria contribute to nutritional and reproductive aspects of wild and laboratory-reared *A. fraterculus* males from the Brazilian-1 morphotype. Following an indirect approach, we tested the effect of AB treatment on several parameters associated to males' reproductive success such as male sexual performance, and sexual communication mediated by chemical signals and behavioral displays. Also, the nutritional status and the starvation resistance of AB treated and non-treated males were evaluated. In parallel, the effect of AB on the gut bacteria diversity was assessed through molecular techniques. As previous studies in other species have shown that the dietary regime, particularly the protein content of the adult diet, interacts with the presence of gut bacteria, we carried out the above experiments providing a complete diet (sugar and a protein source) and a nutritionally poor diet that contained only sugar.

Results

Diet consumption

The presence of AB had no impact on diet consumption, irrespectively of the origin of the flies or the diet given ($F_{1,2}$ = 0.02, P = 0.9107 for S fed lab males; $F_{1,2}$ = 6.52, P = 0.1252 for S+P fed lab males; $F_{1,2}$ = 1.35, P = 0.3655 for S fed wild males; $F_{1,2}$ =0.10, P = 0.7776 for S+P fed wild males) (Fig. 1).

Molecular characterization of gut bacteria

Total DNA from single *A. fraterculus* guts was used to describe the bacterial community associated to male flies from different origin, types of food and AB treatment using molecular tools. The V6-V9 region of the bacterial 16S *rRNA* gene was amplified by PCR using universal primers. 27 bands of approximately 420 bp were excised from the DGGE gels, and 14 PCR fragments were successfully sequenced to identify the associated bacterial taxonomic groups. The nucleotide sequences obtained for the rest of the PCR products (13) presented double peaks and low quality, showing the potential presence of several amplicons in the same sample. The results of differential band sequencing obtained from the different combinations of treatments showed the presence of microorganisms closely related to the Proteobacteria, distributed as: Gamaproteobacteria, 71% and Alphaproteobacteria, 29% of the total bands (Table 1, Additional files 1; Fig. S1). The use of both distance matrix (Fig. 2) and character-based (parsimony, data not shown) methods resulted in the construction of similar phylogenetic trees. All bacterial strains were phylogenetically related to taxonomic groups of Proteobacteria (linked to Enterobactereales, Xanthomonadales and Alphaproteobacteria class) (Fig. 2), in accordance with the closest relatives found using RDP/Blast search (Table 1). The analysis of the sequences revealed that the Enterobacteriaceae family is the dominant bacterial group in the *A. fraterculus* gut, in both wild and lab flies (S or S+P diet). AB treated flies (wild and lab) fed with a S+P diet contained species of the genus *Stenotrophomonas* sp., and Alphaproteobacteria class; whereas AB treated flies (wild and lab) fed with sugar contained only species of the Alphaproteobacteria class (Table 1; Fig. 2).

Male mating competitiveness

Overall, the mean percentage of copulations achieved by wild males was not affected by AB ($F_{1,2}$ = 0.16, P = 0.7299 for S fed males; $F_{1,4}$ = 1.31, P = 0.3163 for S+P fed males). In contrast, for lab males, the effect of AB depended on the diet. AB had a significantly negative impact on percentage of mating for S+P fed males ($F_{1,3}$ = 18.71, P = 0.0228) while for males fed with S diet, the differences were not significant ($F_{1,2}$ = 0.46, P = 0.5689) (Fig. 3A). Latency to mate was not significantly affected by AB neither for wild (W = 366.5, P = 0.1590 for S fed males; W = 4814.5, P = 0.1000 for S+P fed males) nor for lab males (W = 2762, P = 0.5256 for S fed males; W = 3857.5 P = 0.9155 for S+P fed males) (Fig. 3B). Copula duration was also not significantly affected by AB ($F_{1,107}$ = 1.29, P = 0.2587 for S fed lab males; $F_{1,128}$ = 0.12, P = 0.7291 for S+P fed lab males; $F_{1,36}$ = 1.67, P = 0.2048 for S fed wild males; $F_{1,128}$ = 0.90, P = 0.3441 for S+P fed wild males) (Fig. 3C).

Male calling behavior

Behavioral recordings showed that for S fed males, AB affected the mean number of wing fanning and salivary gland exposure (t = 2.148, d.f. = 14, p = 0.024; and t = 1.870, d.f. = 14, p = 0.041, respectively). For the two variables, males without AB performed these courtship-related behaviors more frequently than AB males (Fig. 4A, B). On the other hand, AB did not affect wing fanning or gland exposure in S+P fed males (t = 0.100, d.f. = 14, p = 0.461; and t = 0.387, d.f. = 14, p = 0.352, respectively) (Fig. 4A, B).

Male volatile and cuticle compounds

Ten compounds were quantified in the volatile collections of *A. fraterculus* males. For S fed males, we detected significantly higher amounts of three compounds (E-E-α-farnesene, anastrephin, epianastrephin) in the volatiles' collections compared to S+AB fed males, whereas

the remaining seven compounds showed no significant differences (Table 2). For males S+P males, no significant differences were detected for any of the 10 compounds between AB treated and non-treated males (Table 2). When antennally active compounds were combined, S fed males that were treated with AB released significantly less amount of these compounds than non-treated males whereas no differences between treated and non-treated males were detected for S+P males (Table 2).

Fifteen compounds were quantified in the cuticle extracts of *A. fraterculus* males. We did not detect significant differences between AB treated and non-treated males in any compound for any of the two diets (Table 3). The same result was found when antennally active compounds were added (Table 3).

Starvation resistance

Laboratory males fed on S and treated with AB lived longer under starvation than S-fed non-treated males ($\chi^2 = 5.28$, $p = 0.0215$). For S+P males, AB treatment had no effect ($\chi^2 = 2.28$, $p = 0.1311$) (Fig. 5A). Conversely, S fed wild males treated with AB lived less than non-treated males ($\chi^2 = 4.94$, $p = 0.0263$). Similarly to lab males, AB had no impact on starvation resistance in S+P fed wild males ($\chi^2 = 1.39$, $p = 0.2369$) (Fig. 5B).

Dry weight

Antibiotics did not affect the adult dry weight both for lab and wild males ($F_{1,10} = 1.92$, $P = 0.1962$ for S fed lab males; $F_{1,10} = 0.25$, $P = 0.6263$ for S+P fed lab males; $F_{1,10} = 0.13$, $P = 0.7227$ for S fed wild males; $F_{1,10} = 1.68$, $P = 0.2235$ for S+P fed wild males) (Fig. 6).

Nutritional reserves

Antibiotic treatment had no effect on total sugar content in any combination of male origin and diet ($F_{1,4}$ = 1.19, P = 0.3375 for S fed lab males; $F_{1,4}$ = 3.12, P = 0.1522 for S+P fed lab males; $F_{1,4}$ = 0.001. P = 0.9769 for S fed wild males; $F_{1,4}$ = 1.23, P = 0.3297 for S+P fed wild males) (Fig. 7A). Likewise, AB had no impact on the glycogen content for both origins and type of diets ($F_{1,4}$ = 0.94, P = 0.3876 for S fed lab males; $F_{1,4}$ = 1.35, P = 0.3103 for S+P fed lab males; $F_{1,4}$ = 0.30, P = 0.6144 for S fed wild males; $F_{1,4}$ = 7.23, P = 0.0547 for S+P fed wild males) (Fig. 7B). The analysis of protein content showed a negative effect of AB for S+P fed lab males ($F_{1,4}$ = 53.33, P = 0.002) (Fig. 7C). For the rest of the treatments, no significant differences in protein content were detected between diets containing or not AB ($F_{1,4}$ = 2.90, P = 0.1637 for S fed lab males; $F_{1,4}$ = 0.01, P = 0.9222 for S fed wild males; S+P: $F_{1,4}$ = 0.42, P = 0.5532 for S+P fed wild males) (Fig. 7C). Lipid content was also negatively affected by AB for S+P fed lab males ($F_{1,4}$ = 18.41, P = 0.0127) (Fig. 7D). For the remaining combinations, no differences were found in the lipid content between AB treated and non-treated males ($F_{1,4}$ = 3.62, P = 0.1298 for S fed lab males; $F_{1,4}$ = 0.07, P = 0.8095 for S fed wild males; $F_{1,4}$ = 0.18, P = 0.6938 for S+P fed wild males) (Fig. 7D).

Discussion

Symbiotic bacteria play an important role in the development and biology of many insect species. Recently, an increasing number of studies have focused on the interaction between bacteria and Tephritidae fruit flies [e.g, 5, 14, 19-23, 25, 27]. Our data suggest that bacteria might affect in a positive way several parameters directly related to the mating success of laboratory *A. fraterculus* males, as well as their nutritional status, but would negatively affect their survival under

starvation. Specifically, this is supported by the fact that ingestion of antibiotic was associated to detrimental effect in males fed on both types of diet. In S fed males, AB produced a decrease in their sexual display rate, a decrease in the amount of three pheromonal compounds and a mild reduction in mating competitiveness. For S+P males, AB affected the amount of copulas obtained by males, which was correlated with a decrease of protein content. The effect of AB on fitness related parameters depended on two additional factors: the origin of the males (wild or lab) and the presence of a proteinaceous source in the adult diet. Nonetheless, it is important to mention that our results were obtained by an indirect approach under which males received AB as a means of disrupting symbiotic association with bacteria. Even when we found a drastic change in the gut microbiota, and we associated this with a reduction of the overall fitness of the males, AB could have also affected the mitochondria [50] causing (or at least contributing to) a decrease in mating success and related parameters. This is a limitation of the current experimental approach and should be considered in further studies, for example by inoculating specific bacteria to the diet. This approach has shown promising results in different fruit fly species, such as *Dacus ciliatus* (Loew) [51], *C. capitata* [10, 27, 30, 31] and *B. oleae* [29].

Analysis of the gut's bacterial community and the effect of antibiotic treatment

We found that the incorporation of AB in the adult diet affected the bacterial community of the digestive tract of *A. fraterculus* males. Similar results were obtained for another fruit flies like *C.*

246 *capitata* and *B. oleae* subjected to similar antibiotic trials [5, 19-23] . In our experiments, the
247 presence of AB had no impact on the decision to feed on a given food source. This shows neither
248 a phagostimulant nor a deterrent effect of adding AB into the diet. DGGE followed by
249 sequencing showed a dominant representation of the Enterobacteriaceae family in the *A.*
250 *fraterculus* male gut, as has been previously evidenced for other fruit fly species (see [52] for a
251 review). Some of these microbial taxonomic groups are composed by diazotrophic bacteria (i.e.,
252 nitrogen fixers) with an essential function in the acquisition of nitrogen compounds and carbon
253 metabolism, allowing both sexes to reach their reproductive potential [12, 13, 53-55]. The strong
254 impact of AB on potentially key symbiotic bacteria evidenced in males, suggest a similar
255 approach could provide relevant information on the role of gut bacteria in females as well.
256 Antibiotics appear to have drastically affected the gut enterobacterial diversity, since other
257 taxonomic classes (e.g., *Klebsiella sp., Enterobacter sp.* and *Serratia sp.*) were not detected in
258 adult males' flies under AB treatment. These differences in the gut bacterial community found
259 between AB-treated and non-treated individuals were also supported by the linkage dendrogram
260 analysis of DGGE profiles. This reduction in gut bacterial diversity, associated to physiological
261 changes in the host has been previously reported for Tephritidae fruit flies [5, 19, 20, 21] as well
262 as for other insect species [56].

263 *Impact of antibiotic treatment on reproductive parameters, nutritional status and starvation*
264 *resistance*

265 *Anastrepha fraterculus*, similarly to other tephritid species, presents a lek based mating system
266 [43, 57] in which males aggregate and perform sexual displays (calling behavior) to attract
267 females to a mating arena that has neither resources nor refuges [58]. The sexual display involves
268 acoustic, chemical and visual signals (e.g., wing fanning, the extrusion of the salivary glands and

269 protrusion of the anal tissue) [59], and is therefore an energetically demanding task ([60],
270 reviewed in [61]). This means that adults need to acquire specific nutrients in order to complete
271 their sexual development [54, 61, 62]. Numerous studies have found that protein intake has a
272 positive impact on the reproductive success of *C. capitata* males, affecting their ability to
273 participate in leks [63], to emit pheromone [64, 65], to transfer a substantial ejaculate [66] and to
274 decrease female receptivity [67]. In the same way, studies with other *Anastrepha* species showed
275 that protein intake results in an improvement of male's sexual competitiveness [46, 62, 68, 69],
276 as well as an increase in the amount of pheromone released by males [70]. In the present study
277 we found significant differences in the amount of lipids and proteins between lab males that were
278 fed with AB and those that were not, for S+P treatment. For both nutrients, the addition of AB to
279 the diet had a negative effect on the nutritional reserves compared to males that retained their gut
280 bacteria. The effect of AB on the nutritional reserves of S+P fed lab males correlates with a
281 significant decrease of the amount of copulas reached by these males compared to non-treated
282 males. Ben-Yosef et al. [19] also observed for S+P fed males a decrease (although not
283 significant) in the reserves of protein after the addition of AB and an impact on mating related
284 variables (see below).

285 The higher mating competitiveness in S+P fed non-treated lab males was not associated to
286 higher rates of sexual displays or sex pheromone emission. Henceforth, it seems that females
287 were able to assess the nutritional status of the males, in spite of the lack of differences in these
288 components of the courtship, maybe using more subtle, close range signals that were not recorded
289 in this study. For several tephritid species, acoustic communication has major implications on
290 mating success. For instance, in several *Anastrepha* species the sound produced by repeated
291 bursts of wing-fanning generates pulse trains that stimulate the females [71-75]. Likewise,
292 behavioral male-male or male-female interactions (e.g., movements, fights or contacts) could be

293 influencing female choice [59]. In our case, females could have used multiple signals to assess
294 males' quality, rejecting those of poor quality related to a low amount of protein as result of a
295 change in their gut bacteria community [52]. Alternatively, males with larger reserves could be
296 more aggressive in defending small territories, a parameter that was not assessed in our
297 experiments. Observations at a finer scale (like video or sound recordings) and also at a higher
298 scale (like field cages with host trees inside) may help to reveal the targets of female choice that
299 could be affected (directly or indirectly) by gut bacteria.

300 Several studies tested the hypothesis that bacteria contribute to mating success of *C.*
301 *capitata*. Most of them followed a direct approach adding specific bacterial strains as probiotics
302 into artificial diets and showed an increase in male mating success [27, 30, 39] with some
303 exceptions [25, 31]. Ben-Ami et al. [39] found that irradiation of *C. capitata* pupae affected the
304 abundance of adult gut bacteria, more specifically *Klebsiella oxytoca*, and this was associated to a
305 reduction of male mating success. Following an indirect approach, as the one used in the present
306 study, Ben Yosef et al. [19] found that *C. capitata* males that were fed antibiotics needed more
307 time to mate (higher latency times) than males that did not received antibiotics, and only when
308 the diet contained protein, as no effect of antibiotics was detected for sugar fed males. According
309 to the same study, bacteria could be involved in the production of a more attractive sexual signal
310 (not analyzed), which may have been mediated by a protein-bacterial interaction [19]. This study
311 on *C. capitata*, and the results of the present one on *A. fraterculus*, showed that the manipulation
312 of symbiotic bacteria in S+P fed males affected their nutritional reserves, and this was associated
313 with a decrease of their mating competitiveness, although the precise mechanism by which
314 females respond to these changes is still unknown and differences in the variable in which this
315 was expressed (i.e., latency or mating percentage) can be attributed to differences in the species
316 under study.

317 Antibiotic treatment also affected parameters associated to the sexual behavior of S fed *A.*
318 *fraterculus* lab males. For these nutritionally stressed males, AB significantly decrease the rate of
319 sexual displays (wing fanning and exposure of salivary glands) and the amount of three
320 antennally active compounds of the male sex pheromone. Additionally, AB treated males fed on
321 sugar obtained numerically less copulas than non-treated males, even though the differences were
322 not statistically significantly. However, in this case there was no significant difference in any of
323 the analyzed nutrients. Although bacteria do not seem to impact on the nutritional status of S fed
324 males when lipids, carbohydrates and protein were measured, they still could be contributing
325 with other essential nutrients that allow fruit flies to fill 'deficiency gaps' (*sensu* [52]) or even to
326 certain essential aminoacids. For example, Ben-Yosef et al. [5, 21] found that the fecundity of
327 females was significantly enhanced by the presence of gut bacteria when flies were fed with a
328 diet containing only non-essential amino acids. This hypothesis needs further research, as it may
329 help to better understand the role of bacteria and even try to supplement artificial diets with
330 specific nutrients as to improve flies' quality with pest management purposes. In any case,
331 through an indirect approach (i.e., antibiotic treatment) it was possible to observe the benefits of
332 symbiotic bacteria in males fed on poor diets.

333 When nutritional reserves and parameters associated to the sexual success of *A.*
334 *fraterculus* were analyzed in wild males, no significant differences were found. However, the
335 addition of AB resulted in a lower, but not statistically different, protein content in S+P fed
336 males, which is similar to what was observed in lab males. It was also observed that the total
337 amount of sugar and glycogen in wild males was much higher in comparison to lab males, which
338 showed larger lipid reserves. All these results showed that removal of gut bacteria (mainly
339 Enterobacteria) at the adult stage was not strongly connected to changes in the nutritional status
340 or mating competitiveness in wild males. This could be the result from at least three different

341 reasons. First, wild males and bacteria could establish an association more similar to a
342 commensalism than to a mutualistic one, being bacteria the only organisms obtaining a benefit, at
343 least when mating is considered. Second, wild flies used in this study had developed in guavas (a
344 primary host for *A. fraterculus*) where the pupal weight is higher than in alternative hosts, such as
345 peach or plum [46]. Guava fruit could provide exceptional nutrients that allow males to reduce
346 the impact of unfavorable conditions, such as the removal of the intestinal microflora. Third, wild
347 flies were provided with an artificial adult diet, which could represent a huge shift compared to
348 natural food sources. This change in environmental and nutritional conditions, associated to the
349 adaptation of wild individuals to artificial rearing conditions, could have produced instability in
350 the microflora constitution and/or a physiological impact on males, adding further complexity
351 and even diluting the contribution of bacteria.

352 Regarding males' ability to endure starvation, we found that the effect of AB depended on
353 the type of diet as well as the origin of the males. First, the starvation resistance of S fed males
354 was higher (i.e., lived longer) than S+P fed males, regardless of the addition of AB and the origin
355 of the flies. Similar results were also observed in previous works [61, 64, 68, 76] where adding
356 protein in the diet (although it increased the sexual performance of males), negatively affected
357 their ability to endure starvation [61]. Second, AB had contrasting results for wild and lab males.
358 While for S fed wild males the presence of bacteria gave males a significant advantage over
359 males fed with AB, the addition of AB allows S fed lab males to significantly live longer than
360 males that were not treated with AB. Ben-Yosef et al. [20] also showed that AB treatment
361 positively affects the longevity of males and females fed on sugar. As mentioned before,
362 nutritionally stressed lab males without their gut bacteria (i.e., S+AB males) were found to
363 perform significantly less sexual signaling than S males (and therefore did not spend great
364 amounts of energy), which may have leave them in better nutritional conditions to endure

365 starvation. Alternatively, the addition of AB could have removed pathogenic bacteria which
366 could be more widespread in laboratory due to the rearing conditions [39]. For example, Behar et
367 al. [22] found that inoculation of sugar diet with *Pseudomonas aeruginosa* reduced the longevity
368 in *C. capitata*.
369

370 **Conclusions**

371 In summary, following an indirect approach (AB treatment) potential contributions of the gut
372 bacteria associated to *A. fraterculus* males was found. These contributions to the fitness of
373 the male were more evident for laboratory flies fed on sugar and protein. This could be
374 mediated by a combination of higher protein reserves and bacteria presence in S+P diets,
375 which leads to a greater male competitiveness; whereas the absence of protein and presence
376 of bacteria in S diets does not improve nutritional reserves but increases the rate of sexual
377 displays, the amount of pheromone emitted and enhances the sexual success of the males.
378 Thus, the evidence suggests that gut microbiota includes beneficial bacterial species that are
379 able to exert a positive contribution. Removal of bacteria had nonetheless a positive effect on
380 starvation resistance in sugar fed lab males, which probably points out to the presence of
381 pathogenic strains in the rearing or the inability of sugar fed to cope with the energetic
382 demand associated to reproduction, or both. Our results have important implications for the
383 development and effectiveness of SIT for *A. fraterculus* although the role of gut bacteria
384 should be confirmed following a more direct approach (i.e., the addition of specific bacterial
385 strains to the diet). Likewise, the characterization of the gut bacterial community associated
386 to females and its potential impact throughout the life cycle should be further addressed.

Materials and methods

Biological material and holding conditions

Experiments were carried out with wild and laboratory-reared *A. fraterculus* flies of the Brazilian-1 morphotype. Wild pupae were recovered from infested guavas (*Psidium guajava* L.) collected at Horco Molle, Tucumán, Argentina. Laboratory flies were obtained from the colony held at INTA Castelar. Rearing followed standard procedures [77, 78] using an artificial diet based on yeast, wheat germ, sugar, and agar for larvae [79] and a mixture of sugar and hydrolyzed yeast (MP-Biomedical®, Santa Ana, California, USA) (3:1 ratio) for adults. Rearing was carried out under controlled environmental conditions (T: 25 ± 2°C, RH: 70 ± 10%, photoperiod 14L: 10D) until adult emergence.

Diets and antibiotics

Males from the two origins (wild or lab) were provided with one of two different diets: sugar (S) or sugar + hydrolyzed yeast (S+P), which in turn could have been supplemented or not with antibiotics (AB). This procedure resulted in four treatments: 1) S; 2) S+AB; 3) S+P; 4) S+P+AB. The S+P diet consisted of 3:1 mixture of sugar and hydrolyzed yeast, which constitutes a rich source of peptides, amino acids, vitamins and minerals, in addition to carbohydrates [5] and is comparable with artificial diets that provide the flies with all their nutritional needs [19, 20, 80]. Because we aimed at comparing the impact of AB between males that had access to protein sources and males that were deprived of protein, S diet was supplemented with NaCl, $MgSO_4$, H_3BO_3 and a complex of vitamins (A, D, B1, B2, B3, B5, B6, B9, B12, C) and minerals ($FeSO_4$, $Ca_3(PO_4)_2$, $CuSO_4$, $Ca(IO_3)2.6H_2O$, $CoSO_4$, $MnSO_4$, $MgSO_4.7H_2O$, $ZnSO_4$, Mo, K_2SO_4) (DAYAMINERAL, Laboratorios Abbot, Buenos Aires, Argentina). This way, S and S+P diets were as similar as possible in terms of micronutrient content. AB treatment consisted of

Ciprofloxacin (10µg mL^{-1}) and Piperaciline (200 µg mL^{-1}), which proved to be the most potent antibiotic combination for the inhibition of bacterial growth in *C. capitata* [19]. The different components of each diet were mixed with distilled water to form a liquid diet. For most experiments, the diet solution was applied to a piece of filter paper and placed inside the cages, and replaced every 48 h. Only when consumption was evaluated (see below), the diets were placed in a container (the lid of a 2 ml Eppendorf vial) and left inside the cage. The diets were colored with a food dye (FLEIBOR, Laboratorios Fleibor, Buenos Aires, Argentina) to allow the differentiation between those males that had been fed with AB and those that had not. This marking system does not present any detrimental effect on *A. fraterculus* [48, 81].

Intake of antibiotic supplemented diets and its effect on gut bacteria diversity

Diet consumption

To evaluate whether the presence of antibiotic affected the rate of food consumption, males were offered either S and S+AB diets, or S+P and S+P+AB diets in a dual choice experiment. For each male origin and type of diet, three replicates were evaluated. In each replicate, 20 recently emerged males (< 24-h old) were confined in a 1 L plastic container and provided with diets as a solution (500 µl of initial volume – V_0) placed in two different vials. Diet consumption was determined every 48 h by removing the vials containing diet and measuring the remaining volume of diet (Vr) with a Hamilton syringe. For each recording, the volume consumed (Vc) was calculated as: V_0 - Vr + Ve (the volume of diet lost due to evaporation). Ve was estimated from control vials which contained the different diets but no flies. Every time a vial was removed for measuring Vr, a new vial with 500 µl of diet was placed in the cage. The number of flies that remained alive at each recording was used to estimate individual consumption (Vci) during the 48 h time interval in which the vial was exposed (Vci = Vc/number of individuals alive in the

433 cage). The experiment lasted 18 days, and the Vci from subsequent 48 h periods were added to
434 obtain the total individual consumption (Vti).

435 *Molecular characterization of gut bacteria*

436 Ten-day-old virgin males from each origin, type of food and treatment were washed 3 times in
437 ethanol 70% and their guts were dissected. Total DNA from single fly guts was extracted
438 following Baruffi et al. [82] protocol with some modifications of volume due to the size of the
439 tissue under study (gut of individual fly), and used as template to amplify the V6-V9 variable
440 region of the bacterial 16S *rRNA* gene by PCR and posterior DGGE fingerprinting, using the
441 primers 968F-GCclamp / 1408R [83].

442 DGGE was conducted using a DcodeTM system (Bio-Rad) and performed in 6%
443 polyacrylamide gels, containing 37.5:1 acrylamide:bisacrylamide and a denaturing gradient of
444 35:70% and 40:60% of urea. The gels were stained for 30 min in 1X TAE buffer containing
445 ethidium bromide and visualized in a UV trans-illuminator. DGGE marker was prepared from a
446 selection of bacterial 16S *rRNA* gene products to enable gel to gel comparison. For the
447 identification and subsequent characterization of DGGE bands, a selection of bands was made
448 according to their position in the electrophoretic profiles. This selection included bands that were
449 shared between individuals (located at the same position in different lanes) and some others that
450 were exclusively present in one individual (differentially located), in order to get a representative
451 sampling of all bands in the DGGE profile. DGGE fragments of interest were numbered and
452 excised with sterile razor blades immediately after staining and visualization of the gels. Gel
453 bands were stored in 50 µl distilled water at –20ºC and eluted at 4°C overnight before PCR
454 reaction. DNA was reamplified using the PCR-DGGE primers without the clamp, and product
455 integrity was checked by agarose gel electrophoresis. The PCR products were purified using the

QIAGEN PCR purification kit (Qiagen Ltd, Hilden, Germany) and directly sequenced with 968F primer.

V6-V9 (approximately 440 bases) 16S *rRNA* gene sequences obtained from DGGE bands were aligned using BioEdit [84] and Clustalw [85]. Sequence similarity searches were performed using the online sequence analysis resources BLASTN [86] of the NCBI (nt database) and Seqmatch provided by the Ribosomal Database Project (RDP) [87]. Alignment of our sequences and the closest related taxa was carried out using the MEGA 6.06 software package. A phylogenetic tree based on distance matrix method was constructed. Evolutionary distances were calculated using the method of Jukes and Cantor [88] and topology was inferred using the ‘‘neighbor-joining’’ method based on bootstrap analysis of 1,000 trees. Phylogenetic tree calculated by maximum parsimony using the PAUP phylogenetic package was also generated.

Nucleotide sequences generated from 16S *rRNA* gene corresponding to *A. fraterculus* gut bacteria, and obtained from DDGE purified bands, were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/index.html). The samples were named as follows: 1 S+P+AB Wild; 10 S+P+AB Wild; 4 S+P Wild; 5 S+P Wild; 6 S+P Wild; 5 S+AB Wild; 3 S Wild; 1 S+P+AB Lab; 2 S+P+AB Lab; 5 S+P Lab; 3 S+P Lab; 4 S+P Lab; 4 S+AB Lab and 5 S Lab. The corresponding accession numbers are: MH250014-27, respectively.

Impact of antibiotics on reproductive parameters

Males' mating competitiveness

To evaluate males' mating competitiveness, one wild sexually-mature virgin female (14 days-old) was released inside a mating arena (a 1 L plastic container), which contained two males from the same origin as well as diet, but only one had received AB. Males were fed on the diets from

478 emergence until sexual maturity (14 days-old), time at which they were tested. After the female
479 was released in the arena, the occurrence of mating was followed by an observer. The type of
480 male, the copula start time and the time at which flies disengaged were recorded. The experiment
481 was conducted under laboratory conditions (T: 25 ± 1ºC and 70 ± 10 % RH) from 8:00 to 11:00
482 am. The experiment was replicated on different days as follows: five days for wild males (both S
483 and S+P diets), three days for S fed lab males and four days for S+P fed lab males. We evaluated
484 667 mating arenas: 191 for S fed wild males and 171 for S+P fed wild males, 145 for S fed lab
485 males, 160 for S+P fed lab males.

486 *Males calling behavior and chemical profile*

487 To evaluate the potential changes in male sexual signaling related to the AB treatment, males'
488 calling behavior was recorded at the same time that male-borne volatiles were collected. Each
489 replicate consisted of ten males from the same combination of diet and AB treatment, placed in a
490 250 mL glass chamber (20 cm length, 4 cm in diameter) [81]. Males were 10 days-old and were
491 kept under the aforementioned treatments until the day of the test. Eight replicates were carried
492 out and only lab males were analyzed.

493 Behavioral recordings and collection of volatiles started at 8:30 am and lasted for 3 h
494 [daily period of sexual activity for this *A. fraterculus* morphotype (43)]. Two components of male
495 courtship associated with pheromone emission and dispersion were considered: wing fanning and
496 exposure of salivary glands [43, 59, 89]. During the observation period, the number of males
497 performing these behaviors was recorded every 30 minutes. At the same time, the volatiles
498 emitted by the calling males were collected by blowing a purified air stream through the glass
499 chambers. Volatiles were collected onto traps made of 30 mg of Hayesep Q adsorbant (Grace,
500 Deerfield, IL, USA) [81]. After collection, the trapped volatile compounds were eluted with 200

µl of methylene chloride and chemically analyzed using an Agilent 7890A gas chromatograph (GC) equipped with a HP-5 column (30 m ± 0.32 mm inner diameter ± 0.25 µm film thickness; Agilent Technologies), and an Agilent 5977 mass spectrometer. The initial oven temperature was 35°C and after 1 min the oven temperature was increased to 100°C at 5 °C min^{-1} and from 100°C to 230°C at 12°C min^{-1}, then held for 10 min. Samples were injected in the splitless mode with the injector purged at 30 s with helium as the carrier gas at 27.6 cm/sec flow velocity. Methyl nonadecanoate (5 ng per 1 µl of methylene chloride) was used as internal standard. The compounds were identified by using their relative retention times and comparison of their mass spectra with libraries. The identity of specific compounds (e.g., limonene, suspensolide, (E,E)-α-farnesene, anastrephin and epianastrephin) was also confirmed with standards.

In order to analyze the effect of AB on the chemical profile of the cuticle, after the pheromone sampling ended males were gently removed from the glass chambers and washed (in groups of ten) with 1 ml of hexane for 1 min in 2 ml glass vials. Methyl nonadecanoate (5 ng per 1 µl of hexane) was used as internal standard. Compounds were identified as described above.

Impact of antibiotics on starvation resistance and nutritional status

Starvation resistance

To evaluate the effect of antibiotics on males' ability to endure starvation, a group of 20 wild or lab males (< 24-h old) was caged in a 1 L plastic container and fed one of the aforementioned diets. Food was replaced every 48 h. After 10 days, food was removed and only water was provided. Every 24 h, the number of dead males was recorded until all individuals had died. For each origin and treatment, the experiment was replicated three times.

Dry weight and nutritional reserves

To evaluate the effect of AB on males' dry weight and nutritional reserves, groups of 20 wild or lab males (< 24-h old) were placed in 1 L plastic containers and provided with one of the aforementioned diets (i.e., S; S+AB; S+P; S+P+AB). Six cages were arranged per diet and origin. Diet was replaced every 48 h. After 14 days, males were removed from the cage and preserved at -20°C. A sample of 10 individuals from each cage was dried out in an oven at 50°C for 5 h and weighed in a precision scale (readability: 0.0001 g) (Ohaus Corporation, Parsippany, NJ, USA). Nutritional reserves were determined following standard biochemical techniques. Protein content was determined with the Bradford [90] method using Coomassie brillant blue G-250 reagent. Lipid and carbohydrate contents were determined with the Van Handel [91] method. Total sugar and glycogen contents were measured with anthrone reagent [92] whereas vanillin reagent was used for lipid measurement [93].

Statistical Analysis

Data were analyzed using InfoStat and R software [94, 95].

To determine whether the presence of AB in the diet affected diet consumption, a mixed effect model analysis for each combination of diet and origin was performed with AB treatment as the fixed factor and the cage from which the flies were taken as the random factor.

To evaluate the AB effect on the percentage of copula achieved by treated and non-treated males, we performed a mixed effect model analysis with AB treatment as the fixed factor and the day of the experiment as the random factor. After verifying lack of heteroscedasticity, the data were analyzed without transformation. For wild males fed on the S diet, two experimental days (replicates) were removed due to the low number of copulations recorded (less than 10 matings). Latency was analyzed by Mann-Whitney test for each category (male origin and diet

545 regimen) separately. Copula duration was analyzed with a mixed effect model where the fixed
546 factor was the AB treatment and the random factor was the day of the experiment.

547 The mean number of males exposing their salivary gland or fanning their wings across the
548 observation period was compared between S and S+AB, or S+P and S+P+AB, by means of
549 Student's *t*-tests. The abundances of volatile and cuticle compounds were obtained by computing
550 the ratio between the area under the peak of each compound and the area under the peak of the
551 internal standard. Then, the abundance of each compound was compared between AB treated and
552 non-treated males (separately for S and S+P males) in two ways. First, a Student's *t*-test was
553 performed for each single compound detected by the mass detector. Second, a new Student's *t*-
554 test was performed by building a new variable that resulted from adding those compounds that
555 showed evidence of electroantennal activity in *A. fraterculus* females of the same laboratory
556 strain we used in this study. These compounds included: E-β-ocimene; Z-E-α-farnesene; E-E-α-
557 farnesene; and epianastrephin [96, 97].

558 To evaluate the effect of AB on starvation resistance, the data were analyzed using a
559 Kaplan-Meier survival analysis for each male origin and diet combination separately. The effect
560 of AB on males' dry weight and nutritional reserves were analyzed by means of mixed effects
561 models in which the AB treatment was the fixed factor and the cage from which the flies were
562 taken was the random factor.

563

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569

570 **Abbreviations**

571 AB: Antibiotic; SIT: Sterile insect technique; S: Sugar; S+P: Sugar + hydrolyzed yeast; DGGE:
572 Denaturing gradient gel electrophoresis; RDP: Ribosomal database project; UPGMA:
573 Unweighted pair-group method with arithmetic averages; GC: Gas chromatograph.

574

575 **Ethics approval**

576 Not applicable.

577

578 **Competing interests**

579 The authors declare that they have no competing interests

580

581 **Consent for publication**

582 Not applicable.

583

584 **Availability of data and materials**

585 All data generated or analyzed during this study are included in this published article (and its
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587

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593

594 **Author contributions**

595 MLJ, MJR, LG and MTV performed the diet consumption, mating competitiveness, dry weight,
596 nutritional reserves and starvation resistance tests. LEP and SBL conducted the molecular
597 analysis. DFS, GEB and PCF carried out the calling behavior, and volatile and cuticle
598 compounds analyses. PMP and FC performed the nutritional reserves analyses. DFS, SBL, KB,
599 JLC and MTV conceived the project and coordinated the activities. MLJ, LEP, SBL, PCF, JLC,
600 KB, MTV and DFS wrote the paper. All authors interpreted the results and commented on the
601 manuscript. All authors read and approved the final manuscript

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879

880

881 **Figure legends**

882 **Figure 1.** Effect of antibiotics treatment on laboratory and wild *Anastrepha fraterculus* males'
883 consumption. Individual total consumption (µl) of males exposed to two different diets with or
884 without the antibiotic addition (AB): S and S+AB diets, or S+P and S+P+AB in a dual choice
885 experiment.

886 **Figure 2.** Phylogenetic tree based on V6-V9 16S *rRNA* gene sequence analysis of *A. fraterculus*
887 gut bacteria and the closest relative taxa. The tree is based on Neighbor-Joining method (Jukes-
888 Cantor distance), using a 50% conservation filter. Numbers on the nodes present % bootstrap
889 values based on 1000 replicates. Scale bar indicates 10% estimated sequence divergence. The
890 16S *rRNA* gene sequences of *Methanogenium marinum* were arbitrarily chosen as an outgroup.

891 **Figure 3.** Effect of antibiotics treatment on laboratory and wild *Anastrepha fraterculus* male
892 mating competitiveness. (A) Percentage of matings (B) Latency to copulate (time elapsed before
893 copulation started) and (C) Duration of copula obtained by males fed with two different diets
894 with or without addition of antibiotic (AB).

895 **Figure 4.** Effect of antibiotics treatment on laboratory *Anastrepha fraterculus* male calling
896 behavior and pheromone release. (A) Number of males fed on S or S+AB and S+P or S+P+AB
897 diets that were detected fanning their wings across the observational period. (B) Number of males
898 fed on S or S+AB and S+P or S+P+AB diets that were detected exposing their salivary glands
899 across the observational period.

900 **Figure 5.** Effect of antibiotics on laboratory (A) and wild (B) *Anastrepha fraterculus* males'
901 starvation resistance. Cumulative proportion of surviving males fed on S or S+P diets with or
902 without the addition of antibiotics (AB).

903 **Figure 6.** Effect of antibiotics on laboratory and wild *Anastrepha fraterculus* males' dry weight.
904 Weight (mg) of males fed on S or S+AB and S+P or S+P+AB diets with or without the antibiotic
905 addition (AB).

906 **Figure 7.** Effect of antibiotic on laboratory and wild *Anastrepha fraterculus* males' nutritional
907 reserves. (A) Sugar, (B) Glycogen, (C) Protein and (D) Lipids content in males fed on S or S+AB
908 and S+P or S+P+AB diets with or without the antibiotic addition (AB).

909

910 **Table 1.** Analysis of V6-V9 16S *rRNA* gene sequences obtained from DGGE profiles and sequencing.

911

DGGE Band Number	Taxonomic group (RDP)	Closest related sequence (BLAST) (Genbank Accession number)	Nucleotide bases compared	Similarity
3 S+P Lab / 4 S+P Lab / 5 S+P Lab	Gamaproteobacteria/ Enterobacteriales/ Enterobacteriaceae/ unclassified_Enterobacteriaceae	*Raoultella planticola* strain Ns8 (MG544105.1) *Serratia marcescens subsp. marcescens* (HG326223.1)	389	99%
4 S+P Wild / 5 S Lab	Gamaproteobacteria/ Enterobacteriales/ Enterobacteriaceae/ unclassified_Enterobacteriaceae	*Enterobacter soli* strain YHBG2 (MG516168.1) *Serratia marcescens subsp. marcescens* (MG516113.1)	384	100%
1 S+P+AB Wild / 2 S+P+AB Lab	Gamaproteobacteria/ Xanthomonadales/ Xanthomonadaceae/ Stenotrophomonas	*Stenotrophomonas maltophilia* (MG546679.1) *Stenotrophomonas maltophilia* (MG546678.1)	388	100%
5 S+P Wild / 6 S+P Wild	Gamaproteobacteria/ Enterobacteriales/ Enterobacteriaceae/ unclassified _Enterobacteriaceae	Uncultured *Enterobacter sp*. clone 03 (KJ526996.1) *Klebsiella aerogenes* strain JMB006 (MG546216.1)	387	99%
10 S+P+AB Wild / 5 S+AB Wild / 1 S+P+AB Lab / 4 S+AB Lab	Alphaproteobacteria/ unclassified_Alpha-proteobacteria	Uncultured alpha proteobacterium (HM111616.1)	390	99%
3 S Wild	Gamaproteobacteria/Enterobacteriales/ Enterobacteriaceae/Citrobacter	*Klebsiella sp*. M5al (CP020657.1) *Klebsiella oxytoca* strain FCX2 16S (KU942497.1)	387	100%

Table 2. Relative abundances (mean ± S.E.) of compounds detected in the volatile collection of *Anastrepha fraterculus* males fed on S or S+P diets (N = 8). Results are shown as mean ± SE for AB treated and non-treated males and compared by means of a Student´s *t*-test.

				Sugar fed males			Sugar + protein fed males		
Ret. time (min)	**Compound**	**KI**	**KI lit.[c]**	**S**	**S+AB**	**p**	**S+P**	**S+P+AB**	**p**
10.76	limonene	1027	1031	0.06 ± 0.004	0.07 ± 0.004	0.45	0.19 ± 0.05	0.15 ± 0.005	0.27
10.94	indane	1033	1034	0	0	-	1.622 ± 0.36	1.34 ± 0.026	0.27
11.00	unknown	1035	-	0.01 ± 0.002	0.05 ± 0.031	0.18	0	0	-
11.40	E-β-ocimene[b]	1049	1050	1.84 ± 0.247	1.68 ± 0.215	0.32	6.554 ± 0.16	6.15 ± 0.098	0.41
14.00	4-Methylindane	1141	1142	0	0	-	3.375 ± 0.08	2.89 ±0.052	0.30
19.90	suspensolide[a]	1496	no data	0.42 ± 0.057	0.33 ± 0.058	0.16	0.543 ± 0.01	0.79 ± 0.018	0.15
19.92	Z-E-α-farnesene[b]	1498	1497	0.56 ± 0.012	0.39 ± 0.052	0.08	0.997 ± 0.03	1.11 ± 0.033	0.39
20.06	E-E-α-farnesene[ab]	1510	1508	9.21 ± 1.497	6.04 ± 0.818	0.04	14.996 ± 0.38	15.71 ± 0.447	0.45
20.98	anastrephin[a]	1596	no data	0.31 ± 0.037	0.21 ± 0.038	0.04	0.571 ± 0.01	0.78 ± 0.025	0.24
21.12	epianastrephin[ab]	1610	1621	0.91 ± 0.128	0.59 ± 0.071	0.04	1.498 ± 0.03	2.21 ± 0.072	0.19
	Sum of EAG+ compounds	-	-	11.93 ± 1.712	8.28 ± 1.09	0.05	23.106 ± 5.43	23.73 ± 5.641	0.23

a Compound identified by comparison with authentic standards.
b Compound that triggers a positive EAG response in female´s antennae (Brizova et al. 2013; Bachmann 2016).
c: KI: Kovats index obtained for a DB5 / HP5 column and similar chromatographic conditions from www.pherobase.com and webbook.nist.gov.

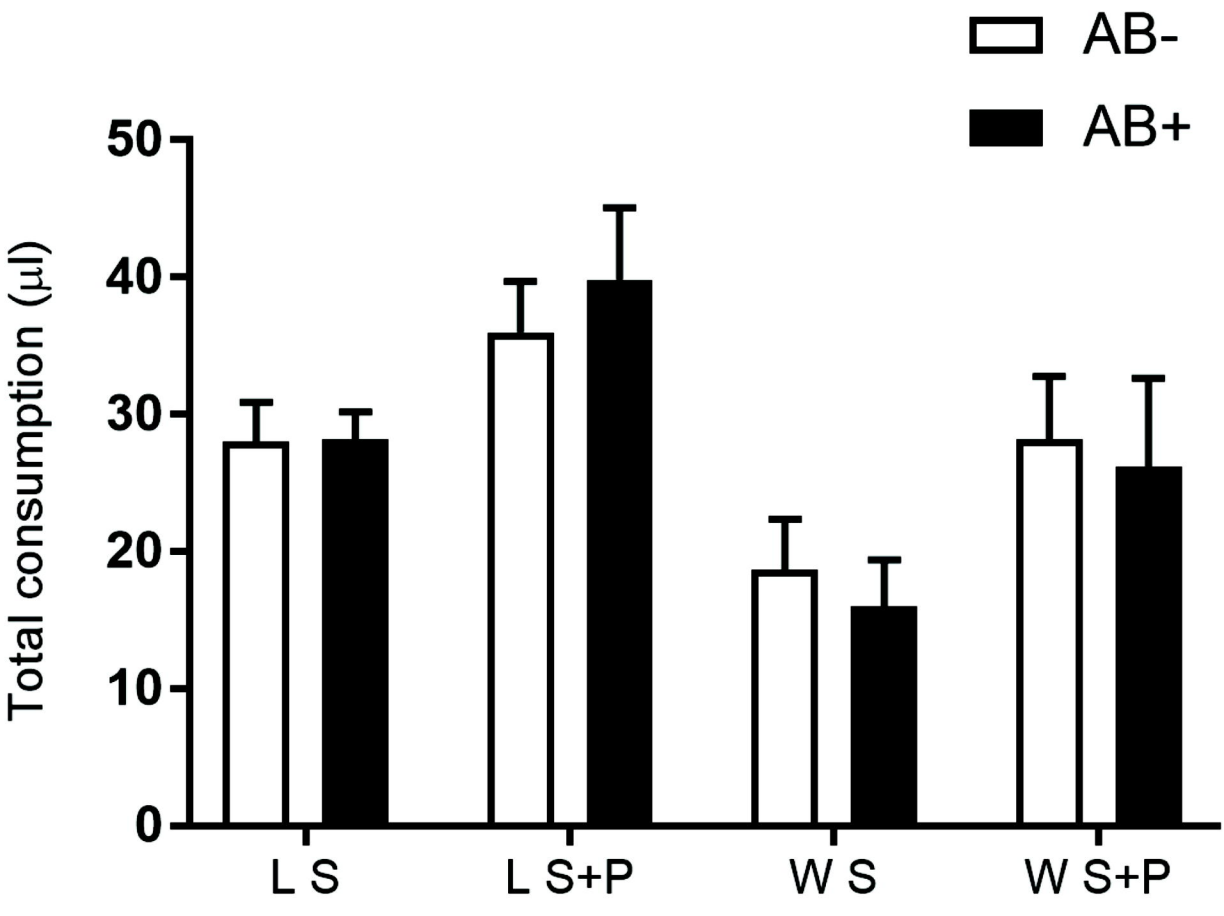
Table 3. Relative abundances (mean ± S.E.) of compounds detected in the cuticle extracts of *Anastrepha fraterculus* males fed on S or S+P diets (N = 8). Results are shown as mean ± SE for AB treated and non-treated males and compared by means of a Student´s *t*-test.

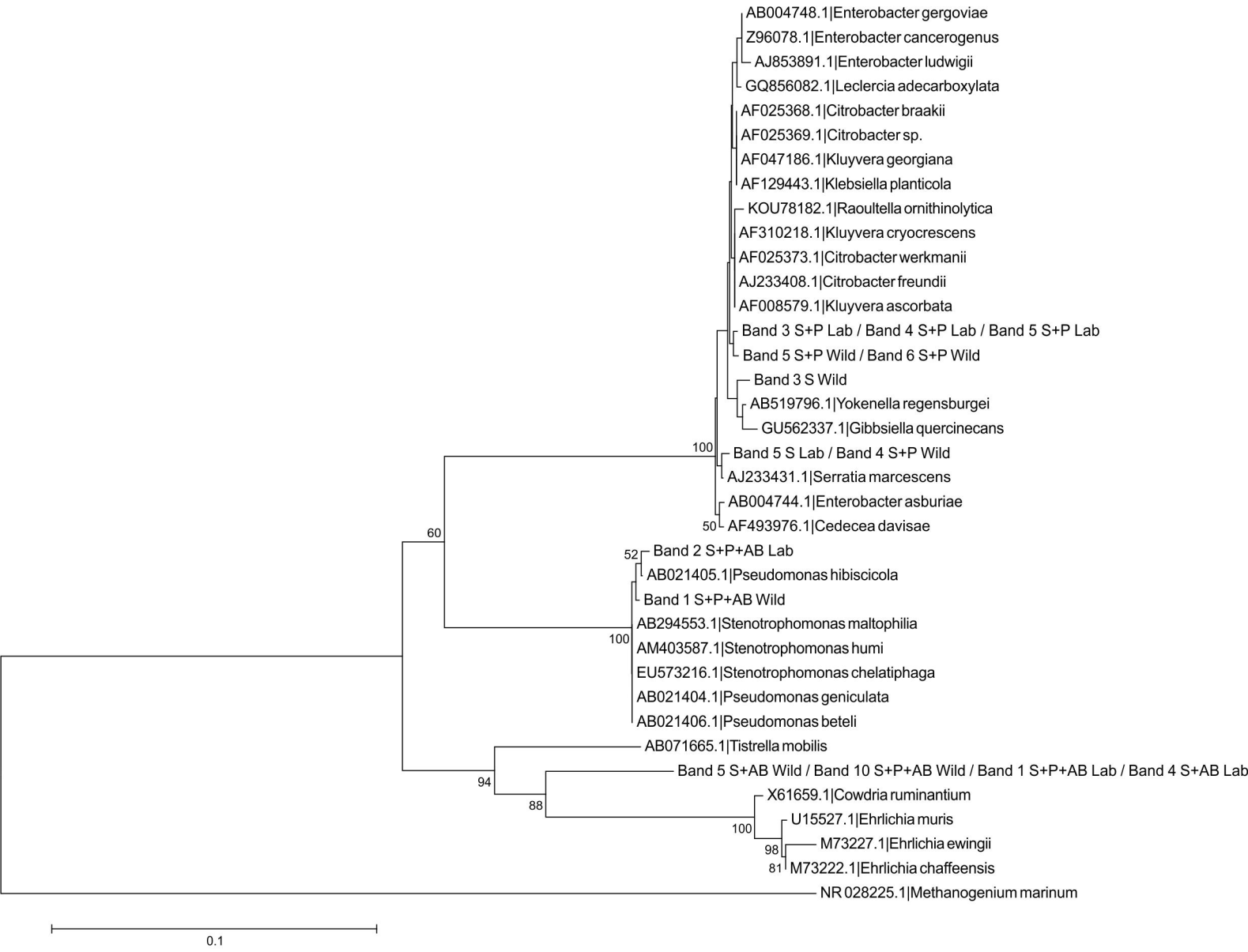
				Sugar fed males			Sugar + protein fed males		
Ret. time (min)	**Compound**	**KI**	**KI lit.[c]**	**S**	**S+AB**	**p**	**S+P**	**S+P+AB**	**p**
19.90	suspensolide[a]	1496	no data	0.11 ± 0.001	0.12 ± 0.002	0.31	0.07 ± 0.002	0.06 ± 0.001	0.33
20.06	E-E-α-farnesene[ab]	1510	1508	0.60 ± 0.014	0.44 ± 0.007	0.16	0.57 ± 0.035	0.91 ± 0.053	0.31
20.98	anastrephin[a]	1596	no data	0.11 ± 0.002	0.12 ± 0.004	0.43	0.10 ± 0.006	0.27 ± 0.019	0.22
21.12	epianastrephin[ab]	1610	1621	0.55 ± 0.013	0.52 ± 0.016	0.44	0.46 ± 0.026	1.00 ± 0.066	0.25
23.79	nonadecane*	1900	1900	0.47 ± 0.011	0.45 ± 0.006	0.43	0.33 ± 0.008	0.66 ± 0.032	0.18
24.46	monounsaturated alkene (C_{20})	1981	-	0.11 ± 0.002	0.11 ± 0.003	0.47	0.12 ± 0.004	0.29 ± 0.016	0.18
25.23	monounsaturated alkene (C_{21})	2079	-	1.02 ± 0.029	1.12 ± 0.028	0.40	0.78 ± 0.022	1.87 ± 0.094	0.16
25.28	monounsaturated alkene (C_{21})	2084	-	14.24 ± 0.417	15.16 ± 0.376	0.44	19.24 ± 0.621	44.65 ± 2.460	0.18
25.44	heneicosane[a]	2100	2100	20.53 ± 0.431	20.94 ± 0.352	0.48	10.50 ± 0.344	9.87 ± 0.245	0.45
26.23	monounsaturated alkene (C_{22})	2180	-	0.81 ± 0.025	0.86 ± 0.011	0.44	0.64 ± 0.022	1.10 ± 0.044	0.20
27.32	monounsaturated alkene (C_{23})	2274	-	1.96 ± 0.061	2.11 ± 0.037	0.42	0.64 ± 0.016	1.33 ± 0.051	0.13
27.40	monounsaturated alkene (C_{23})	2280	-	22.89 ± 0.066	21.83 ± 0.346	0.44	18.59 ± 0.473	34.27 ± 1.600	0.20
27.64	tricosane[a]	2300	2300	3.40 ± 0.582	3.57 ± 0.058	0.44	1.65 ± 0.047	1.81 ± 0.053	0.42
29.20	tetracosane[a]	2400	2400	2.49 ± 0.029	2.25 ± 0.022	0.27	1.04 ± 0.023	0.74 ± 0.016	0.18
31.25	pentacosane[a]	2500	2500	3.28 ± 0.058	3.23 ± 0.040	0.48	1.62 ± 0.038	1.64 ± 0.039	0.48
	Sum of EAG + compounds	-	-	1.15 ± 0.021	0.96 ± 0.020	0.50	1.03 ± 0.032	1.91 ± 0.024	0.38

a Compound identified by comparison with authentic standards.
b Compound that triggers a positive EAG response in female´s antennae (Brizova et al. 2013; Bachmann 2016).
c: KI: Kovats index obtained for a DB5 / HP5 column and similar chromatographic conditions from www.pherobase.com and webbook.nist.gov.

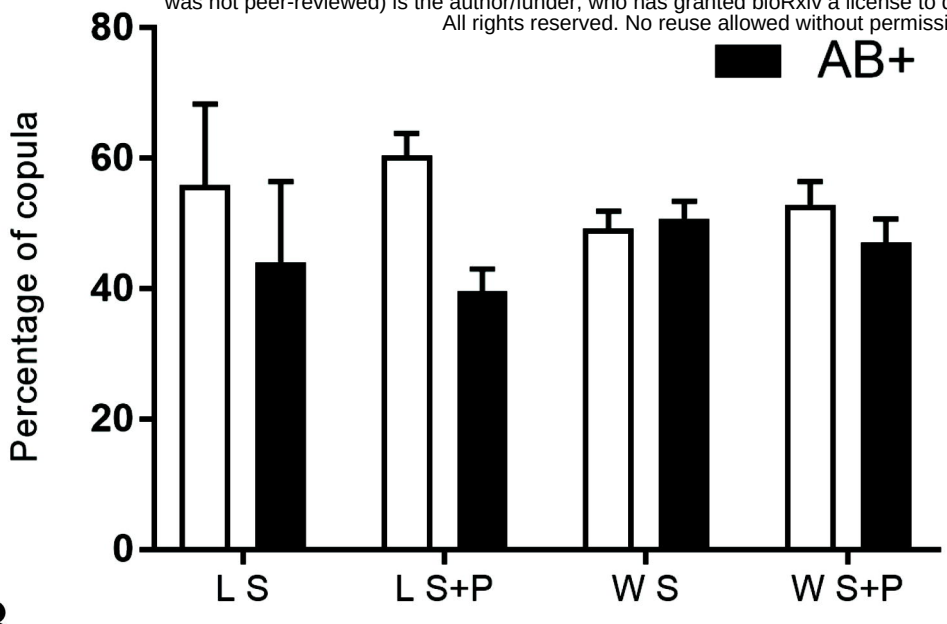
Additional files

Additional files 1:Figure **S1.** Alignment of V6-V9 16S *rRNA* nucleotide sequences (420 bases) obtained from DGGE profiles. Af V6-V9 Seq 1-14 correspond: Band 1 S+P+AB Wild, Band 10 S+P+AB Wild, Band 4 S+P Wild, Band 5 S+P Wild, Band 6 S+P Wild, Band 5 S+AB Wild, Band 3 S Wild, Band 1 S+P+AB Lab, Band 2 S+P+AB Lab, Band 5 S+P Lab, Band 3 S+P Lab, Band 4 S+P Lab, Band 4 S+AB Lab, Band 5 S Lab respectively (WORD 51.5 KB).


AB-
AB+
Total consumption (µl)
50
40
30
20
10
0
L S
L S+P
W S
W S+P


AB004748.1|Enterobacter gergoviae
Z96078.1|Enterobacter cancerogenus
AJ853891.1|Enterobacter ludwigii
GQ856082.1|Leclercia adecarboxylata
AF025368.1|Citrobacter braakii
AF025369.1|Citrobacter sp.
AF047186.1|Kluyvera georgiana
AF129443.1|Klebsiella planticola
KOU78182.1|Raoultella ornithinolytica
AF310218.1|Kluyvera cryocrescens
AF025373.1|Citrobacter werkmanii
AJ233408.1|Citrobacter freundii
AF008579.1|Kluyvera ascorbata
Band 3 S+P Lab / Band 4 S+P Lab / Band 5 S+P Lab
Band 5 S+P Wild / Band 6 S+P Wild
Band 3 S Wild
AB519796.1|Yokenella regensburgei
GU562337.1|Gibbsiella quercinecans
100
Band 5 S Lab / Band 4 S+P Wild
AJ233431.1|Serratia marcescens
AB004744.1|Enterobacter asburiae
50
AF493976.1|Cedecea davisae
60
52
Band 2 S+P+AB Lab
AB021405.1|Pseudomonas hibiscicola
Band 1 S+P+AB Wild
AB294553.1|Stenotrophomonas maltophilia
100
AM403587.1|Stenotrophomonas humi
EU573216.1|Stenotrophomonas chelatiphaga
AB021404.1|Pseudomonas geniculata
AB021406.1|Pseudomonas beteli
AB071665.1|Tistrella mobilis
Band 5 S+AB Wild / Band 10 S+P+AB Wild / Band 1 S+P+AB Lab / Band 4 S+AB Lab
94
88
X61659.1|Cowdria ruminantium
100
U15527.1|Ehrlichia muris
98
M73227.1|Ehrlichia ewingii
81
M73222.1|Ehrlichia chaffeensis
NR 028225.1|Methanogenium marinum
0.1

A



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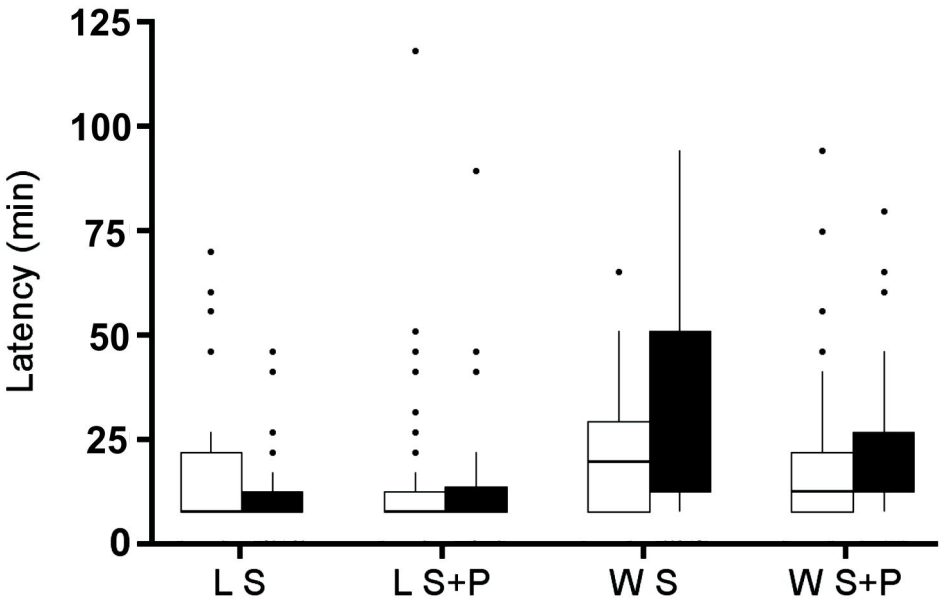

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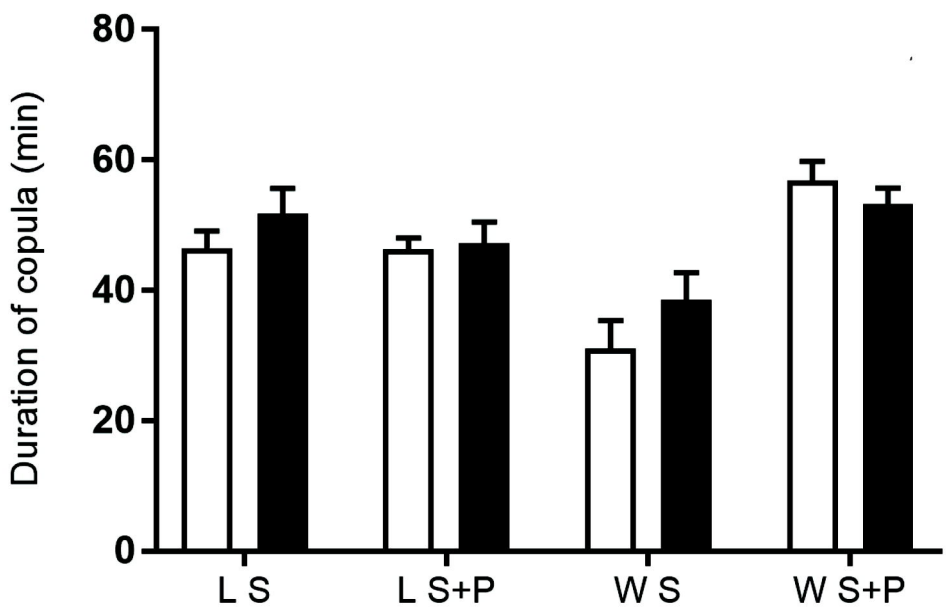

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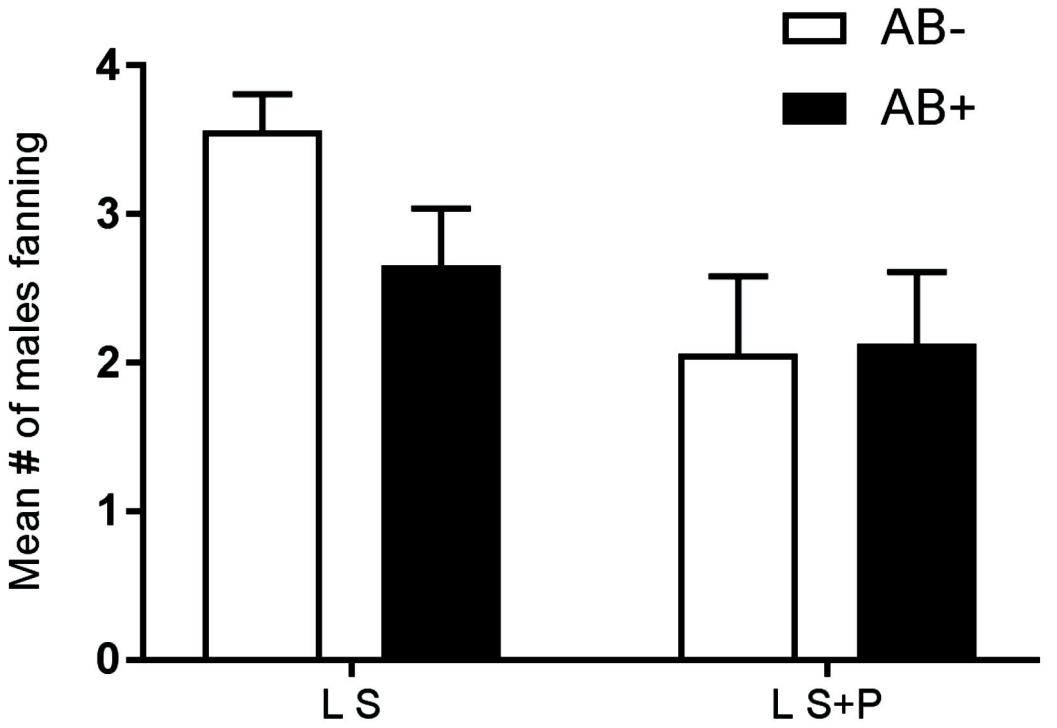
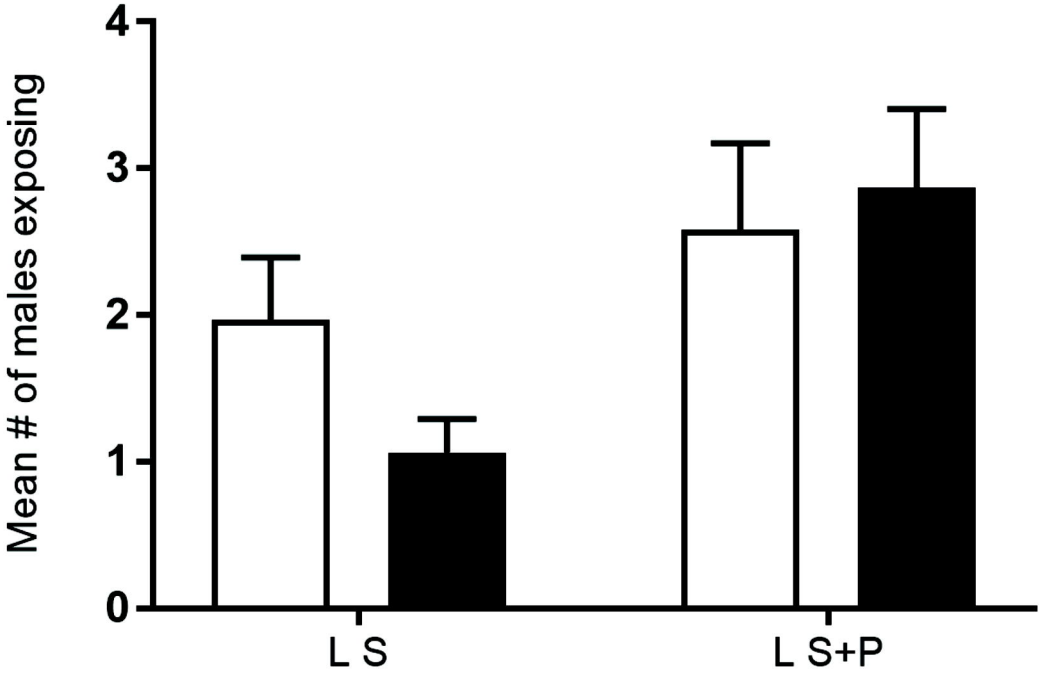
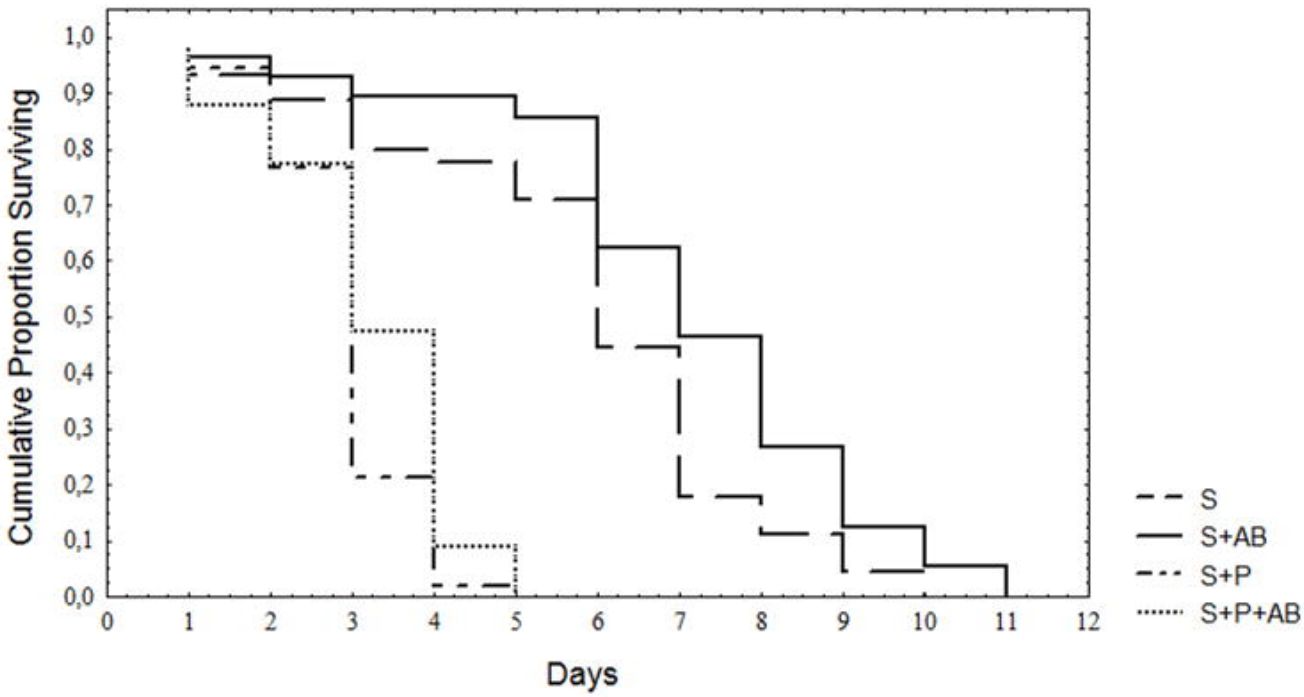

AB-
AB+
Mean # of males fanning
4
3
2
1
0
L S
L S+P

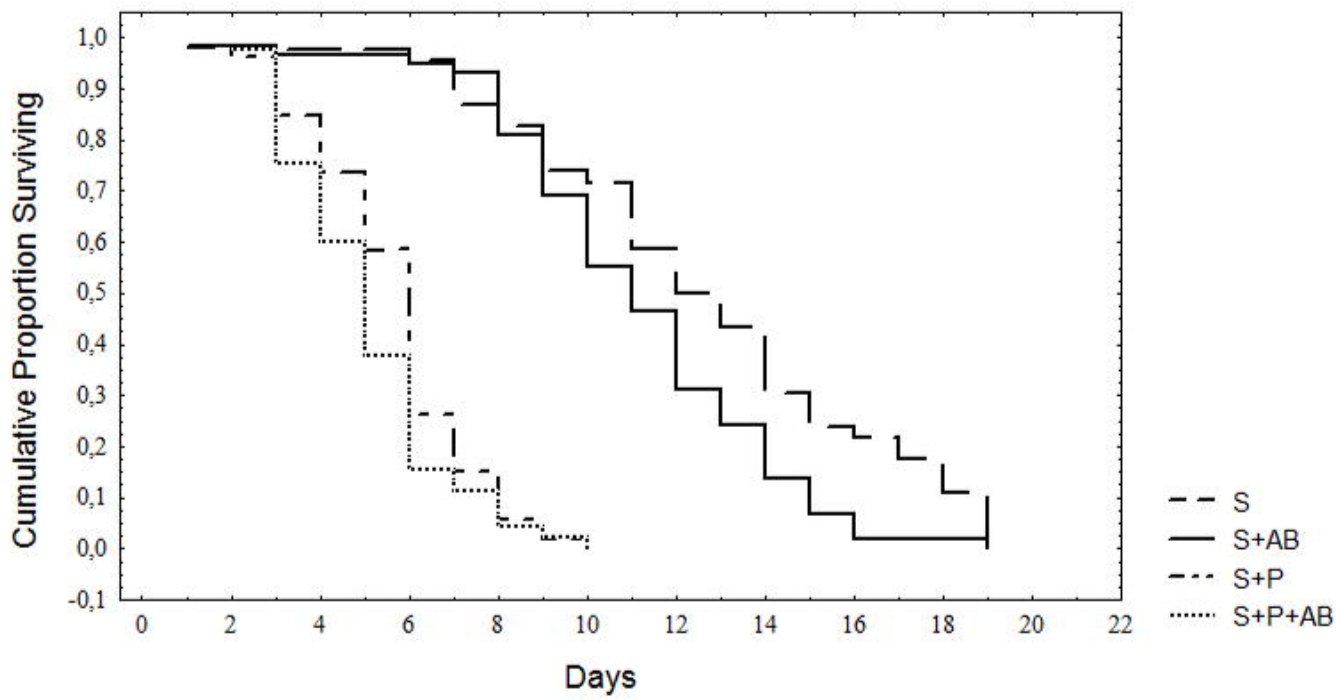
B

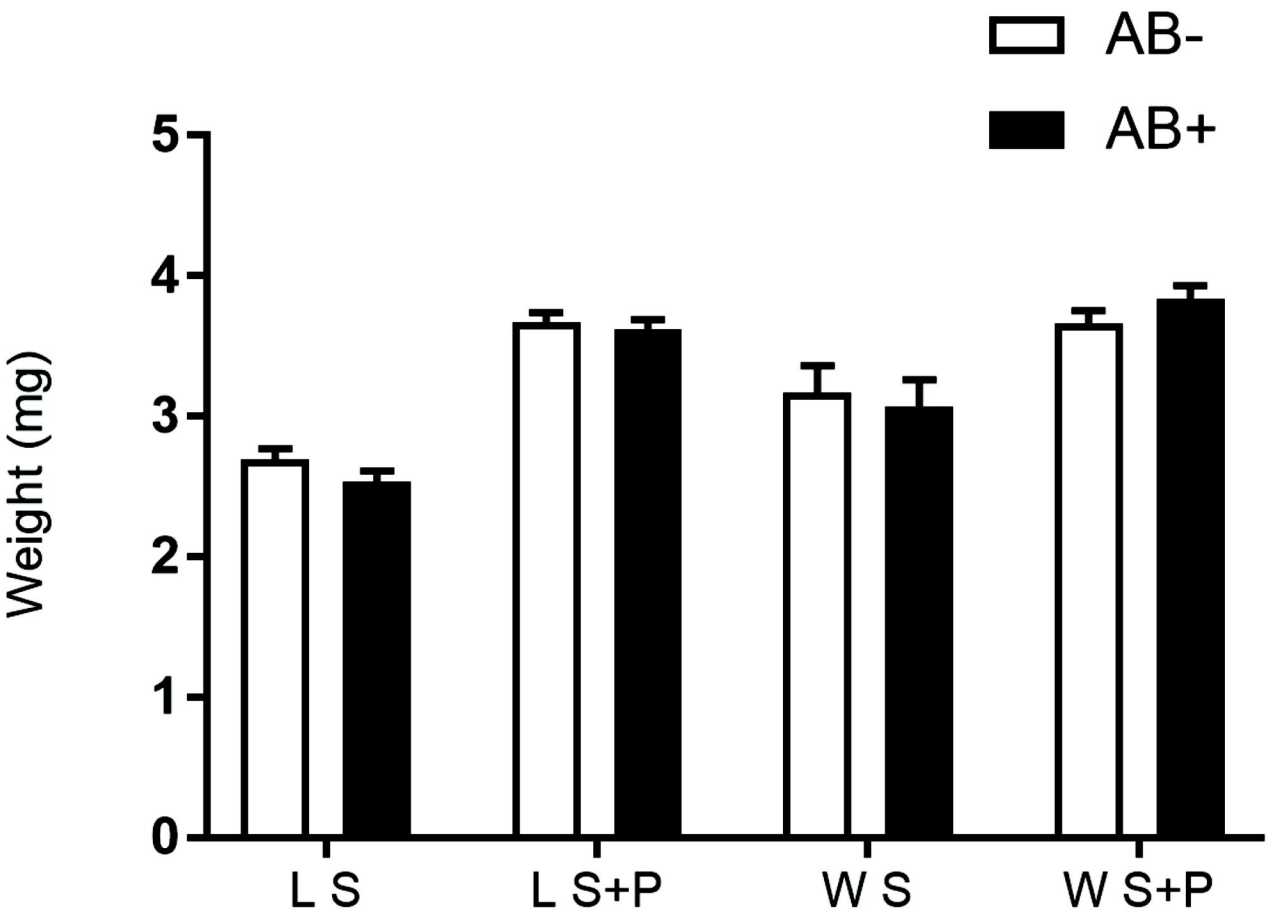

Mean # of males exposing
4
3
2
1
0
L S
L S+P

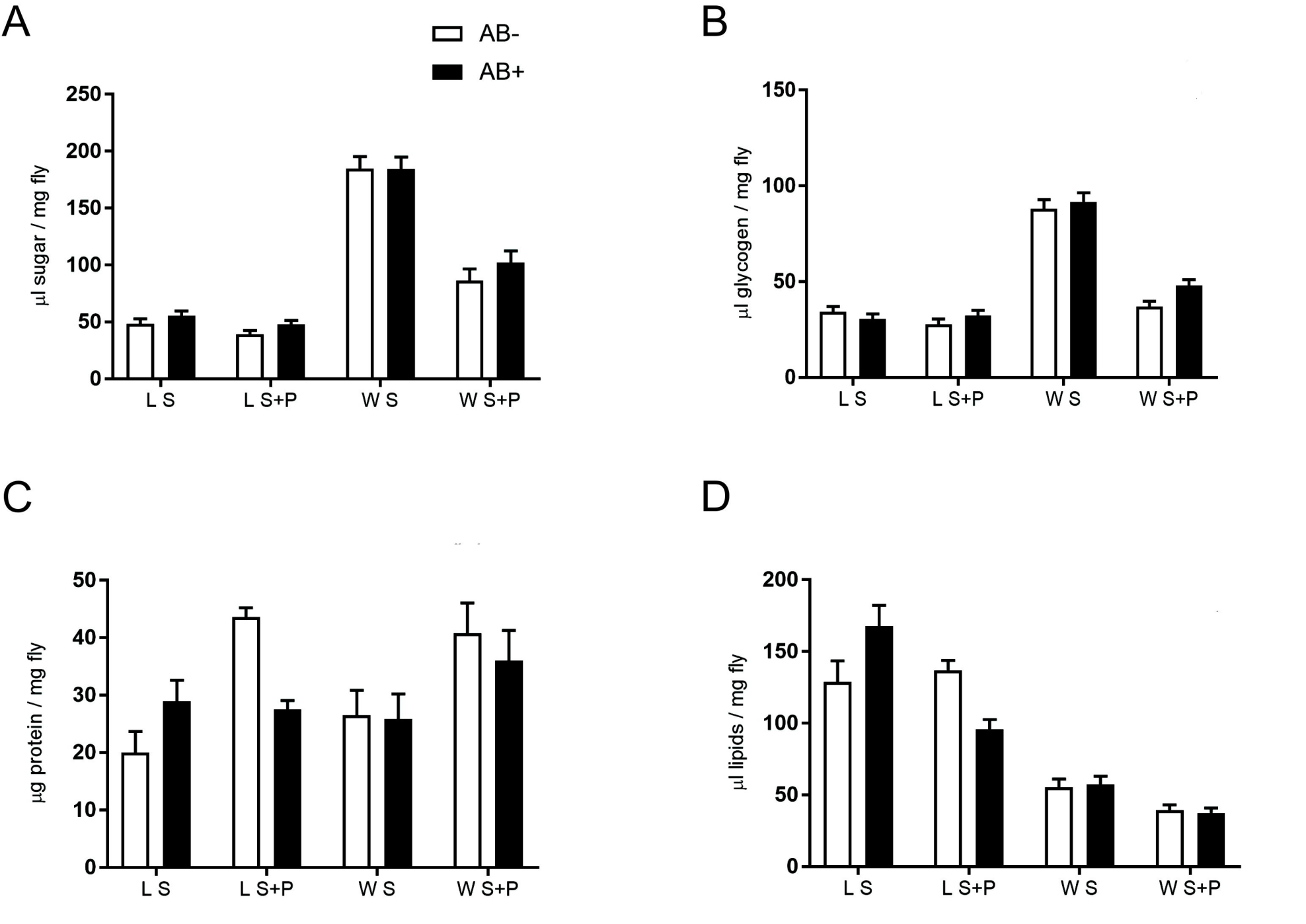
A


Cumulative Proportion Surviving
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
0
1
2
3
4
5
6
7
8
9
10
11
12
Days
S
S+AB
S+P
S+P+AB

B


Cumulative Proportion Surviving
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
-0,1
0
2
4
6
8
10
12
14
16
18
20
22
Days
S
S+AB
S+P
S+P+AB


AB-
AB+
Weight (mg)
5
4
3
2
1
0
L S
L S+P
W S
W S+P


A
AB-
AB+
µl sugar / mg fly
250
200
150
100
50
0
L S
L S+P
W S
W S+P
B
µl glycogen / mg fly
150
100
50
0
L S
L S+P
W S
W S+P
C
µg protein / mg fly
50
40
30
20
10
0
L S
L S+P
W S
W S+P
D
µl lipids / mg fly
200
150
100
50
0
L S
L S+P
W S
W S+P