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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 co-dependence (Ishikawa 2003; Dillon & Dillon 2004; Baumann *et al.* 2006). Bacteria may contribute to host immunity, thermoregulation and communication (reviews in Bourtzis & Miller 2003, 2009). However, the major interface whereby mutualistic, beneficial associations have evolved is nutritional (e.g. Buchner 1965; Douglas 2009). 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).

Insects that are less restricted to feeding on one particular diet may also derive nutritional benefits from microbial symbionts (Dasch *et al.* 1984; 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 *Blochmania* (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 &

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Lloyd 1991; Lauzon 2003; Behar *et al.* 2009), and many species evince morphological adaptations for housing bacteria in the digestive tract (Stammer 1929; Girolami 1973; Mazzon *et al.* 2008). The olive fly, *Bactrocera oleae* (Gmelin), was the first member of this family in which an association with bacterial symbionts was described (Petri 1910; see also Buchner 1965; Manousis & Ellar 1988), and may be the most specialized in this regard among fruit-infesting members of the Dacinae and Trypetinae (Stammer 1929; Girolami 1973). In adult olive flies, a diverticulum of the oesophagus accommodates a large and proliferating population of extracellular bacteria (Petri 1910; Stammer 1929; Girolami 1973). This organ periodically releases bacteria into the oesophagus, which inoculate and densely colonize the anterior midgut (Petri 1910; Girolami 1973; Capuzzo *et al.* 2005). The association extends via the egg and continues in the larval stage where bacteria heavily populate the four midgut caeca during the entire larval development within the olive fruit (Petri 1910; Stammer 1929; Mazzini & Vita 1981). Although recent studies have identified several species of bacteria in the digestive tract of wild olive flies (Kounatidis *et al.* 2009), the most common and widespread of these is *Candidatus Erwinia dacicola* (Capuzzo *et al.* 2005; Estes 2009; Estes *et al.* 2009).

Adult olive flies are opportunistic feeders that are not restricted to feeding on one particular diet and exploit various substrates such as honeydew, nectar, fruit and plant exudates, and occasionally bird droppings and pollen (Christenson & Foote 1960; Drew & Yuval 2000). The nutritional value of these food sources may vary greatly; however, the ones considered most important (e.g. honeydew, nectar and plant-derived exudates; Downes & Dahlem 1987; Vijaysegaran *et al.* 1997) are generally rich in carbohydrates but relatively poor in amino acids (Wackers 2005; Lundgren 2009). Moreover, in some cases, the small amounts of amino acids that are available may be highly unbalanced in composition and contain mainly those that are considered non-essential (NE; Wackers 2005; Douglas 2006). Thus, despite their seemingly varied choice of foodstuffs, these flies rely on food sources that are poor and unbalanced in their amino acid composition.

The low nitrogen content of such a diet seems to contrast with the nutritional demands of adult olive flies, which (like many other long-lived tephritid fruit flies) require a continuous external supply of protein in order to achieve their reproductive potential (Tsitsipis 1989; Drew & Yuval 2000). This requirement is particularly important in females, whose fecundity greatly depends on the nitrogenous contents of their diet (Economopoulos *et al.* 1976; Tsiropoulos 1980*a*, 1981*a*). When peptides or proteins are not available, a source of essential amino acids is obligatory for oogenesis as olive flies (like other eukaryotes) are unable to synthesize these compounds (Tsiropoulos 1984). Indeed, in the laboratory, females are unable to adequately synthesize protein and to mature eggs if maintained on diets lacking essential amino acids (Tsiropoulos 1983).

The proliferation of bacteria in the gut and the dependence of adults on essential amino acids suggest that bacteria have a role in nitrogen metabolism. Previous investigations aimed to determine the relationship 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 1981*b*; 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 vermiculite-filled trays in which the mature larvae could pupate. Trays containing pupae were then placed in 100 l mesh cages supplied with sucrose and water, which housed the newly ecdysed flies. Subsequently, 1–3-day-old adults were separated by sex and maintained in groups of approximately 150 individuals in 5 l 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 ($^{w}/_{w}$) 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, $25 \pm 1.5^{\circ}$C, $65 \pm 10\%$ RH).

Table 1. Composition of the diets offered to females. (S, sugar; NE, sugar and non-essential amino acids; F, full diet.)

	amount (mg)		
constituents/diet	S	NE	F
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			
$FeCl_3$	1	1.133	—
Na_2MoO_4	0.20	0.226	—
H_3BO_3	0.28	0.317	—
$MnSO_4$	0.21	0.237	—
$ZnSO_4$	0.0240	0.02716	—
$CuSO_4$	0.0025	0.00283	—
$MgSO_4$	20	22.675	—
Na_2HPO_4	16	18.136	—
$CaCl_2$	2	2.267	—
NaCl	10	11.337	—
antibiotics			
piperacillin	20/—	10/—	20/—
sucrose	18 000	20 000	18 000
yeast hydrolysate	—	—	9000
DDW	100 000	100 000	100 000

(b) *Diets and antibiotics*

Females were maintained on three artificial diets (table 1), as specified below. Two diets represented two extremes in terms of their nutritional composition, with one lacking most nutritional groups and the other being nutritionally complete. A third diet contained sugar, minerals and NE amino acids as the sole source of amino nitrogen. Antibiotics (piperacillin, Fluka), which had previously been used effectively to suppress the gut bacterial population in the Mediterranean fruit fly (Ben-Yosef *et al.* 2008*a*), were added to the diet of half of the females in each of the three groups in order to eliminate the bacterial component of the gut microbiota.

Diet S (sugar) was composed of a 17 per cent ($^{w}/_{v}$) sucrose solution supplemented with minerals needed to sustain proper metabolism in bacteria and insect alike (Tsiropoulos 1980*a*; Dadd 1985; Garrity 2001). The females of this treatment group were not provided with an external source of protein nor amino acids.

Diet NE (non-essential amino acids) was similar in composition to diet S but additionally contained the eight NE amino acids. Ratios between the different amino acids were adopted from the chemically defined diet described by Chang *et al.* (2001) that supported high fecundity in the Mediterranean fruit fly. However, their concentration was modified according to the maximal solubility of tyrosine in water at 25°C.

Diet F (full) consisted of a mineral-free sucrose solution, supplemented with yeast hydrolysate (Difco). This complete diet constitutes a rich source of peptides, amino acids, vitamins and minerals, in addition to carbohydrates, and is known to support high fecundity in female tephritids (reviewed by Tsitsipis 1989).

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 μ 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 $\mu g\,ml^{-1}$, 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.* 2008*a*,*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 $\mu g\,l^{-1}$ 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).

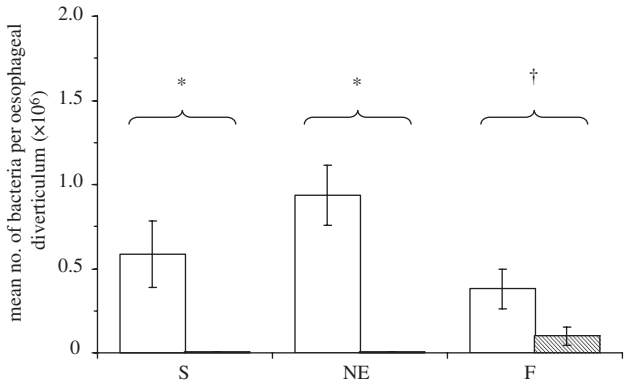

Figure 1. Effect of the antibiotic treatment on the bacterial population housed within the oesophageal diverticulum as estimated by total bacteria counts. Incorporating the antibiotic piperacillin into all three diets (S, sugar; NE, sugar and non-essential amino acids; F, full diet) decreased the number of bacteria in the gut. Antibiotic-treated females: shaded box. Non-treated females: unshaded box. $^{*}p < 0.05$; $^{\dagger}p = 0.065$.

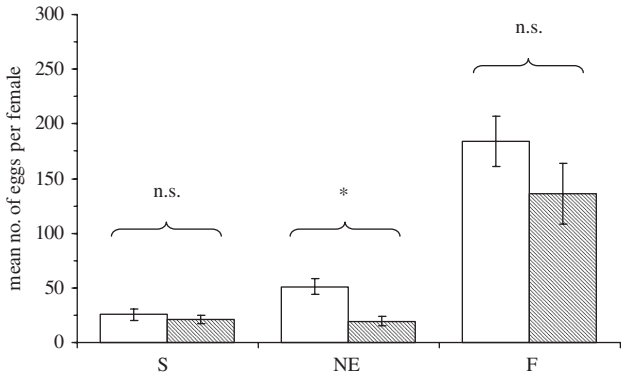

Figure 2. Mean number of eggs laid by females as affected by the presence (shaded box) or absence (unshaded box) of antibiotics in the diet. Fecundity significantly depended on the presence of bacteria only when females were offered NE amino acids as the sole source of nitrogen in their diet (diet NE). $^{*}p < 0.001$.

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 analysed 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 ($4.2 \times 10^3 \pm 2.7 \times 10^3$ and $4.3 \times 10^3 \pm 1.3 \times 10^3$ 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 ($5.8 \times 10^5 \pm 1.9 \times 10^5$ and $9.3 \times 10^5 \pm 1.7 \times 10^5$ 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 ($9.9 \times 10^4 \pm 5.5 \times 10^4$ and $3.7 \times 10^5 \pm 1.1 \times 10^5$ 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

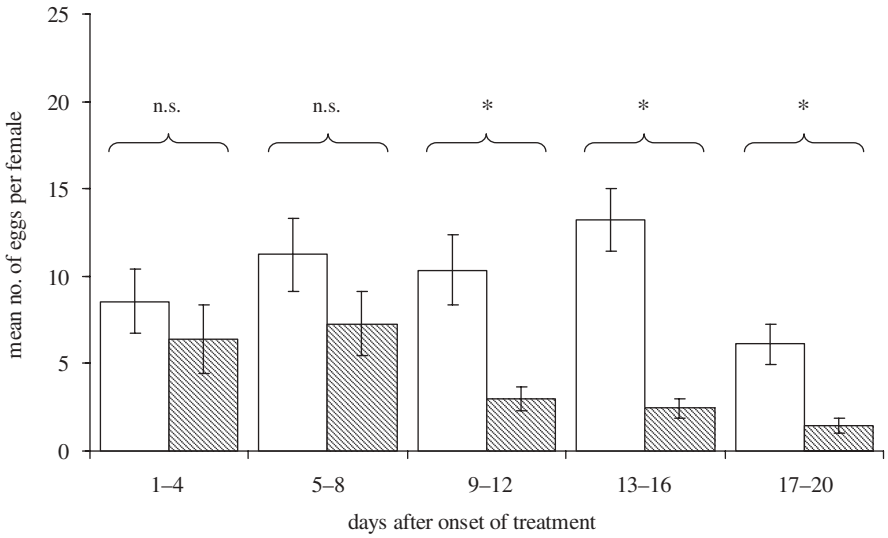

Figure 3. Oviposition pattern of females fed on sugar and non-essential amino acids (diet NE) throughout the 20 day treatment period. Antibiotic-treated females: shaded box. Non-treated females: unshaded box. $*p < 0.0005$.

(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).

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.

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.* 2008*a*,*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 1980*a*,*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 1980*a*). 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 demand 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 existent 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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