Adonia variegata (Coleoptera: Coccinellidae) bears maternally inherited Flavobacteria that kill males only

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SUMMARY

Inherited bacteria that parasitically distort the pattern of sex allocation of their host, biasing allocation towards female progeny, are found in many arthropods. One such manipulation is male-killing, where male progeny of infected females die during embryogenesis. We here provide evidence for a male-killing bacterium in the coccinellid beetle, *Adonia variegata*. We then address 3 questions. First, is this male-killing bacterium one that is found in other hosts, or does it represent a new transition to male-killing within the eubacteria? Using the sequence of the 16S rDNA of the bacterium, we found that the male-killing bacterium is a member of the Flavobacteria–Bacteroides group, most closely related to the male-killing bacterium in another ladybird beetle, *Coleomegilla maculata*. Secondly, is there any evidence that this bacterium affects female host physiology? In a paired test under nutritional stress, we found no evidence for a physiological benefit to infection, and weak evidence of a physiological cost, in terms of reduced fecundity. Thirdly, is there any evidence of host involvement in the transmission of the bacterium to the germ line? We found no evidence of host involvement. Rather, bacteria migrated to the ovariole independently of host cells. We conclude that the bacterium is a parasite, and discuss how 2 different species of ladybird come to be infected with 1 lineage of bacterium, and why case studies of male-killing bacteria have generally found little evidence of any symbiont contribution to host physiological functioning.

Key words: inherited parasite, Coccinellidae, Flavobacteria, male-killing, symbiosis, biodiversity.

INTRODUCTION

Many species of arthropod are host to bacteria that are vertically transmitted through eggs. These bacteria enter into a variety of relationships with their host. In many cases, the presence of bacteria increases the fitness of the host. These host-beneficial bacteria play a role in host anabolic pathways, and have been implicated in amino acid, vitamin and sterol biosynthesis, and in nitrogen metabolism (Douglas, 1994). In other cases, the bacteria distort the reproductive behaviour of the host, and in particular the pattern of sex allocation. Being maternally inherited, these bacteria are at an evolutionary dead end in the male line. Thus, selection has favoured strains that bias sex allocation towards the production and resourcing of daughters. In consequence, inherited symbionts convert hosts to asexuality (Stouthamer, 1997), bias the primary sex ratio towards females (Dunn *et al*. 1995; Rigaud, 1997), and kill male offspring during embryogenesis (Hurst, Hurst & Majerus, 1997*a*). The death of male offspring (male-killing) is adaptive where the host exhibits sib–sib competition for resources, deleterious sib–sib inbreeding, or sib–sib cannibalism (Skinner, 1985; Werren, 1987; Hurst, 1991), or where male death is accompanied by horizontal transmission (Hurst, 1991).

Male-killers are known from 5 different orders of insect and occur most commonly in taxa such as the coccinellid beetles, where the host behaviour of sibling egg cannibalism favours the evolution of male-killing by bacteria (Hurst & Majerus, 1993). There are 5 records of infection with male-killing bacteria in this group, with prevalence varying from 7 to 50% of female hosts (Lus, 1947; Shull, 1948; Matsuka, Hashi & Okada, 1975; Niijima & Nakajima, 1981; Hurst, Majerus & Walker, 1993; Hurst *et al*. 1996*a*; Majerus *et al*. 1998). To date, 3 different male-killing agents have been recognized: a member of the genus *Rickettsia* (Werren *et al*. 1994; Hurst, Walker & Majerus, 1996*b*), a member of the Flavobacteria–Bacteroides group (Hurst *et al*. 1997*b*), and a member of the genus *Spiroplasma* (Hurst *et al*. 1998).

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We here provide evidence for the existence of a male-killing bacterium in a female from a Turkish population of the coccinellid beetle *Adonia variegata*. We then ask 3 questions. First, is this male-killing agent related to a known male-killing bacterium, or does it represent a new transition to male-killing behaviour within the eubacteria? Second, does the symbiont have any beneficial role in host physiology, or does possession of the bacterium have a physiological cost? Whilst male-killing bacteria with a cost can be maintained in a population by virtue of the increase in survivorship of female hosts following the death of their brothers, this does not preclude the possibility that the bacteria enhance female host fitness through a role in host physiology. We thus examine the effect of the bacterium on host physiological performance. Third, is there any evidence of host involvement in the transmission process, or does the bacterium control its own transmission to the germ line?

MATERIALS AND METHODS

Nature of trait, susceptibility to antibiotics

Adonia variegata adults were collected from a cotton field in Adana, Turkey, during July 1996. Eight females were fed pea aphids, and the eggs produced collected. The number of eggs in each clutch and the proportion of these eggs successfully hatching were recorded. The progeny were reared to maturity and then sexed. Sex was determined using abdominal morphology; the male possessing a distinct ventral 'notch' in the posterior margin of the posterior abdominal tergite, through which the penis protrudes during copulation, compared to the more rounded margin of the posterior tergite of the female. Sexing criteria were checked by dissection of the genitalia, and found to be 100% accurate ($n = 70$).

In a total of 7 crosses, females from the biased sex ratio family L–4 were separately crossed to a male from 1 of the normal families, such that each female had mated with males from a different family. In turn, 1 female from each of the normal sex ratio families (L–1, 2, 3, 5, 6, 7, 8) was crossed to males from normal families, such that each female had mated to a male from a different family from the others. The egg hatch-rate and sex ratio produced by these crosses were recorded as before.

The susceptibility of the trait to antibiotics was tested as described by Hurst, Majerus & Walker (1992). In short, after their initial period of reproduction, females from the above crosses were allowed to feed upon tetracycline in golden syrup (10% w/w) for 4 h daily, over a period of 14 days. The hatch-rate of eggs and sex of progeny was thence recorded. In addition to these, a 'no antibiotic' control was set up where 3 F1 females from family L–4 were allowed to breed over a period of 2 weeks before being treated with golden syrup alone (no tetracycline). This controls against endogenous effects of golden syrup on the sex-ratio and hatch-rates produced by individuals showing the male-killing trait, and also against the effect of host age on the penetrance of the male-killing trait.

Analysis of affiliation of associated microorganisms

Genomic DNA was extracted from ovaries of 3 females from line L–4 (1 F1 female, 2 F2 females) using the methodology detailed in Hurst *et al.* (1997*b*).

Template from these females and 2 water controls were subject to amplification with primers that amplify a wide range of eubacteria (Weisburg *et al.* 1991). In short, the primers 27f (5′-GAGAGTTT-GATCCTGGCTCAG-3′) and 1495r (5′-CTACG-GCTACCTTGTTACGA-3′) were used in a PCR using the reagents and cycle conditions given by Hurst *et al.* (1997*b*). The resultant PCR product was examined on a 1% agarose gel, and agarose containing the DNA excised under long wave length U.V. light. The DNA was liberated from the gel segment using gene-clean (Biorad). This DNA was then subjected to cycle-sequencing with dye-labelled terminators using the primers above, and primers internal to the 16S rDNA fragment, and the products visualized on an ABI automated sequencing machine. Both strands were sequenced, and the coverage was such that over 85% of the sequence is based on information from both strands.

The sequence obtained was subjected to a Blast search (Altschul *et al.* 1990). It was then manually aligned with a group of pre-aligned 16S rDNA sequences from the 5 main groups of the Flavobacteria–Bacteroides lineage (Gherna & Woese, 1992), the genus *Blattabacterium* (Bandi *et al.* 1994, 1995), and the *Coleomegilla maculata* symbiont (Hurst *et al.* 1997*b*). Phylogenetic analysis was effected using TREECON (Van de Peer & De Wachter, 1993) after both including or excluding the 16S rDNA variable regions and any insertions/deletions; the robustness of the result was evaluated by bootstrap analysis.

The hypothesis that this sequence represented that of the *A. variegata* male-killer was tested through the use of a PCR reaction that specifically amplifies this bacterial clade, originally designed for the closely related bacterium in *C. maculata* (Hurst *et al.* 1997*b*). In addition to the genomic DNA samples above, samples were prepared likewise for individuals from each of the 7 uninfected lineages, and from antibiotic-cured individuals. The presence of close relatives of the *C. maculata* male-killer in these samples was then tested. One microlitre of a 10% solution of the genomic DNA was subjected to amplification with the primers specific for the *C.*

Table 1. The proportion of eggs hatching, and the sex-ratio of families produced by 8 wild-caught *Adonia variegata* females

(The female producing a biased sex-ratio is in bold.)

Cross	Eggs laid	Proportion eggs hatched	Progeny reared	Sex ratio (proportion male)
L–1	83	0·903	39	0·565
L–2	23	0·913	7	0·582
L–3	133	0·879	83	0·578
L–4	**193**	**0·507**	**72**	**0·000**
L–5	26	0·846	19	0·368
L–6	35	0·857	23	0·565
L–7	53	0·773	21	0·619
L–8	32	0·968	21	0·381

maculata male-killer, 5′-ATTGTTAAAGTTCCG-GCG-3′ (forward) and 5′-CTGTTTCCAGCTTA-TTCGTAGTAC-3′ (reverse), using the conditions given by Hurst *et al*. (1997*b*). The resultant PCR product was run out on a 1% agarose gel against a size standard. The expectation was of a product of 762 bp in length in samples bearing the bacterium, with no product from other samples.

As a control against poor quality of genomic DNA causing amplification failure, the quality of the DNA extractions that failed to amplify in the specific reaction was verified by attempting amplification with a pair of general primers which amplifies a portion of the beetle *COI* gene of mtDNA (Howland & Hewitt, 1995).

Direct effect of bacterium upon female host fitness

We assessed the effect of infection on fitness in the absence of an egg meal (i.e. physiological effect of the bacterium) by comparing the demography of infected and uninfected females following the method of Hurst *et al*. (1994).

Pairwise comparison of the demography of an uninfected female with that of an infected female was conducted, where the 2 individuals shared common grandparents. Eggs from 2 females (infected and uninfected) were collected, allowed to hatch, and prevented from cannibalizing unhatched eggs by removal of such eggs from clutches with a fine needle. A single neonate larva from the infected female was then placed in a Petri dish with a neonate larva from the comparator strain. This pair of larvae was then fed on excess pea aphids, *Acyrthosiphum pisum*, until pupation. On emergence as adults, the individuals were placed in separate dishes, marked with the pair number and the subscript A or B, depending on order of emergence (A for first to emerge, B for second). If 1 of the pair was male, then the pair was discarded. The members of the pair were weighed to the nearest milligram between 6 and 12 h after emergence.

Longevity and fecundity were then assessed. The females were fed 3 large aphids/day for 9 days. On the tenth day, each adult was placed in a new dish, mated once, and provided with 4 large aphids. This level of food is enough to maintain a low rate of oviposition in normal laboratory lines without inducing high rates of cannibalism of the eggs by the adults. The adults were removed to a new dish containing 4 large aphids daily and the eggs laid on the previous day counted. Pairs where either female consistently failed to produce fertile eggs were typed for infection and discarded from analysis of physiological performance. The individuals were re-mated every 10 days to prevent sperm depletion leading to infertility. The regime of aphids and dish-changing was maintained until 40 days after first mating, after which time the adults were fed daily on artificial food (see Majerus & Kearns, 1989 for recipe). The date of death was recorded.

The infection status of each member of the pair was ascertained *post-hoc* by testing of progeny for presence of the male-killing bacteria through PCR using the *C. maculata* male-killer specific primers (method above).

Process of transmission to the ovariole

We examined the process of bacterial transmission to the ovariole, with particular reference to the role of host cells in bacterial transmission, using electron microscopy. Ovaries of *A. variegata* were removed and fixed for 3 h in cold 2·5% glutaraldehyde in 0·1 M cacodylate buffer (pH 7·2), rinsed in the same fixative, and post-fixed in 1% buffered osmium tetroxide for 1·5 h. They were thence dehydrated through ethanol and embedded in an Epon-Araldite mixture. Ultrathin sections were thence examined and photographed on a Zeiss EM 900.

RESULTS

Nature of trait, susceptibility to antibiotics

The hatch-rates of eggs, and the sex ratio of families produced by each of the 8 females is given in Table 1. The 8 crosses are heterogeneous with respect to sex ratio produced ($\chi^2 = 70{\cdot}9$; 7 D.F.; $P < 0{\cdot}001$). Seven of the crosses (L1–3, L5–8) fall into 1 homogeneous class on the basis of sex ratio ($\chi^2 = 5{\cdot}587$; 6 D.F.; $P > 0{\cdot}1$), and there is no evidence of a sex ratio bias in these crosses (Test *vs* 1:1: $\chi^2 = 1{\cdot}36$; 1 D.F.; $P > 0{\cdot}1$). The remaining cross, (L–4) produced a strongly female-biased sex ratio ($\chi^2 = 36$; 1 D.F.; $P < 0{\cdot}001$). The group of 7 individuals producing a 1:1 sex-ratio produced eggs with a homogeneous egg hatch-rate ($\chi^2 = 8{\cdot}82$; 6 D.F.; $P > 0{\cdot}1$), with 87·5% of eggs hatching. This

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Table 2. The proportion of egg hatching, and the sex ratio of families produced by daughters from the 'female-biased' sex-ratio cross L–4 before and after treatment with tetracycline in golden syrup

(In any cross, the first number indicates the parentage of the female, the second the parentage of the male. Sex ratio given as proportion male; numbers in parentheses detail sample sizes.)

	Before antibiotics		After antibiotics	
Cross	Egg hatch-rate	Sex ratio	Egg hatch-rate	Sex ratio
4·1	0·407 (201)	0·000 (58)	0·663 (261)	0·496 (137)
4·2b	0·477 (218)	0·000 (88)	0·717 (113)	0·580 (31)
4·3	0·446 (193)	0·011 (85)	0·798 (178)	0·500 (106)
4·5	0·250 (148)	0·000 (30)	0·510 (200)	0·555 (63)
4·6	0·432 (185)	0·014 (71)	0·806 (160)	0·472 (127)
4·7	0·436 (78)	0·000 (32)	—	—
4·8	0·396 (139)	0·000 (46)	0·696 (102)	0·272 (44)

Table 3. The proportion of egg hatching, and the sex ratio of families produced by daughters from the 'normal' sex-ratio crosses (L1–3, L5–8) before and after treatment with tetracycline in golden syrup

(In any cross, the first number indicates the parentage of the female, the second the parentage of the male. Sex ratio given as proportion male; numbers in parentheses detail sample sizes.)

	Before antibiotics		After antibiotics	
Cross	Egg hatch-rate	Sex ratio	Egg hatch-rate	Sex ratio
1·2	0·873 (158)	0·471 (121)	0·833 (168)	0·394 (104)
2·7	0·883 (145)	0·431 (102)	0·906 (139)	0·402 (92)
3·1	0·724 (69)	0·454 (44)	—	—
5·6	0·947 (115)	0·494 (79)	—	—
6·3	0·874 (87)	0·516 (62)	0·798 (164)	0·425 (101)
7·8	0·917 (84)	0·529 (68)	0·820 (156)	0·449 (98)
8·7	0·957 (117)	0·470 (100)	0·911 (101)	0·537 (67)

differs significantly from the sex ratio biased individual (L–4), where 50·7 % of eggs hatched ($\chi^2 = 93{\cdot}3$; 1 D.F.; $P < 0{\cdot}001$). Thus, the individual producing a female-biased sex ratio is also producing eggs with higher embryonic mortality.

All 7 crosses involving females from biased sex ratio clutches produced female-biased families, 5 producing females only, and the other 2 just 1 male progeny each (Table 2). In no case did more than half of the eggs from these crosses hatch, and the hatch-rate of eggs from the 7 crosses taken together was 41·1 % (Table 2). In contrast, crosses involving females derived from normal sex ratio families produced families with a normal sex ratio (summed data, test *vs* 1 : 1 sex ratio: $\chi^2 = 1{\cdot}17$; 1 D.F.; $P > 0{\cdot}1$). Egg hatch-rates were high, with 89·0 % of eggs from these crosses hatching (Table 3).

Following treatment with tetracycline in golden syrup, the hatch-rate of eggs from each of the sex ratio females increased (Two-tailed Sign test: $P < 0{\cdot}05$), and all sex-ratio females produced an increased proportion of male progeny (Two-tailed Sign test: $P < 0{\cdot}05$) (Table 2). The sex ratio produced by these females after administration of tetracycline was consistent with equality ($\chi^2 = 0{\cdot}503$; 1 D.F.; $P > 0{\cdot}5$). Normal females allowed to feed upon golden syrup bearing tetracycline showed no consistent changes in either the sex ratio produced, nor in the hatchability of eggs (Table 3). The changes in egg hatch-rate and sex ratio observed following tetracycline treatment of sex ratio females were not apparent when golden syrup alone was administered. In none of the 3 crosses were males produced after golden syrup treatment ($n = 52$, 37 and 27 progeny), and in each case the hatch-rates remained below 50 % in all cases ($n = 131$, 96, 84 eggs).

Analysis of affiliation of associated microorganism

The 16S rDNA sequence produced was 1453 bases in length (EMBL accession no. AJ009687). Blast

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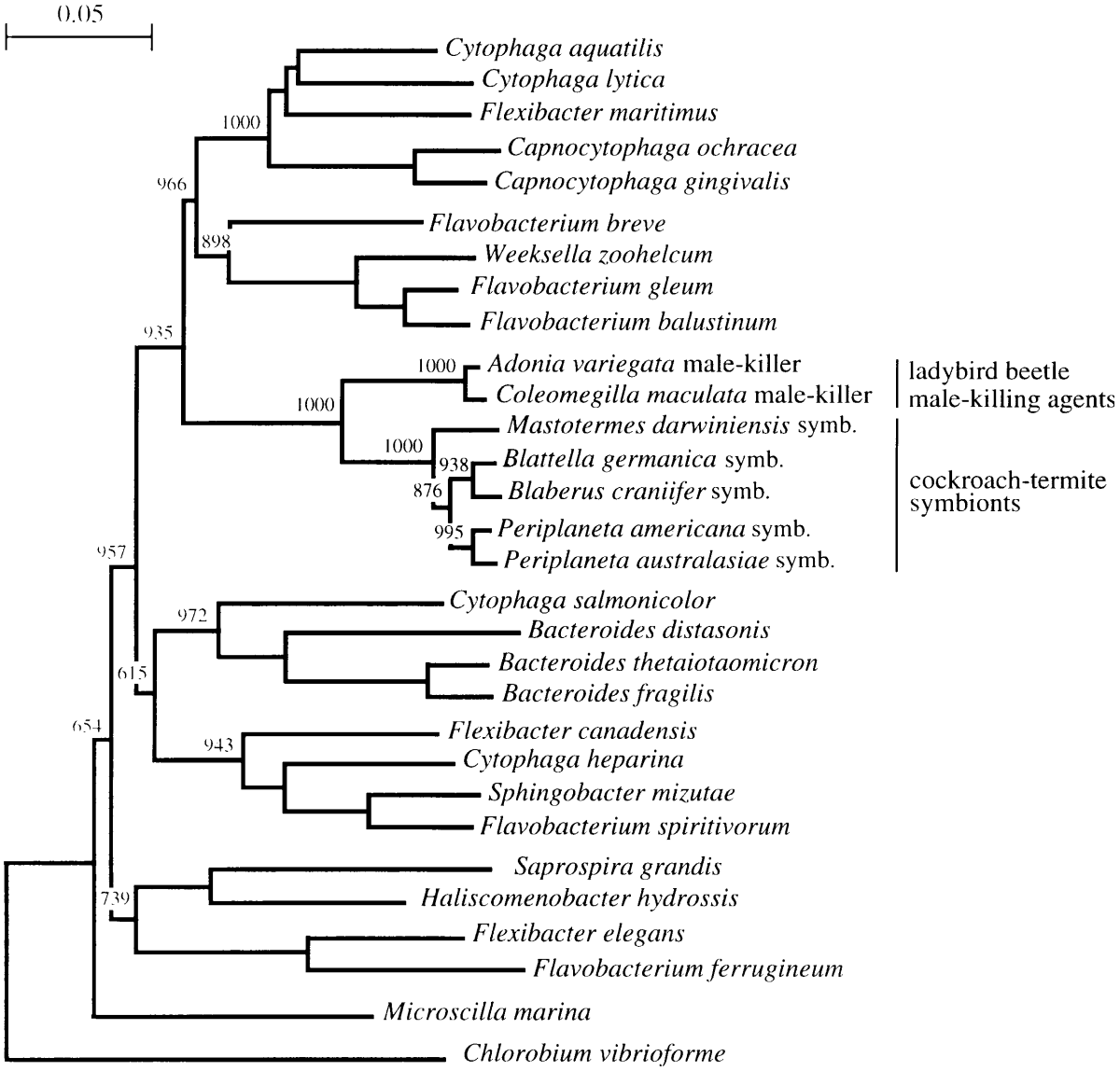

Fig. 1. The phylogenetic affiliation of the male-killing symbiont of *Adonia variegata*, as determined from 16S rDNA sequence. Tree was constructed on TREECON by neighbour joining. Bootstrap values indicate the results of 1000 bootstrap replicates.

search suggested affiliation with the Flavobacteria–Bacteroides clade, and phylogenetic analysis confirmed this conclusion (Fig. 1). The bacterium forms a monophyletic clade with the male-killing bacterium of *Coleomegilla maculata*, which itself is related to the genus *Blattabacterium*, beneficial symbionts of cockroaches. There is a 1·2% divergence between the 16S rDNA sequence of the *A. variegata* male-killer and that of the male-killer in *C. maculata*.

The hypothesis that this represented the sequence of the male-killing agent was tested across infected and uninfected lines. PCR amplification was successful with template derived from beetles from the line L–4, and with infected *C. maculata* (positive control). Amplification was not successful with template from beetles from the other 7 lines, nor with template from beetles from line L–4 derived after the administration of antibiotics (Fig. 2). The failure of amplification was not due to poor DNA quality: control amplifications were successful (results not shown).

Direct effect of bacterium upon female host fitness

We detected no diferences between infected and uninfected individuals in any of the characters assayed (Table 4). No difference in development rate was observed: the uninfected individual emerged first in 8 of the 17 pairs of beetles, and the infected beetle first in 9 (Two-tailed Sign test; $P > 0{\cdot}90$). There was also no observable difference in body mass at the point of entry to the adult stage. In 7 cases, the uninfected individual was heavier, in 10 the infected individual (Wilcoxon signed rank test for difference: $n = 17$; $T = 55$; $P > 0{\cdot}40$). Further, we could find no difference in the fecundity of

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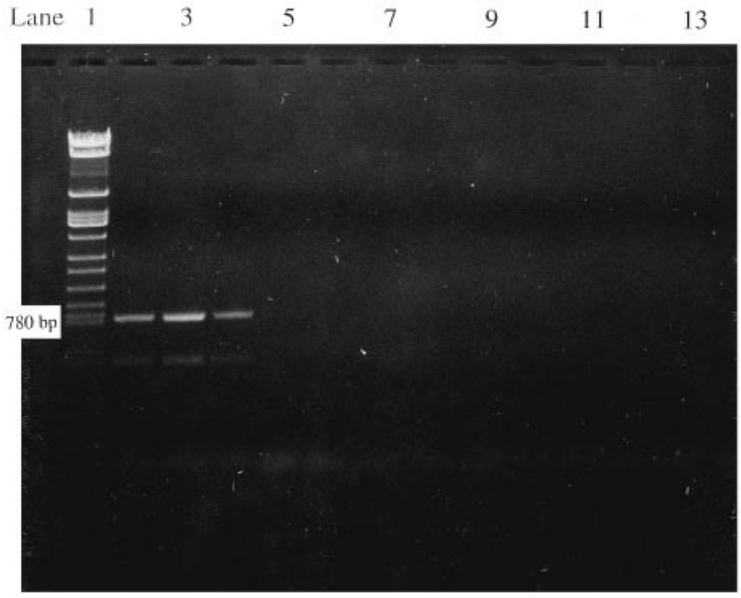

Fig. 2. Amplification products produced in the *Coleomegilla maculata* symbiont-specific PCR detailed in text, with templates from infected and uninfected *Adonia variegata*. Lane 1: λ *Hin*dIII/*Eco*RI/*Bam*HI marker; Lane 2: template from infected *C. maculata* (positive control); Lanes 3–4: template from F1 and F2 females from line L–4; Lanes 5–11: template from each uninfected matriline; Lanes 12–13: template from 2 females from line L–4 laid after the administration of antibiotics as described in the text.

Table 4. The order of emergence, mass on emergence as adults, fecundity (eggs laid over 40 days) and longevity (days) of 17 pairs of concurrently reared infected and uninfected females

Pair	Infection status of first female to emerge	Body mass (infected female) (mg)	Body mass (uninfected female) (mg)	Fecundity (infected female) (eggs/40 days)	Fecundity (uninfected female) (eggs/40 days)	Life-span (infected female) (days)	Life-span (uninfected female) (days)
B–2	Uninfected	6·6	10·3	27	105	56	65
B–7	Uninfected	10·2	10·3	317	132	84	81
G–2	Infected	10·4	9·7	120	194	84	98
E–4	Infected	10·3	11·2	43	238	68	84
E–2	Infected	9·5	9·3	187	240	88	89
E–9	Infected	10·1	10·4	250	301	94	79
E–8	Uninfected	9·8	9·5	114	44	72	68
E–11	Uninfected	8·4	7·5	63	324	60	91
E–12	Uninfected	8·4	10·4	137	96	82	72
D–8	Uninfected	8·0	10·4	146	317	80	89
D–6	Infected	7·0	7·1	350	496	86	86
D–3	Infected	11·7	8·9	344	485	62	83
E–14	Uninfected	9·4	9·0	131	285	75	69
E–16	Infected	8·9	7·0	253	396	76	85
D–19	Uninfected	9·0	7·6	409	485	66	86
D–21	Infected	8·1	7·1	327	359	77	85
E–23	Infected	10·7	10·4	299	126	78	76

infected and uninfected individuals. In 13 of 17 pairs, the uninfected individual was more fecund, whilst in 4 cases the infected individual produced more eggs during the experiment. Although this simple statistic suggests a cost to infection (Two-tailed Sign test: $P < 0{\cdot}05$), consideration of the data incorporating the magnitude of observed differences does not allow us to reject the null hypothesis of no difference between infected and uninfected individuals in a pair (Wilcoxon signed rank test: $n = 17$; $T = 37$; $0{\cdot}10 < P < 0{\cdot}05$). With respect to longevity, the infected individual lived longer in 5 of the 17 pairs, and the uninfected individual lived longer in 11 (1 pair with equal longevity). There is no statistical evidence to reject the null hypothesis of equal longevity (Wilcoxon signed rank test: $n = 16$; $T = 34$; $0{\cdot}10 < P < 0{\cdot}05$). There was no obvious correlation between infertility and infection. In 3 of

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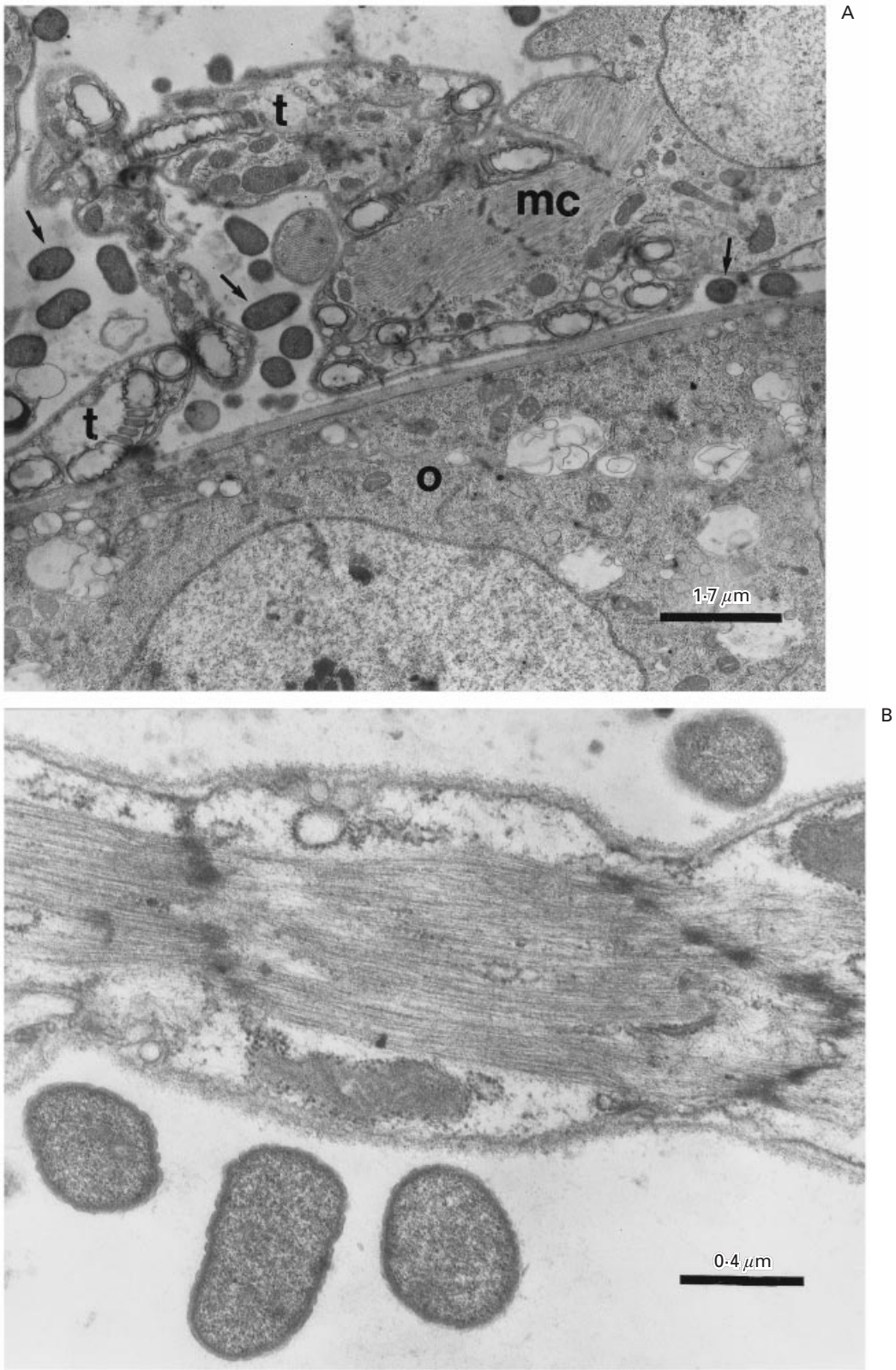

Fig. 3. (A) Transmission electron micrograph of an ovariole of an *Adonia variegata* female infected with a male-killing bacterium. Bacteria, indicated by arrows, are extracellular, and are found between the ovariole (o) and the envelope formed by the tracheocytes (t) and musculoconnective cells (mc). (B) Detail of bacteria near the musculoconnective envelope.

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the 5 pairs in which 1 individual was infertile, it was the infected individual that failed to produce viable eggs.

Process of transmission to the ovariole

Bacteria were not observed in the cells surrounding the ovariole. Rather, bacteria were observed in the space between the cells forming the ovariole sheath and the ovariole (Fig. 3A). The bacteria were approximately 0·4 μm in diameter by 1·5 μm in length (Fig. 3A and B).

DISCUSSION

In this paper we record the presence of a male-killing bacterium in the ladybird *Adonia variegata*. This bacterium is most closely related to the male-killing bacterium in another ladybird, *C. maculata*, and these form a sister group to *Blattabacterium*, the beneficial symbiont of cockroaches.

We have no definitive evidence for either a physiological benefit or cost to infection to female hosts. The data suggest that any physiological effects are likely to be negative, but an experiment with a larger sample size and greater number of strains would be required to verify this. Our data do show that if this bacterium plays a role in host physiology, then this role is a minor one. With the caveat that we investigated interaction with a single strain of the bacterium, we conclude that when the male-killing phenotype is taken into account, the bacterium causes a reduction in the fitness of its host. We therefore conclude that it is a parasite rather than a beneficial symbiont.

The mode of transmission of these micro-organisms differs from that of a beneficial symbiont. Whereas studies of *Blattabacterium* in the cockroach have shown transmission to the germ line to be in part host mediated, with host cells bearing bacteria (bacteriocytes) adhering to the ovariole (Sacchi *et al.* 1988), we found no evidence for host-mediated transmission of our organism, the bacteria arriving at the ovariole surface independent of host cells. This difference is expected. Whereas a host is selected to actively transmit beneficial symbionts (e.g. *Blattabacterium*), it is selected to exclude parasitic ones (like that in *A. variegata*).

Our study raises 2 questions. First, how is it that 2 species of coccinellid bear related male-killing bacteria? Second, why is it that few if any case studies of male-killing bacteria show them to make any physiological contribution to their female host?

The presence of 1 male-killing bacterium in 2 species of ladybird may be accounted for in one of two ways. First, the bacterium may be present in the 2 species by virtue of their common ancestry. Passage of inherited bacteria between mother and daughter over periods of hundreds of millions of years, such that all derived host species are infected, is typical of beneficial symbionts such as *Buchnera* or *Blattabacterium* (Moran *et al.* 1993; Bandi *et al.* 1994, 1995). However, this pattern is less well recognized for inherited parasites. The second possibility is that the bacterium may have moved horizontally from one host species to the other at some point in time. This is the pattern shown by *Wolbachia*, the most common inherited bacterium in insects, which moves horizontally between different species of host at a rapid rate (O'Neill *et al.* 1992; Werren, Windsor & Guo, 1995; Schilthuizen & Stouthamer, 1997).

At present it is impossible to gauge which of these hypotheses is correct. Our estimate of the date of divergence of the male-killing bacterium in *C. maculata* and *A. variegata*, 20–40 Ma BP, is compatible with the divergence time of the 2 host species. However, this compatibility does not exclude the possibility of horizontal transmission. In order to fully assess the possibility that it has passed down lineages, it will be necessary to investigate whether other members of this host clade also bear this male-killing bacterium, and thence assess whether the phylogeny of the host recapitulates that of the bacterium. A good case study in this regard would be to identify the nature of the male-killing agent in *Hippodamia 15-punctata*, a relative of these species. Further study would be appropriate, as the finding of a long-lived relationship between host and parasite would challenge the view that host–parasite interactions are typically short-lived over evolutionary time.

The second issue raised by the study is the absence of evidence for any role of the bacterium in host physiology. Within the constraints of our experiment, our data suggest that any physiological effects of infection are likely to be negative. This conclusion reflects other studies of male-killing bacteria, where evidence of physiological effects are either equivocal (Ebbert, 1991), or show a cost to male-killer infection (Ikeda, 1970; Hurst *et al.* 1994). Are these results representative of all male-killing bacteria?

Consideration of the ecology of the hosts suggests that such a conclusion would be premature. Bacteria making a beneficial contribution to host physiology generally occur in hosts with depauperate diets: obligate phloem, xylem and blood feeders, and insects feeding on decaying wood. Here, the bacteria add to the anabolic capacity of the host through the manufacture of essential amino acids or vitamins missing from the diet, or through aiding nitrogen recycling (Douglas, 1994). The case studies of male-killing bacteria to date have focused on ladybirds and fruit flies. Ladybirds feed upon an insect diet, one that is nutritionally sufficient. In ladybirds, there may be little scope for bacterial contribution to host physiology, and thus there is likely to be a net physiological cost to infection associated with the energetic requirements of the bacteria. Similarly, fruit flies feed upon a generalist plant diet, deriving

particular nutrients from yeasts. This again is a fairly complete diet, not obviously lacking amino acids or vitamins. So although we can state that male-killing bacteria in these host groups have a fairly minor role in host physiology, it would be premature to extrapolate this conclusion to male-killing bacteria in other hosts.

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