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Give us the tools and we will do the job: symbiotic bacteria affect olive fly fitness in a diet-dependent fashion

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Olive flies (Bactrocera oleae) are intimately associated with bacteria throughout their life cycle, and both larvae and adults are morphologically adapted for housing bacteria in the digestive tract. We tested the hypothesis that these bacteria contribute to the adult fly's fitness in a diet-dependent fashion. We predicted that when dietary protein is superabundant, bacterial contribution will be minimal. Conversely, in the absence of protein, or when only non-essential amino acids are present (as in the fly's natural diet), we predicted that bacterial contribution to fitness will be significant. Accordingly, we manipulated diet and the presence of bacteria in female olive flies, and monitored fecundity—an indirect measure of fitness. Bacteria did not affect fecundity when females were fed a nutritionally poor diet of sucrose, or a protein-rich, nutritionally complete diet. However, when females were fed a diet containing non-essential amino acids as the sole source of amino nitrogen, egg production was significantly enhanced in the presence of bacteria. These results suggest that bacteria were able to compensate for the skewed amino acid composition of the diet and may be indispensable for wild adult olive flies that subsist mainly on nitrogen-poor resources such as honeydew.

Keywords: Tephritidae; bacterial symbionts; nutritional ecology

1. INTRODUCTION

Insects are the dominant multicellular animals in terrestrial habitats, and maintain intricate and complex interactions with other organisms. Chief among these are the intimate symbioses that have evolved with bacteria and other micro-organisms. These interactions are widespread and range from casual associations to complete and other micro-organisms. These interactions are wide-ranging and complex. Insects are the dominant multicellular animals in terrestrial habitats, and maintain intricate and complex interactions with other organisms. Chief among these are the intimate symbioses that have evolved with bacteria and other micro-organisms. These interactions are widespread and range from casual associations to complete and other micro-organisms. These interactions are wide-ranging and complex. In these associations, insects harness the unique metabolic pathways of bacteria to gain access to resources that are otherwise inadequate for supporting development and reproduction. The most developed of these associations are found in insects that specialize in feeding on nutritionally poor, monotonous diets (e.g. sap-feeding homopterans and some blood feeders). In these cases, the physiology and life cycle of the host and symbiont are so intimately intertwined that the elimination of the latter completely impairs the ability of the insect to subsist on its natural food source (Baumann et al. 2006; Douglas 2009). However, owing to the varied choice of nutrients available in complex diets, a symbiont's contribution may only be apparent when the host is nutritionally compromised. For example, Carpenter ants (Camponotus) are opportunistic feeders that carry the intracellular symbiont Blochmannia (reviewed by Zientz et al. 2005). These bacteria compensate for dietary deficiencies in essential amino acids and sustain the fitness of the colony in their absence (Feldhaar et al. 2007). Other ants harbour extracellular bacteria in their hindguts, which are assumed to recycle nitrogenous waste and thus nutritionally upgrade the diet of their host (reviewed by Cook & Davidson 2006). By associating with bacteria, these ants, although capable of occasionally obtaining protein through scavenging or predation, gain an important advantage by having the ability to subsist on nitrogen-poor diets such as plant exudates and honeydew without experiencing protein deficiencies (Davidson et al. 2003; Stoll et al. 2007; Russell et al. 2010). Similarly, many cockroaches, which are also typical omnivores, harbour intracellular bacteria (Blattabacterium sp.; Bandi et al. 1994). These bacteria apparently recycle uric acid reserves, providing the insect with usable nitrogenous compounds during times of nitrogen famine (Dasch et al. 1984; Ishikawa 1989; Lopez-Sanchez et al. 2009). Thus, when feeding on complex diets, symbiotic bacteria may act as a buffering agent—filling up the nutrient voids in the varied landscape of their host's diet and consequently optimizing fitness.

Fruit flies (Diptera: Tephritidae) are well known for their association with bacteria (reviewed by Drew &
between nutrition, gut bacteria and fecundity in other fruit flies suggest that gut bacteria compensate, at least partially, for amino acid and vitamin deficiencies in the diet (Miyazaki et al. 1968; Boush et al. 1969; Hagen & Tassan 1972; Tsiropoulos 1981b; Tamashiro et al. 1990). The apparent dependence of wild olive flies on resources that are poor and unbalanced in their dietary nitrogen substantiates this suggestion. However, as flies feed on various substrates, bacterial contribution may be dependent on the nutritional value of the diet. Accordingly, we tested the hypothesis that the gut bacteria of the olive fly contribute to fitness in a diet-dependent fashion. Specifically, we predicted that when dietary protein is superabundant, bacterial contribution will be minimal and thus will have no consequences for fitness. Conversely, in the absence of protein, or when only NE amino acids are present (as in the fly’s natural diet), we predicted that bacterial contribution to fitness will be more significant and apparent. Accordingly, we manipulated diet and the presence of bacteria in female olive flies, and monitored fecundity—an indirect measure of fitness.

2. MATERIAL AND METHODS
(a) Fly origin and maintenance
Experiments were conducted either with wild flies or with their F1 progeny, all of which completed their larval development in olives and pupated in the laboratory. Wild flies were reared out of infested, green/semi-ripe Manzanillo or Suri olives picked in late November 2008 in Moshav Kidron, Israel. Some of the adults obtained thereby were then used to establish a small breeding colony, which reproduced in unripe, green Suri olives, picked in December 2008 in Rehovot, Israel, and generated the F1 progeny. Infested olives were incubated over vermicultile-filled trays in which the mature larvae could pupate. Trays containing pupae were then placed in 1001 mesh cages supplied with sucrose and water, which housed the newly ecysed flies. Subsequently, 1–3-day-old adults were separated by sex and maintained in groups of approximately 150 individuals in 51 cages for the next 12–14 days—a period required to mature sexually (Zervas 1983). During this period, males were offered a standard diet consisting of 3:1 (v/v) mixture of sucrose and yeast hydrolysate, respectively, while females were provided only with sucrose, in addition to water. On their 12–17th day, females were joined with the males in 30 cm cubical cages, where mating took place. Couples were carefully collected and confined in glass tubes and left undisturbed overnight. Mated females, collected over the course of the next 2–4 days, were maintained on sucrose and water, then randomly assigned to treatment groups and fed the appropriate diet for the next 20 days.

During this period, females were maintained individually in 100 ml transparent plastic cages and fed through a sterile glass capillary that contained the diet solution. Each capillary was replaced every 24 h in order to minimize the occurrence of micro-organisms in the diet and to ensure the bactericidal effect of the antibiotic. Females intended for microbiological examinations were maintained in groups of six individuals per cage but were otherwise exactly as described above. At the end of the treatment period, all flies were frozen (−80°C) until further processing. All experiments and rearing procedures were conducted in a controlled environment (LD 16 : 8, 25 ± 1.5°C, 65 ± 10% RH).
Table 1. Composition of the diets offered to females. (S, sugar; NE, sugar and non-essential amino acids; F, full diet.)

| constituents/diet    | amount (mg) |
|----------------------|-------------|
| NE amino acids       |             |
| l-alanine            | 73.69       |
| l-aspartic acid      | 106.57      |
| l-cysteine           | 38.55       |
| l-glutamic acid      | 370.72      |
| glycine              | 85.03       |
| l-proline            | 117.90      |
| l-serine             | 73.69       |
| l-tyrosine           | 45.35       |
| minerals             |             |
| FeCl₃                | 1           |
| Na₂MoO₄              | 0.20        |
| H₃BO₃               | 0.28        |
| MnSO₄                | 0.21        |
| ZnSO₄                | 0.0240      |
| CuSO₄                | 0.0025      |
| MgSO₄                | 20          |
| Na₃HPO₄              | 16          |
| CaCl₂                | 2           |
| NaCl                 | 10          |
| antibiotics          |             |
| piperacillin         | 20/—        |
| sucrose              | 18 000      |
| yeast hydrolysate    | 9000        |
| DDW                  | 100 000     |

Diet stock solutions were prepared under microbiologically controlled conditions using double-distilled water (DDW) as follows: sucrose and mineral salts were dissolved separately in water, then mixed and sterilized by autoclaving. After cooling to room temperature, an appropriate amount of autoclaved water or 0.2 μl filter sterilized solutions of amino acids or yeast hydrolysate was added to yield diets S, NE and F, respectively. The antibiotic was added to half of the volume of each of the resulting diet solutions to a final concentration of 100–200 μg ml⁻¹, depending on the diet (table 1). These concentrations were previously found to effectively clear the gut of bacteria (M. Ben-Yosef 2009, unpublished data; see also Ben-Yosef et al. 2008a,b). Finally, solutions were aliquoted and frozen (−20°C) until use and for a maximum period of one month.

(c) Effect of antibiotic treatment on gut bacteria

In the wild populations we worked with, the most prominent bacterial species is Ca. E. dacicola (Estes 2009). To estimate the effectiveness of the antibiotic in reducing the bacterial populations in individual females, we counted the bacteria housed within the oesophageal diverticulum of females from all treatment groups (n = 6 in each group).

Initially, females were surface sterilized as follows: insects were suspended for 1 min in a mild detergent solution, washed in sterile distilled water and resuspended in 70 per cent ethanol for 1 min. Ethanol was removed by a final wash in sterile phosphate buffered saline (PBS). The oesophageal diverticulum of each fly was then aseptically dissected out of the head using sterile forceps and homogenized in 50 μl of sterile PBS. A 3 μl sample of each homogenate was subsequently spread within the boundaries of a 6 mm diameter well on a sterile, gelatin-coated, Teflon-laminated slide (MAGV, Germany). Samples were then stained with DAPI (4',6-diamidino-2-phenylindole; 20 μl of a 4.6 μg l⁻¹ solution) for 10 min on ice and in the dark. Excess DAPI was removed with cold distilled water, and the slide was air dried in the dark and subsequently treated with an antifadent (Citifluor, Canterbury, UK). A maximum number of 600 bacterial cells were then counted in 5–50 randomly chosen fields using an Olympus BX51 epifluorescence microscope (Glockner et al. 2000). Finally, the average number of bacteria per field was used to calculate the total number of bacteria per oesophageal diverticulum.

(d) Effect of antibiotic treatment on female fecundity

During the 20 day treatment period, the eggs laid by each female were collected and counted every 4 days. Paraffin domes, prepared as described in Hagen et al. (1963), were placed on the floor of the cages, serving as olive mimics and a substrate through which oviposition took place. Nonetheless, not all the eggs accumulated inside the domes and, in order to minimize count errors, females were transferred to a new cage every time the eggs were counted. Two replicates were conducted for each diet with a total of 20 and 24 females used in each treatment group of the F and S diets, respectively (11–12 females per replicate). For the NE amino acid diet, a total of 30 females were used in each treatment group (15 females per replicate).

(e) Statistical analysis

Differences in mean population size of gut bacteria were established between the two treatment groups of each diet using non-parametric analyses (Wilcoxon signed-rank test).
Female body size (estimated by wing length, measured from the tip of the third radial vein to the alular notch) was not significantly correlated with fecundity, nor was it significantly different between the two treatment groups of each diet, and was therefore not included in the analysis. The datasets of females fed all three diets were log-transformed (log_{10} (n + 1); n = number of eggs) to obtain homogeneous variances (Bartlett’s test) and the effects of two fixed factors, ‘diet’ and ‘antibiotic treatment’, on female fecundity were analysed by two-way analysis of variance (ANOVA) in a full factorial design. In another model, the effects of the fixed factors ‘antibiotic treatment’ and ‘time after onset of treatment’ on egg-laying patterns throughout the experimental period were established by ANOVA using a factorial hierarchical design with ‘female’ as a third, random factor nested within ‘antibiotic treatment’. An additional random factor—‘replicate’—was initially included in these models; however, all the interactions involving replicate were found to be highly non-significant (p > 0.24 and p > 0.18, respectively), and it was therefore omitted from the analyses. Within the models, a priori comparisons (t-test) were used to establish differences between the mean fecundity of females in the two treatment groups of each diet, or in the number of eggs laid by females in the two treatment groups at different time periods.

Mortality was uncommon during the treatment period, occurring only in F-fed females (n = 3 in both the antibiotic-treated and non-treated groups) and NE-fed females (n = 3 and 2, antibiotic-treated and non-treated females, respectively). Data obtained from females who died during the experimental period were not included in the analyses. All data were analyzed using JMP 7 statistical package (SAS, Cary, NC). Means and their standard errors are reported.

3. RESULTS
(a) Effect of antibiotic treatment on the abundance of gut bacteria
Supplementing the diet with antibiotics significantly reduced the size of the bacterial population housed within the oesophageal diverticulum (figure 1). This organ, which normally appears milky-white as a result of the large bacterial mass it contains, was found to be reduced in size and translucent in antibiotic-treated females. Correspondingly, very few bacteria were detected in the oesophageal diverticulum of females fed on sugar with or without NE amino acids (diets S and NE, respectively) after 20 days of exposure to the antibiotic (4.2 × 10^5 ± 2.7 × 10^4 and 4.3 × 10^3 ± 1.3 × 10^2 bacteria per female, diets S and NE, respectively). Bacteria were significantly more abundant in S-fed and NE-fed females whose microbiota was not manipulated and surpassed the population in antibiotic-treated females by several orders of magnitude (5.8 × 10^3 ± 1.9 × 10^5 and 9.3 × 10^5 ± 1.7 × 10^5 bacteria per female, diets S and NE, respectively; Wilcoxon signed-rank test: Z = −2.8, p = 0.0051 in both analyses; figure 1). The bacterial population in females maintained on the nutritionally complete diet (diet F) was also reduced by the antibiotics (9.9 × 10^3 ± 5.5 × 10^4 and 3.7 × 10^3 ± 1.1 × 10^3 bacteria/female, antibiotic-treated and non-treated females, respectively; Wilcoxon signed-rank test: Z = −1.84, p = 0.065; figure 1).

(b) Effect of antibiotic treatment on female fecundity
Feeding on antibiotics affected female fecundity in a diet-dependent fashion (two-way ANOVA; full model: F = 18.08, p < 0.0001, r^2 = 0.39). Post hoc comparisons between antibiotic-treated and non-treated females fed the same diet revealed that mean fecundity was not affected by the antibiotic when females were maintained on sugar or on the nutritionally complete diet respectively (two-way ANOVA followed by t-test; t = 0.26, p = 0.78, and t = 1.19, p = 0.23, for S and F, respectively; figure 2). Within these diet groups, females produced similar numbers of eggs regardless of the antibiotic treatment (S-fed females: 21.04 ± 3.95 and 25.58 ± 5.17; F-fed females: 136.3 ± 27.89 and 183.95 ± 22.61 eggs/female, antibiotic treated and non-treated females, respectively). However, females whose diet contained NE amino acids as the sole source of nitrogen (diet NE) suffered a significant reduction in egg production when bacteria were absent from the gut.
after onset of treatment; during the last three egg collections (at 12, 16 and 20 days egg production associated with antibiotic-treated females difference in fecundity resulted from a sharp decrease in (forms the characteristic population of the midgut serves as the main source of the bacterial inoculum that organ is a site of intense bacterial reproduction and Our use of a broad-spectrum antibiotic effectively cleared 4. DISCUSSION

12.6 than non-treated females (days 9–12: 2.77 + 6.92 eggs/female, antibiotic-treated females consistently produced fewer eggs (two-way ANOVA followed by t-test; t = 3.63, p = 0.0004; figure 2). The fecundity of these females was reduced by more than half when treated with antibiotics (19.62 ± 4.17 and 51.21 ± 6.92 eggs/female, antibiotic treated and non-treated females, respectively).

We next attempted to understand the temporal manner of the contribution of bacteria to the NE-fed females. Comparing the number of eggs produced by these females at each of the five egg collection periods showed a gradual decrease in egg production associated with antibiotic-treated females (ANOVA; full model: $F = 6.15$, $p < 0.0001$, $r^2 = 0.64$). While similar numbers of eggs were produced by antibiotic-treated and non-treated females at the beginning of the treatment period (days 1–4: 6.4 ± 1.91 and 8.57 ± 1.87 eggs/female, respectively; $t = 1.58$, $p = 0.11$; days 5–8: 6.55 ± 1.99 and 10.67 ± 1.96 eggs/female, respectively; $t = 1.53$, $p = 0.12$; ANOVA followed by t-test; figure 3), the main difference in fecundity resulted from a sharp decrease in egg production associated with antibiotic-treated females during the last three egg collections (at 12, 16 and 20 days after onset of treatment; figure 3). During this time, antibiotic-treated females consistently produced fewer eggs than non-treated females (days 9–12: 2.77 ± 1.53 and 12.6 ± 1.5 eggs/female, respectively; days 13–16: 2.44 ± 1.36 and 13.25 ± 1.34 eggs/female, respectively; days 17–20: 1.44 ± 0.9 and 6.1 ± 0.89 eggs/female, respectively; ANOVA followed by t-test: $t = 4.85$, $p < 0.0005$; $t = 6.11$, $p < 0.0005$; $t = 3.7$, $p < 0.0005$, respectively).

![Figure 3. Oviposition pattern of females fed on sugar and non-essential amino acids (diet NE) throughout the 20 day treatment period. Antibiotic-treated females: shaded box. Non-treated females: unshaded box. *p < 0.0005.](image)

4. DISCUSSION

Our use of a broad-spectrum antibiotic effectively cleared the female oesophageal diverticulum of bacteria. This organ is a site of intense bacterial reproduction and serves as the main source of the bacterial inoculum that forms the characteristic population of the midgut (Capuzzo et al. 2005; Estes 2009; Estes et al. 2009). Therefore, by quantifying the bacterial population in the oesophageal diverticulum, we provide a reasonable measure of the effectiveness of the antibiotic in eliminating bacteria from the entire intestinal tract. The bactericidal effect of the antibiotic was especially prominent in females maintained on the S and NE diets, and somewhat less so in females maintained on the F diet (figure 1). It is possible that the conditions of ample nutrients and presence of antibiotics in the gut of F-fed females favoured the more resistant bacterial types and shifted the composition of the bacterial community, eventually leading to a gut microbiota that is less susceptible to the antibiotic. Exactly how the gut bacterial community is affected by diet and antibiotics, the interaction between them and the mechanism of host regulation need to be further clarified.

In this study, we demonstrate a contribution of the intestinal microbiota to egg production—an indirect fitness measure of female olive flies. This contribution, however, was diet-dependent: insignificant when females were maintained on poor (S) or complete (F) diets while eminent when fed a diet unbalanced in amino acid content (NE; figure 2). The fact that female fecundity was sustained only in the presence of bacteria when essential amino acids were absent from the diet suggests that these missing nutrients were supplied to the females by their intestinal bacteria. In order to substantiate this suggestion, we would like to address two issues. First, to eliminate the gut bacterial population, we used an antibiotic which in addition to its bactericidal properties may also exert a direct adverse effect on egg production. Such an effect, however, must have been small enough or non-existent in order for antibiotic-treated, S-fed and F-diet-fed females to remain as fecund as their non-treated counterparts, especially when considering the relatively high antibiotic content of their diets (figure 2 and table 1). Additionally, the postponed effect of the antibiotic treatment on egg production (figure 3) implies that the antibiotic itself was not the direct cause of the decline in fecundity because then this effect would already be evident in the first days after onset of treatment. It is more probable that the gut bacterial population of antibiotic-treated females gradually decreased during the first week of the experiment, and with it the ability of females to compensate for the missing nutritional components in their diet and to mature eggs.

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Finally, when tested on another tephritid fruit fly—
*Ceratitis capitata*—this antibiotic was found to have no
detrimental effects on diet consumption, longevity, weight or nutritional status (Ben-Yosef et al. 2008a,b). For these reasons, we feel it is safe to assume that consuming the antibiotic affected the flies only indirectly, by decreasing the gut bacterial population, and not directly by detrimentally affecting the insect’s food consumption or metabolism.

A second issue that needs consideration is the bacterial provisioning of nutrients other than amino acids. NE-fed females were deprived of essential nutrients other than amino acids, such as vitamins, for which the importance to female fecundity was previously demonstrated (Tsiropoulos 1980a,b). However, the contribution of vitamins to egg production when added to a basal diet of sucrose and minerals was found to be negligible compared with that of amino acids (Tsiropoulos 1980a). Additionally, if vitamins were a major bacteria-derived nutrient affecting fecundity, we would have expected bacteria to encourage egg production in S-fed females as well. We thus suggest that the intestinal bacteria of olive flies contribute mainly to the nitrogen budget of their host, probably by supplementing the diet with protein or amino acids. Specifically, we suggest that bacteria served as a source of the missing essential amino acids in diet NE. However, in addition to amino acids, other essential nutrients such as vitamins may have been supplied as well.

The significance of gut bacteria to the biology of adult olive flies is apparent when considered in light of their nutritional ecology. Adult flies feed mainly on plant-derived exudates such as nectar and various leachates, or on honeydew. While these substrates easily fulfil the daily energetic needs by providing ample carbohydrates, the reproductive demands for protein, especially in females, is unlikely to be satisfied by the poor or unbalanced amounts of utilizable nitrogen in these foods (Tsiropoulos 1977; Drew & Yuval 2000). This nutritional gap may be bridged by occasionally feeding on nitrogen-rich substrates such as pollen and animal excreta, or on leaf surface and saprophytic bacteria (Drew et al. 1983; Drew & Yuval 2000; Sacchetti et al. 2008). Alternatively, the intestinal microbiota can provide the metabolic capability to generate the nitrogenous components missing from the diet. This may be achieved by fixing atmospheric nitrogen, recycling nitrogenous waste or by using the existing nitrogen in the diet (Drew & Lloyd 1991; Behar et al. 2005). The last two possibilities seem more relevant to our study as the only case in which bacteria may have experienced nitrogen shortage, and thus engaged in nitrogen fixation, was in S-fed females, where no contribution to egg production was detected. Apparently, these bacteria require some nitrogenous building blocks, either from the ingested diet (e.g. NE amino acids) or from metabolic waste (e.g. uric acid) in order to synthesize amino nitrogen. Amino acids may then be secreted by bacteria and directly assimilated by the fly as demonstrated in other insect–bacteria associations (e.g. aphids; Douglas 1998). Alternatively, when conditions in the gut support a high rate of bacterial reproduction, the flies may be realizing their need for protein simply by digesting the excessive bacterial biomass in the gut (Drew et al. 1983; Drew & Lloyd 1991; Lemos & Terra 1991).

Close associations with micro-organisms are also found among other insects that resemble fruit flies in their nutritional preferences. Many ants, for example, are associated with symbiotic bacteria, probably because honeydew and plant exudates are a major part of their diet (Zientz et al. 2005; Stoll et al. 2007; Russell et al. 2010). In certain ants of the genus *Tetraponera* where honeydew has become the main source, if not the sole source, of nutrients, large masses of extracellular bacteria are harboured in a unique pouch in the anterior hindgut (Billen & Buschinger 2000). These bacteria are probably involved in the nutritional enhancement of the ants’ diet by recycling nitrogenous waste into amino acid precursors (van Borm et al. 2002; Russell et al. 2010). Similarly, some Chrysopids (e.g. *Chrysoperla carnea*), which feed primarily on honeydew and plant exudates as adults, are assumed to overcome the nutritional limitations of this diet by using symbiotic yeasts housed in a large diverticulum of the foregut (reviewed by Lundgren 2009). These insects, which have been referred to as ‘secondary’ or ‘cryptic’ herbivores by some authors, may experience the similar low-nitrogen diet characteristic of true herbivores and cope with nitrogen deprivation by associating with bacteria (Cook & Davidson 2006). Olive flies and other tephritids, who share a similar nutritional niche, may be associated with bacteria for the same reason. Thus, by ingesting a varied diet in the wild, olive flies have the potential for acquiring the nitrogen needed for reproduction. However, by simultaneously nurturing a beneficial intestinal microbiota, they gain the ability to continuously subsist on food sources such as honeydew that are more marginal in terms of their nitrogenous composition.

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