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Bacterial Infections of Hemocytes Associated with the Maternally Inherited Male-Killing Trait in British Populations of the Two Spot Ladybird, *Adalia bipunctata*

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Adalia bipunctata, **the two spot ladybird, carries a vertically transmitted bacterial agent which kills male progeny during embryogenesis. Some matrilines of *A. bipunctata* give rise to strongly female-biased sex ratios. 16S rDNA sequence analysis revealed a bacterium of the genus *Rickettsia* associated with this trait, a conclusion which is corroborated here. Using light microscopy, an association between a bacterium located in *A. bipunctata* hemocyte cytoplasm and matrilines which show the sex ratio trait was found. This element was not found in hemocytes taken from females from normal sex ratio lines, nor in hemocytes taken from males. The association is confirmed by study of the inheritance of the sex ratio trait. Only daughters of sex ratio crosses that bear this cytoplasmic bacterium also show the sex ratio trait, with other daughters being normal with respect to sex ratio. Transmission electron microscopy of hemocytes revealed a walled bacterium, bearing features of members of the genus *Rickettsia,* free in the cytoplasm of hemocytes taken from infected lines, but not in those taken from uninfected lines.**

KEY WORDS: ***Adalia bipunctata;*** **male-killing;** ***Rickettsia;*** **symbiont.**

INTRODUCTION

Many species of insects have maternally inherited genetic elements which produce strongly female-biased sex ratios by causing the death of male progeny early in life (Hurst, 1991). In only two cases has the taxonomic identity of the agent responsible been definitively demonstrated (Koch's postulates fulfilled): *Arsenophonus nasoniae,* a member of the gamma subgroup of the proteus-like bacteria in *Nasonia vitripennis* (Gherna *et al.,* 1991; Werren *et al.,* 1986), and members of the genus *Spiroplasma,* with associated viruses, among members of the *Drosophila willistoni* group (Hackett *et al.*, 1985; Williamson and Poulson, 1979). Recent work on the coccinellid beetle, *Adalia bipunctata,* has shown some females of this species to exhibit a similar sex ratio trait. Sex ratio females produce clutches in which approximately half the eggs fail to hatch, and in which nearly all the individuals raised are female. The trait is heritable down the female line, transmits on average to around 87% of female progeny, and is sensitive to oral administration of tetracycline (Hurst *et al.*, 1992; Hurst *et al.,* 1993). Amplification of the 16S rDNA using general bacterial primers showed an association between the presence of a member of the genus *Rickettsia* and the trait (Balayeva *et al.,* 1995; Werren *et al.,* 1994). We here investigate these conclusions through microscopy, examining infected and uninfected lines for bacterial presence using both light and electron microscopy.

MATERIALS AND METHODS

Maintenance of Lines of Known Sex Ratio Status

Matrilines of *A. bipunctata* established from individuals from Surrey, Sussex, and Cambridgeshire (UK) were maintained in the laboratory over the period September–December 1991, with continual checks on sex ratio and egg hatch-rate to maintain pure sex ratio and non-sex ratio stocks. Individuals from seven matrilines which displayed the sex ratio trait were maintained by selection for the individuals showing the trait. These lines are presumed to be unrelated due to the difference in location and time of collection of the founding females. Fifteen matrilines showing a normal sex ratio were also maintained.

Individuals from these matrilines were tested for the presence of a cytoplasmic element as outlined below. Further to these trials, crosses were set up from individuals from sex ratio matrilines of known status with respect to the putative element. The sex ratios

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exhibited by these crosses and the status of their progeny with respect to the element were recorded. Sexing was performed using the criteria of Randall *et al.* (1992).

Preparation for Light Microscopy

The individuals to be examined were anesthetized with ether, and hemolymph was removed by placing a drawn out capillary tube between the junction of the abdomen and thorax, where there is a break in the hard cuticle. The hemolymph was ejected onto a grease-free slide to form a smear by applying gentle pressure on the contents of the capillary tube using a large syringe connected to the capillary tube by means of thin plastic tubing. The material was allowed to dry for 5 sec and then fixed with a drop of 4% v/v formaldehyde in balanced salt solution (10 mM Tris, pH 7.5, 55 mM NaCl, 40 mM KCl, 7 mM $MgCl_2$, 5 mM $CaCl_2$, 20 mM glucose, 50 mM sucrose) (Ashburner, 1989) for 5 min. Excess fixative was removed by tapping the slide gently on tissue paper. The fixed material was then stained for 10 min with a drop of 1% 4′,6-diamidino-2-phenylindole (DAPI) in phosphate-buffered saline (PBS) (10 mM NaP_i, pH 7.2, in 0.9% w/v NaCl) placed on the smear. The stain was removed, and the specimen was mounted in 70% glycerol v/v in PBS. A coverslip was applied and the hemocytes were examined under ultraviolet light at high magnification. DAPI fluoresces blue under ultraviolet light when bound to DNA, but such fluorescence is too weak to be detectable by eye if the DNA is in small amounts. The nucleus and any microbial elements will fluoresce under ultraviolet light. However, animal mtDNA is not visualized using DAPI, the genome of animal mitochondria being too small to produce significant fluorescence. Where only limited quantities of hemolymph were removed, or staining was of poor quality such that fewer than 50 hemocytes could be examined, a new hemolymph smear was prepared from the individual. Slides were scored blind with respect to knowledge of the sex ratio status of the originator individual.

Preparation for Electron Microscopy

Hemolymph from female *A. bipunctata* from infected lines was removed as above, and the capillary tube containing the hemolymph was placed immediately into an Eppendorf tube containing the fast penetrating Karnofsky's fixative (2% paraformaldehyde/2% glutaraldehyde in 0.2 M sodium cacodylate/10% w/v sucrose, pH 7.2, buffer) to prevent damage to the ultrastructure of the hemocytes through the peroxisome (blood clot) reaction of hemocytes on contact with air. The hemolymph from a further six individuals was placed in the same vial. This process was repeated for a further seven sex ratio individuals to create a duplicate sample.

The vials were agitated for 2 hr on an orbital shaker to allow full permeation of the fixative. The material was then centrifuged to create a pellet (1 min on low in an MSE mushroom centrifuge). The supernatant was carefully removed, the samples were washed three times in buffer to remove excess fixative, and the material was placed in 1% osmium tetroxide for 1 hr. The pellet was washed three times in a 0.9% w/v solution of NaCl to remove any surplus osmium tetroxide. The tube was covered with foil to prevent light entry, and the pellet was immersed in 1% w/v uranyl acetate in a 0.9% w/v NaCl solution for 1 hr.

The material was then dehydrated by serially placing the pellet in 50% ethanol (15 min), 70% ethanol (15 min), 90% ethanol (15 min), and absolute ethanol (3× 30 min). The alcohol was then removed, first by physically pipetting and then by washing the pellet with propylene oxide (2× 5 min). The dehydrated pellet was placed in a 50% propylene oxide–Araldite mix on a rotor for 30 min and then left overnight on the rotor, sealed, save for a small hole punched in the lid to allow evaporation to occur. The propylene oxide–Araldite mix was removed, and the material was embedded in Araldite. This was polymerized by placing the Eppendorf in an oven at 60°C for 4 days. The embedded sample was removed, and sections thinner than 100 µm thick were cut on a moving-arm microtome. These sections were placed on copper grids, and the material was stained by floating the grids face down in fresh uranyl acetate for 15 min. Excess uranyl acetate was removed by washing with alcohol, and the material was then stained further by floating the grids face down in lead citrate for 15 min.

The above process was repeated for hemolymph from individuals from lines known to be normal with respect to the sex ratio trait. The material was then examined at 60 kV on a Phillips EM300 machine.

RESULTS

Light Microscopy

Fluorescence microscopy of DAPI-stained blood smears revealed the presence of cytoplasmic elements in a proportion of hemocytes from the majority of individuals taken from sex ratio lines. The proportion of hemocytes containing cytoplasmic elements varied, with some individuals showing evidence of infection in over 50% of cells, others in fewer than 5% of hemocytes. The element can be seen as a small rod-like element, typical of a bacterium (Fig. 1). The loading of infected hemocytes with the element also varied, with a few showing massive infections, others just one or two bacteria. Of the 20 individuals examined, two did not show any evidence of containing cytoplasmic elements (Table 1). Forty-one individuals from the 15 lines with no history of a sex ratio bias were examined. There was

no evidence suggesting nonhost cytoplasmic DNA in smears from any of these individuals. Fifty male individuals (40 from normal lines, 10 from sex ratio lines) were also scored for the presence of cytoplasmic DNA. In none of these was there any evidence of cytoplasmic DNA.

The 20 females from the seven sex ratio lines tested above were mated, and the hatch-rate of eggs and the progenic sex ratio produced were recorded. The two individuals showing no evidence of containing cytoplasmic elements produced a normal sex ratio, whereas the 18 females which were scored as positive for the cytoplasmic element displayed the sex ratio trait (Table 1).

To further test the correlation between the sex ratio trait and cytoplasmic elements, a sample of 29 of the female progeny from the sex ratio individuals above were in turn scored with respect to the presence of the cytoplasmic elements and mated to determine status with respect to the sex ratio trait. Among these 29 females, 10 individuals were found which showed no evidence of cytoplasmic elements. All of these were revertant individuals, showing no evidence of the sex ratio trait (Table 2). With one exception, individuals which scored positive for cytoplasmic elements also displayed the trait (Table 2). The exception (cross B:O in Table 2) was a female which scored positive for the cytoplasmic elements, but was phenotypically normal, with egg hatch-rate being high and (on a very limited sample) more males than females being produced. Significantly, neither of the daughters of this individual scored positive for the presence of cytoplasmic elements, suggesting no transmission of the bacterium to progeny.

TABLE 1

The Status with Respect to Cytoplasmic Elements of the 20 Females Scored to Assess Correlation across Lines, with the Hatch-Rate of Eggs and the Sex Ratio of Families Produced by Such Individuals

Matriline of female	Source	Element present?	Egg hatch-rate	Number of adult progeny obtained	Sex ratio (proportion male)
H10	Cambridge	Y	0.43	54	0.15
H10	Cambridge	Y	0.41	15	0.07
H10	Cambridge	Y	0.52	32	0.16
H10	Cambridge	N	0.79	45	0.38
AP	Cambridge	Y	0.49	42	0.17
AP	Cambridge	Y	0.41	39	0.00
AP	Cambridge	N	0.87	25	0.40
B	Cambridge	Y	0.42	14	0.00
B	Cambridge	Y	0.54	14	0.14
FC25	Cambridge	Y	0.53	44	0.05
FC25	Cambridge	Y	0.51	31	0.13
FC25	Cambridge	Y	0.43	48	0.10
FC25	Cambridge	Y	0.55	52	0.15
M69	Cambridge	Y	0.38	31	0.00
M69	Cambridge	Y	0.42	73	0.01
M69	Cambridge	Y	0.35	40	0.00
SX3	Sussex	Y	0.53	21	0.00
JH34	Surrey	Y	0.48	60	0.05
JH34	Surrey	Y	0.60	49	0.12
JH34	Surrey	Y	0.62	69	0.33

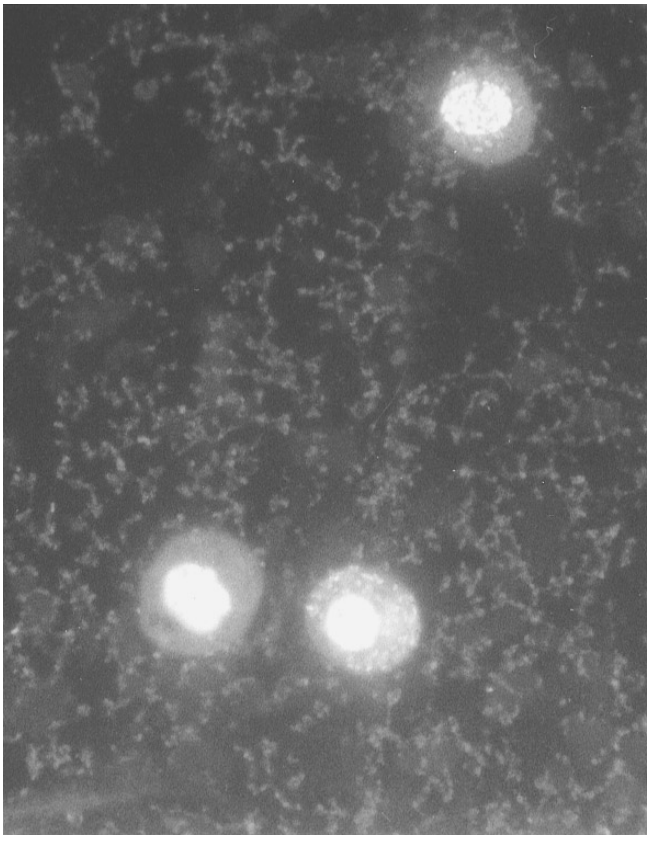

FIG. 1. The appearance of hemocytes from a sex ratio line when stained with DAPI, illuminated under UV light, and viewed at ×1000 original magnification. Each cell is approximately 10 μm in diameter. Leftmost hemocyte is uninfected; the other two hemocytes show high levels of infection.

The sex ratio and transmission rates of the 19 females scored as infected were analyzed. The proportion of sons dying was estimated by 1 minus the number of sons raised divided by the number of daughters raised. This estimate contains an error associated with the binomial nature of the sex ratio. When the death rate of sons is plotted against the rate of transmission of the element to daughters, a correlation is observed ($r = 0.892$, 17 df; $P < 0.001$) (Fig. 2). Females which transmitted the element to a high proportion of their progeny produced few if any males, whereas females which transmit the element to fewer of their progeny produced significant numbers of males.

Electron Microscopy

As found under the light microscope, a proportion of hemocytes from both samples containing hemolymph

TABLE 2

The Status with Respect to the Presence of the Cytoplasmic Elements of 29 Females Derived from Sex Ratio Crosses, the Egg Hatch-Rate and Sex Ratio of Families Produced by These Females, and the Proportion of Progeny Bearing Cytoplasmic Elements within Hemocytes (Revertant Individuals in Boldface)

Female	Element present?	Egg hatch-rate	Number of adult progeny obtained	Sex-ratio (proportion male)	Number of daughters scored for element	Proportion of daughters bearing element
B:C	N	0.86	48	0.48	13	0.00
B:R	N	0.85	11	0.36	7	0.00
B:V	N	0.80	15	0.33	10	0.00
B:X	N	0.83	27	0.48	10	0.00
B:AA	N	0.91	47	0.47	10	0.00
B:AB	N	0.87	80	0.53	10	0.00
B:AD	N	1.00	2	1.00	—	—
B:AF	N	0.86	28	0.71	8	0.00
B:AH	N	0.90	49	0.55	10	0.00
B:AJ	N	0.90	62	0.44	10	0.00
B:F	Y	0.43	28	0.14	19	1.00
B:H	Y	0.24	33	0.09	26	0.88
B:I	Y	0.44	40	0.08	25	0.96
B:J	Y	0.55	16	0.25	11	0.64
B:K	Y	0.42	4	0.00	4	1.00
B:L	Y	0.44	25	0.00	21	1.00
B:M	Y	0.45	15	0.00	13	0.92
B:N	Y	0.48	10	0.00	8	1.00
B:O	Y	0.88	5	0.60	2	0.00
B:P	Y	0.33	2	0.00	2	1.00
B:Q	Y	0.41	12	0.00	12	1.00
B:S	Y	0.55	6	0.33	4	0.75
B:U	Y	0.33	34	0.00	18	1.00
B:W	Y	0.55	25	0.40	13	0.77
B:Z	Y	0.48	62	0.02	16	1.00
B:AC	Y	0.39	46	0.07	20	0.95
B:AE	Y	0.53	25	0.00	15	1.00
B:AG	Y	0.34	38	0.08	25	0.92
B:AI	Y	0.55	108	0.24	29	0.55

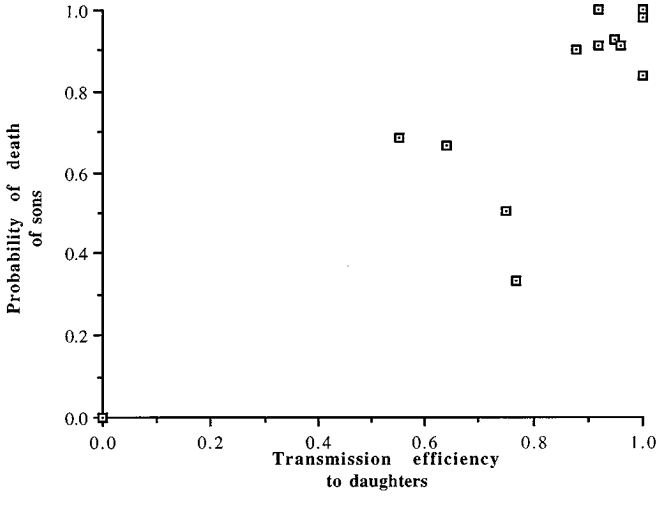

FIG. 2. The relationship between the observed rate of transmission of bacteria to daughters and the estimated death rate of sons for the 19 crosses involving infected females as described in Table 2. Note point at (1,1) represents seven individuals. There is a significant correlation between the two variables ($r = 0.892$, 17 df; $P < 0.001$).

from infected lines showed the presence of one to many cytoplasmic elements (Fig. 3). These cytoplasmic elements are bacteria and are all of similar appearance. They are rod shaped and surrounded by a translucent layer. The bacterium has similar dimensions to a mitochondrion, being a cylinder of approximately 2 μm in length and 0.75 μm in diameter. On close examination, this bacterium can be seen to be walled (Fig. 3) and to possess a distinct electron-lucent capsule layer. There is no surrounding host membrane that would indicate that the element was encapsulated in a host vacuole. No cell from hemolymph extracted from individuals that were normal with respect to the sex ratio trait showed evidence of bacterial infection.

DISCUSSION

We here present evidence to corroborate the conclusion that there is a *Rickettsia* associated with this disease. By means of light microscopy, we have displayed the association of a cytoplasmic bacterium in hemocytes with the heritable production of female-biased sex ratios due to the death of male individuals during embryogenesis. Infected hemocytes are only

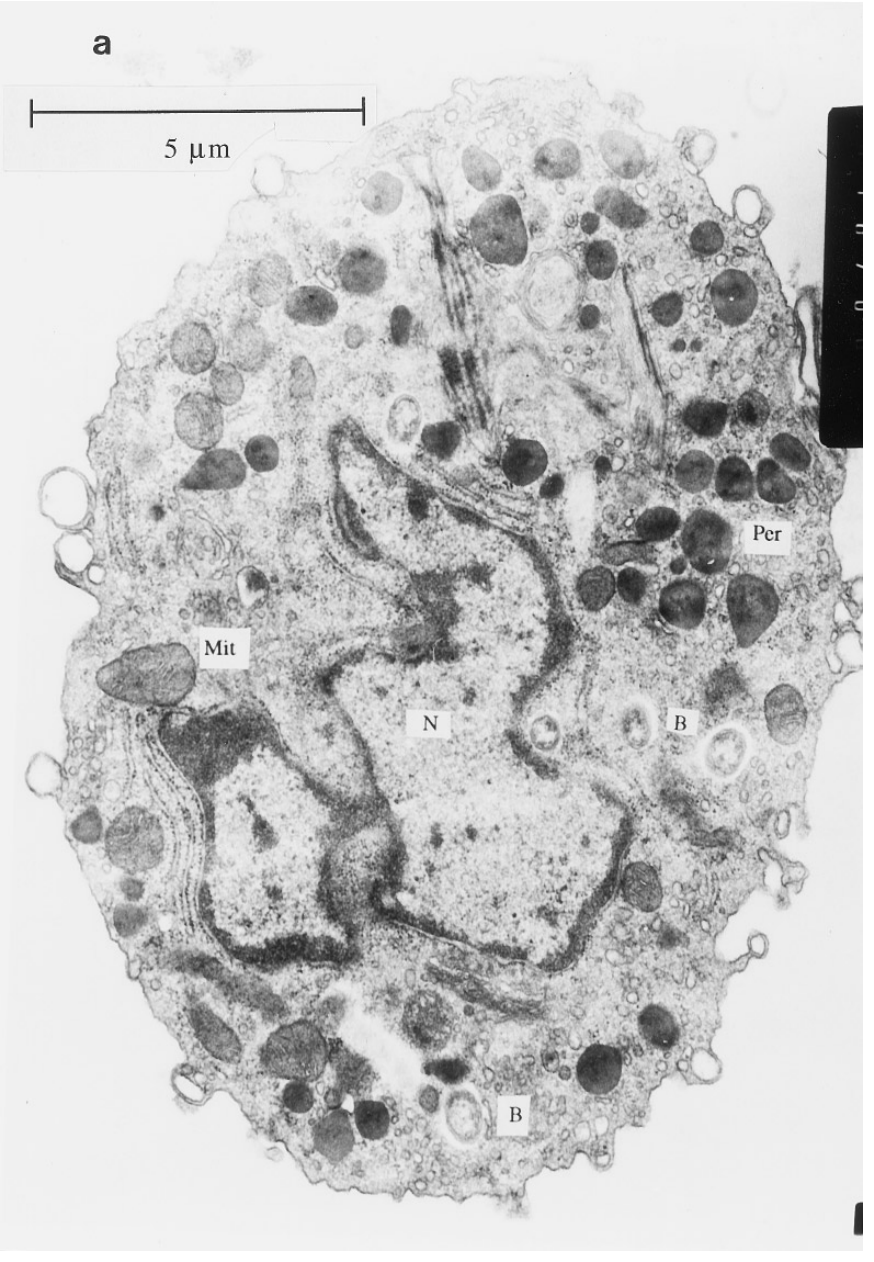

FIG. 3. Electron micrographs of (a) a whole hemocyte and (b) part of a hemocyte from an infected ladybird (Mit, mitochondria; B, bacterium; Per, peroxisome; N, nucleus).

found in individuals from lines showing the sex ratio trait. Further, within a sex ratio line, loss of the trait correlates strongly with loss of cytoplasmic bacteria in hemocytes. The correlation between crosses which show the sex ratio trait and crosses which show this bacterium in both parents and offspring is exact. These observations, taken with the known effect of the antibiotic tetracycline on the trait, strongly indicate that this organism is the cause of the trait, either alone, or in tight association with some element. A formal display will require the fulfillment of Koch's postulates, which will be difficult to perform. Cytoplasmic bacteria are generally fastidious organisms, and attempts to culture this element using simple media have failed (Hurst, personal observation).

Electron microscopy revealed that the associated organism is rod-shaped and is present free in the cytoplasm of the cell. Further, it may be seen that the organism has a thick outer coat, which gives it the apppearance of being haloed in the cytoplasm. These observations are consistent with data from sequence analysis of PCR-amplified 16S rDNA genes and further

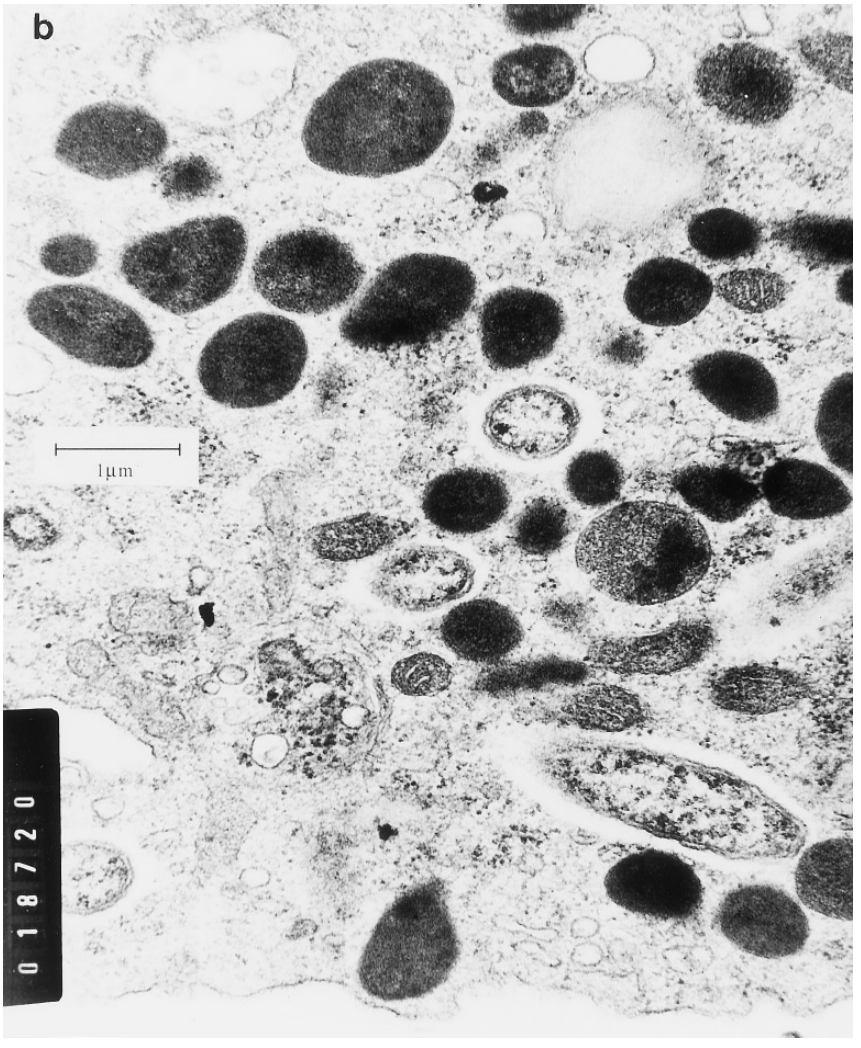

FIG. 3—*Continued*

PCR tests, which show an association between a bacterium affiliated to the genus *Rickettsia* and the trait (Balayeva *et al.*, 1995; Werren *et al.*, 1994). Members of the genus *Rickettsia* are classically found free in the cytoplasm (Winkler, 1990) and characteristically possess a "slime layer" of polysaccharide on their exterior, which makes them appear to be haloed when inside cells (Silverman *et al.*, 1978; Todd *et al.*, 1983).

The observation of a correlation (and approximately unitary relationship) between the proportion of female progeny of a cross that are infected with the bacterium and the proportion of male progeny which die rules out the possibility that male survival is associated with a particular resistance of male embryos to the action of the bacterium. The correlation may have two explanations. First, the survival of sons may be associated with the failure of the bacterium to transmit to the male embryo. Male embryos survive only if they do not inherit the bacterium. The proportion of male embryos which die is thus equivalent to the rate of infection of female embryos, and thus daughters. The alternative explanation is that male survival is associated with resistance in the progeny. To explain the similarity of infection rates of daughters and death rate of sons would require that resistance acted very early in embryogenesis (otherwise males would die) and that the same resistance causes loss of the bacterium in both daughters and sons. Distinguishing these hypotheses awaits precise comparison of transmission to early embryos and adults. A PCR-based test should allow infection status to be measured and this comparison to be made.

The infection rate of hemocytes bears comment in the light of the virulence of this bacterium. Theory predicts that vertically transmitted symbionts should evolve toward avirulence in the female line (Bull *et al.*, 1991; Ewald, 1987; Ewald and Schubert, 1989). However, the male-killer of *A. bipunctata* does cause a small but

significant decrease in the fecundity and longevity of its female host (Hurst *et al.*, 1994). This observation of virulence accords with the observation of large numbers of bacteria outside of the ovarian tissue. Even if not directly disturbing host physiology, the energetic cost imposed by this load of bacteria probably contributes significantly to the decreased performance of the adult female beetle.

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