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LADYBIRDS AS A MODEL SYSTEM FOR THE STUDY OF MALE-KILLING SYMBIONTS

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Maternally inherited bacteria that kill male but not female hosts during embryogenesis occur in a number of aphidophagous coccinellids. Work on English *Adalia bipunctata* (L.), has shown the causative agent of male-killing to be a member of the bacterial genus *Rickettsia*. In coccinellids, the primary advantage of male-killing behaviour to the bacterium has been identified. Following male death, resource reallocation occurs through sibling egg cannibalism: female neonate larvae of infected mothers gain a significant survival advantage by eating the soma of their dead male siblings. In addition, daughters of infected females suffer a reduced risk of cannibalism as a result of the lower egg hatch rate in infected clutches.

Predictions as to which species of coccinellid are liable to harbour male-killers may be made on the basis of the selective advantages of male-killing identified in *A. bipunctata*. Species which may harbour male-killers are likely to lay eggs in clutches, show sibling egg cannibalism, and exhibit high neonate mortality.

Recent work has shown male-killing to occur in a number of other aphidophagous coccinellids with the predicted characteristics. Molecular genetic analysis has putatively identified three bacterial symbionts associated with male-killing, coming from three phylogenetically distant bacterial taxa. We therefore suggest that within coccinellids that possess these features, male-killing may evolve in a taxonomically diverse range of inherited bacteria.

The implications of the presence of male-killing bacteria on the population demography of host coccinellids, and on host mitochondrial DNA variability are discussed. The aphidophagous coccinellids are proposed as a model system for studying the evolution and consequences of infection with male-killers.

KEY-WORDS: Coccinellidae, female-biased sex ratio, cytoplasmic bacteria, male killing, sibling egg cannibalism, mitochondrial DNA.

All or predominantly female families produced by some female *Adalia bipunctata* (L.) from Russia were first reported by Lus almost fifty years ago (Lus, 1947a). Although he did not recognise it, Lus's data showed the trait to be maternally inherited. Subsequently, maternally inherited all female broods have been been reported in a number of other coccinellids (*Hippodamia quinquesignata* Kirby, Shull 1948; *Harmonia axyridis* Pallas, Matsuka *et al.*, 1975; *Menochilius sexmaculatus* (Fabricius), Niijima & Nakajima, 1981; *Coleomegilla maculata* (Der Geer), Hurst *et al.*, 1996a). In these cases, it has been noted that the egg hatch-rates from females producing female-biased sex ratios were low, suggesting that the primary sex ratio was normal, but that the secondary sex ratio was distorted because male eggs failed to hatch. The trait is thus called male-killing.

Recent work on these coccinellids has endeavoured to uncover the identity of the causative agent of male-killing in these beetles, and understand why this trait has evolved. The discoveries made suggest that male-killing bacteria are common in aphidophagous coccinellids, and that these may be used as a model system for investigating these 'ultra selfish' genetic elements. In addition, consideration of the effect of the interaction between these male-killing microorganisms and their hosts suggest them to have a wide range of side effects on the biology of their hosts.

THE IDENTIFICATION OF THE MALE-KILLER IN ENGLISH POPULATIONS OF *A. BIPUNCTATA*

Studies of *A. bipunctata* from several British populations revealed a maternally inherited trait that resulted in the death of most, but not all male embryos. Treatment of infected females with the antibiotic tetracycline, administered in golden syrup, cured the trait, increasing both the egg hatch-rate and the proportion of male progeny produced (Hurst *et al.*, 1992). Microscopic examination of haemolymph cells stained with the DNA stain 4',6'-diamidino-2-phenylindole (DAPI) showed the presence of a bacterium in most haemolymph cells of infected females, but not in the cells of uninfected females, or in those of sons of infected females. Transmission electron microscopy of haemocytes revealed a bacterium, bearing features of members of the genus *Rickettsia,* free in the cytoplasm of haemocytes from infected females (Hurst *et al.*, 1997). Amplification of part of the 16S rDNA gene, using general bacterial primers, showed a correlation between the presence of a member of the genus *Rickettsia* and the female-biased sex ratio trait (Werren *et al.*, 1994; Balayeva *et al.*, 1995).

Population genetic studies in Cambridge showed that the male-killing *Rickettsia* was present in approximately 7% of Cambridge females and the trait was vertically transmitted to, on average, 87% of daughters (Hurst *et al.*, 1993).

WHY ARE APHIDOPHAGOUS COCCINELLIDS PRONE TO INVASION BY MALE-KILLERS?

The strategy of this bacterium in killing male hosts requires an evolutionary explanation. The bacterium, in killing male hosts, effectively commits suicide: there is no evidence of any horizontal transmission from the dead male eggs. In explaining why this bacterium kills male hosts, it is of key importance to remember that inheritance of the bacterium is through the female line only. For male-killing to be adaptive, the death of males (through which the bacterium cannot pass) must result in an increased survivorship of sibling females (in which it is present, and through which it can pass). This will occur if male and female siblings interact, and this interaction is detrimental to the females. If this is the case, then male death enhances sibling female survival, and thus bacterial transmission. Two types of detrimental interaction have been considered: inbreeding (where males mating with their sisters decrease their fitness) and competition between siblings for resources (Hurst, 1991).

Despite the high inbreeding depression reported in captive lines of this species (Lus, 1947b), assessment of inbreeding rates in the field gave levels too low to provide a sufficient selective advantage through the avoidance of inbreeding to account for the maintenance of the male-killer (Hurst *et al.*, 1996b).

The alternative explanation, that of resource reallocation, is more tenable. Like many other aphidophagous coccinellids, *A. bipunctata* is highly cannibalistic. In particular, neonate larvae are known to indulge in sibling egg cannibalism before dispersing from their egg clutch to seek aphids (Banks, 1956). Eating a single unhatched egg increases the survival time of a neonate larva deprived of other food and water by over 50% (Hurst *et*

al., 1996b). Larvae fed on an egg are also larger and move more rapidly when they disperse from the egg clutch (Walker, 1962 unpublished data; Hurst, 1993 cited in Majerus 1994) than larvae deprived of such an egg meal. This will enhance the ability of larvae from infected clutches to catch and subdue their aphid prey. Given that the mortality of neonate larvae between dispersal from their egg clutch and finding their first aphid prey may be very high (Banks, 1955; Dixon, 1970; Wratten, 1973; Wratten, 1976), the additional resources that surviving neonate larvae from infected clutches gain from consuming dead eggs are likely to lead to a substantial survival advantage (Hurst & Majerus, 1993).

A second advantage may accrue to infected female progeny from sibling egg cannibalism. It is known that the first larvae that hatch from a clutch will, once their mandibles have hardened, consume any unhatched eggs in the clutch, including infertile eggs, male-killed eggs, and late-hatching eggs. The level of cannibalism of late-hatching eggs is likely to be lower in infected than uninfected clutches, partly because the number of larvae that hatch is reduced by about a half, so there are less cannibalisers, and partly because as the male eggs do not hatch, there will be more unhatched eggs for the early-hatchers to chose from (Hurst *et al.*, 1992).

The ecological reasons behind the evolution of the male-killing habit of the *Rickettsia* found in British populations of *A. bipunctata* can thus be summarised:

i) eggs are laid in tight clutches,

ii) neonate larvae consume unhatched eggs bearing siblings,

iii) neonate larvae are not very efficient at finding, catching and subduing aphid prey,

iv) aphid populations are ephemeral and unpredictable,

v) as a consequence of iii) and iv), mortality of neonate larvae due to starvation can be very high.

WHICH COCCINELLIDS ARE LIKELY CANDIDATES FOR MALE-KILLER INFECTION?

If resource reallocation is the main reason for the evolution and maintenance of male-killing in coccinellids, we may make predictions about the characteristics of coccinellids which are likely to harbour male-killers, and those which are not. Candidates for invasion by male-killers, or in which symbionts may evolve the male-killing habit, will be:

i) aphidophagous,

ii) lay eggs in tight clutches,

iii) exhibit sibling egg cannibalism,

iv) exhibit significant levels of neonate larval mortality due to starvation.

In addition, we may predict that a range of inherited symbionts may evolve the male-killing habit, because any symbiotic organism that is transmitted maternally will gain the types of advantage described above.

TESTING THE PREDICTIONS

The number of coccinellids that are known to harbour maternally inherited male-killing endosymbionts is now five, and there is circumstantial evidence to suggest that at least two others have male-killers. These are summarised in table 1.

All the coccinellids in which maternally inherited male-killing has been found are aphidophagous, lay eggs in tight batches, show sibling egg cannibalism and are liable to be prone to high levels of neonate starvation, at least intermittently. The predictions for the

TABLE 1

Evidence for male-killing bacteria in coccinellids

Species and location	Type of male-killer	Evidence for maternally inherited male-killer	References
Harmonia axyridis Japan, Mongolia	Spiroplasma	Maternal inheritance of all-female broods with half egg hatch-rates in infected crosses. Partial cure of trait with antibiotics. Artificial infection of uninfected lines by haemolymph transfer.	Matsuka *et al.*, 1975; Hu, 1979; Gotoh, 1982; Majerus *et al.*, in prep.
Menochilius sexmaculatus Japan	Unknown	Maternal inheritance of all-female broods with half egg hatch-rates in infected crosses. Complete cure of trait with antibiotics. Artificial infection of uninfected lines by haemolymph transfer.	Niijima & Nakajima, 1981; Niijima, 1983
Adalia bipunctata England, Holland	*Rickettsia*	Maternal inheritance of all-female broods with half egg hatch-rates in infected crosses. Cure of trait with antibiotics. Exact association of presence of bacterium with male-killing trait.	Hurst *et al.*, 1992, 1993, 1994, 1997; Werren *et al.*, 1994; Balayeva *et al.*, 1995
Adalia bipunctata Russia, E. Germany	Spiroplasma	Maternal inheritance of all-female broods with half egg hatch rates in infected crosses. Cure of trait with antibiotics; artificial transmission of trait. Exact association of presence of bacterium with male-killing trait.	Lus, 1947a, Hurst *et al.* in prep.; Zakharov *et al.*, 1996
Coleomegilla maculata USA	Flavobacteria	Maternal inheritance of all-female broods with half egg hatch rates in infected crosses. Cure of trait with antibiotics. Exact association of presence of bacterium with male-killing trait.	Hurst *et al.*, 1996a
Hippodamia quinquesignata, USA	Unknown	Maternal inheritance of all or predominantly female broods through eight generations with some crosses showing spontaneous reversion to normal sex ratio.	Shull, 1948
Coccinella septempunctata, England	Unknown	Low egg hatch-rate with concomitant significantly female-biased progenic sex ratio.	Slogett, pers. comm.
Adonia variegata, Turkey	Unknown	Low egg hatch-rate with concomitant significantly female-biased progenic sex ratio.	Hurst, pers. obs.

circumstances for invasion are thus met. In addition, the bacteria associated with male-killing which have been identified in three of the species (see table 1) are phylogenetically diverse. This corroborates the prediction that a variety of cytoplasmic bacteria may develop the male killing habit if specific circumstances pertain. Further corroboration comes from investigations of female-biased sex ratio lines of *A. bipunctata* from Russia. These had similar characteristics to the English *A. bipunctata* infected with the male-killing *Rickettsia*, apart from the fact that the vertical transmission efficiency was greater than 99%. Molecular analysis found no evidence of rickettsial infection in the Russian lines that showed a female-biased sex ratio. However, another bacterium (a spiroplasma), was found to be exactly associated with the male-killing trait (Hurst *et al.*, in prep.).

If our predictions of which coccinellids are prone to male-killers are being confirmed, additional predictions of coccinellids which should not harbour such symbionts may also be made. So, non-predatory coccinellids can be excluded. Any species which lays eggs singly, in very small groups, or in loose clutches so that the eggs do not touch, which does not show sibling egg cannibalism, or in which food is not normally limiting for neonate larvae, is unlikely to harbour a male-killer. These include the vegetarian species of the Epilachninae, and mycophagous species such as members of the Psylloborini and Tytthaspini. In these species, eggs are typically laid on or amongst food that does not move and does not have to be actively subdued. Coccidophagous species, such as many members of the Chilocorini, may also be excluded for two reasons. First, they lay eggs singly or in batches of just two or three, typically under a coccid, so food is readily available to neonates. Second, coccid populations are very much less ephemeral and unpredictable than most aphids. The same may be true of adelgids, with the consequence that species such as *Aphidecta obliterata* (L.) which feed mainly on this group are unlikely to be prone to male-killers.

WHY ARE MALE-KILLERS IMPORTANT?

Aphidophagous coccinellids are of considerable interest and importance for their potential in biological pest control. This group has thus received considerable attention. Our thesis, that male-killing bacteria may be a wide-spread feature of aphidophagous coccinellids, has consequences for other fields of study of these beetles. We here give two disparate examples.

First, presence of male-killers could have considerable effects on reproductive strategies and so population demography. In samples of *Harmonia axyridis* from Sapporo, Japan, 48% of females were infected with a male-killing agent with a high vertical transmission efficiency. The sex ratio in this population was almost 2:1 female: male ($n = 287$). Such a distorted sex ratio is bound to have consequences on sexual selection; male competition is likely to be weak or non-existent, females may simply not be able to afford to be highly choosy over their choice of mates. On the other hand, given that females are highly promiscuous, mating far more frequently than is required to maintain high egg fertility, a reduction in the relative number of males available is unlikely to have a significant effect on fertility levels. Indeed, it is possible that as population increase is at least partly limited by the number of eggs laid by females, the female-bias in the sex ratio may give scope for abnormally rapid population increases, partly as a result of the population containing a high proportion of females, and partly because females may spend more time ovipositing rather than mating if fewer males are encountered (Webberley, pers. comm.).

Second, much work on population structure, gene flow and taxonomy is now conducted through examining variation at the level of DNA sequences. In this work, mitochondrial DNA (mtDNA) sequences are frequently used. The presence, now or in the past, of a male-killing bacterium in a lineage under investigation, will have a profound effect on the data obtained and its interpretation.

The most obvious effect of male-killer presence is on mtDNA variability. When a male-killer invades a new population of coccinellids, it immediately becomes linked in its heredity to the mtDNA of its first female bearer. As the male-killer spreads through the population, the mitotype that is linked to it spreads with it. Eventually an equilibrium frequency of male-killer infection is reached. The equilibrium is maintained by a balance between the loss of infection due to inefficiency in vertical transmission, and selection favouring infected individuals. At some point after the equilibrium is reached, the population will consist only of individuals which either carry the male-killer, or are descendants

of lineages that have had the male-killer but have lost it as a result of the vertical transmission efficiency being less than 100%. The variability in mtDNA in such a population will be a consequence of the initial infection level of the male-killer, its vertical transmission efficiency, the equilibrium level of the male-killer, and the mutation rate (Johnstone & Hurst, 1996). Any analysis of mtDNA to investigate, for example past gene-flow between populations, would be invalid.

A second possibility is that a male-killer will have effects upon the DNA sequence of the mtDNA. The carrying of a male-killer will be detrimental to its host. There will be at least intermittent selection pressures on the host to reduce the vertical transmission efficiency of the male-killer. One possible way of achieving this is an increase in the replication rate of germ line cells, to reduce the probability of each daughter cell receiving at least one copy of the bacterium. When cells divide, either mitotically or meiotically, mitochondria also have to be replicated. Faster cell division may produce selection for a reduction in the size of the mitochondrial genome. Selection may favour loss of those parts of the mitochondrial genome that do not affect the function of gene products (e.g. the small regions of intergenic spacer). There may also be effects on the sequence of DNA bases, apart from such deletions, to 'accomodate' such changes. If this occurs, it will have implications for the use of branch lengths as estimators of genetic distance. Faster evolution of mtDNA may occur in male-killer infected lineages.

The effect on mtDNA genome size outlined here is purely speculative. However, the effect on mtDNA variability is an unavoidable consequence of the presence of a male-killer in a population, and one which currently requires the attention of coccinellid biologists. The recent massive spread and increase in *Harmonia axyridis* in North America, with the concomitant stress and financial loss due to the invasion of large numbers of these ladybirds into private homes and business premises, may lead to legal suits for damages (Horn pers. comm.). It is feasible that data from genetic analyses that bear upon the origin of the ladybirds will comprise part of the evidence in such cases. Should this be the case, the effect of the presence of a male-killer in *H. axyridis* should render any analysis employing mtDNA ambiguous. Imagine that the majority of founding *H. axyridis* in North America came from Siberia (a population that appears to be free of male-killer infection: Majerus *et al.*, in prep.), but that one individual from another region, say Japan, bearing a male-killer was also introduced (accidentally or intentionally) at about the same time. If the male-killer spread, its associated mtDNA would also spread. Analysis of the mtDNA of *H. axyridis* from North America subsequently could lead to the deduction that the (theoretical) introduction from Siberia had not been successful, all blame for damage being unfairly laid on Japanese introductions. Our conclusion is that mtDNA may be an inappropriate marker for many types of population study of aphidophagous coccinellids, and that deductions therefrom may be unsound unless endorsed by analyses using nuclear genetic markers.

In summary, male-killing bacteria appear to be common amongst aphidophagous coccinellids. The reasons why these beetles appear to be particularly prone to male-killers are known. Male-killers are also known from a diverse range of other insect taxa (Hurst, 1991). It is our thesis that the presence, past or present, of male-killing bacteria in any species will have effects that impinge upon the interpretation of scientific investigations of that species in a number of widely disparate fields. It is likely that not all such effects have yet been identified. The group in which these fascinating and potentially important microbes are most understood and amenable to investigation are the aphidophagous coccinellids.

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RÉSUMÉ

Les coccinelles en tant que modèle de l'étude des symbiontes tueurs de mâles

Il existe des bactéries transmises maternellement et qui tuent leurs hôtes mâles mais pas leurs hôtes femelles au cours de l'embryogénèse dans de nombreuses coccinelles aphidiphages. Un travail sur *Adalia bipunctata* (L.) a montré que l'agent responsable de cette élimination des mâles était un *Rickettsia*. Chez les coccinelles, on a trouvé l'avantage de base de l'élimination des mâles par les bactéries. Suite à la mort du mâle, la redistribution des ressources a lieu par le cannibalisme des œufs entre membres d'une même ponte : les larves femelles nouvellement écloses des mères infestées obtiennent un avantage significatif en terme de survie en dévorant leurs congénères mâles. En outre, les filles de femelles infestées souffrent d'un risque de cannibalisme réduit, résultat d'un taux d'éclosion des œufs plus bas dans les pontes infestées. La prédiction des espèces de coccinellides susceptibles de comporter des tueuses de mâles peut être faite sur la base des avantages sélectifs d'éliminer le mâle chez *A. bipunctata*. Les espèces susceptibles de comporter des tueuses de mâles pondent probablement leurs œufs groupés, présentent un cannibalisme entre œufs d'une même ponte et une forte mortalité néonate.

Un travail récent a montré que le comportement d'élimination des mâles existe chez de nombreuses autres coccinelles aphidiphages présentant les caractéristiques que l'on attendait. L'analyse génétique moléculaire a permis de distinguer trois symbiontes bactériens associés à ce phénomène, provenant de trois taxa de bactéries phylogénétiquement éloignés. Nous suggérons donc que parmi les coccinelles qui possèdent ces caractéristiques, l'élimination des mâles peut évoluer dans une gamme taxonomiquement diverse de bactéries transmises maternellement.

Les implications de la présence de bactéries tueuses sur la démographie des coccinelles hôtes et sur la variabilité de l'ADN mitochondrial de l'hôte sont discutées. Les coccinelles aphidiphages sont proposées comme modèle pour l'étude de l'évolution et les conséquences de l'infection par des bactéries tueuses de mâles.

REFERENCES

Balayeva, N. M., Eremeeva, M. E., Tissot-Dupont, H., Zakharov, I. A. & Raoult, D. — 1995. Genotype characterization of the bacterium expressing the male-killing trait in the ladybird beetle *Adalia bipunctata* with specific Rickettsial molecular tools. — *Appl. Env. Micro.*, 61, 1431-1437.

Banks, C. J. — 1955. An ecological study of Coccinellidae associated with *Aphis fabae* on *V. faba*. — *Bull. Ent. Res.*, 46, 561- 587.

Banks, C. J. — 1956. The distribution of coccinellid egg batches and larvae in relation to numbers of *Aphis fabae* on *Vicia faba*. — *Bull. Ent. Res.*, 47, 47-56.

Dixon, A. F. G. — 1970. Factors limiting the effectiveness of the coccinellid beetle *Adalia bipunctata* (L.) as a predator of the sycamore aphid, *Drepanosiphum platanoides* (Schr.). — *J. Anim. Ecol.*, 39, 739-751.

Gotoh, T. — 1982. Experimental transfer of abnormal "Sex-Ratio" in the Lady-bird beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). — *Appl. Ent. Zool.*, 17, 319-324.

Hu, K. — 1979. Maternally inherited "sonless" abnormal SR condition in the ladybeetle *Harmonia axyridis*. — *Acta Genetica Sinica*, 6, 296-304.

Hurst, G. D. D., Hammarton, T. C., Obrycki, J. J., Majerus, T. M. O., Walker, L. E., Bertrand, D. & Majerus, M. E. N. — 1996a. Male-killing bacteria in a fifth ladybird beetle, *Coleomegilla maculata* (Coleoptera: Coccinellidae). — *Heredity*, 77, 177-185.

Hurst, G. D. D., Hurst, L. D. & Majerus, M. E. N. — 1996b. Cytoplasmic sex ratio distorters. Influential passengers: microbes and invertebrate reproduction, In **S. L. O'Neill. A. A. Hoffmann & J. H. Werren** (eds) O.U.P., Oxford, U.K.

Hurst, G. D. D. & Majerus, M. E. N. — 1993. Why do maternally inherited microorganisms kill males? — *Heredity*, 71, 81-95.

Hurst, G. D. D., Majerus, M. E. N. & Walker, L. E. — 1992. Cytoplasmic male killing elements in *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae). — *Heredity*, 69, 84-91.

Hurst, G. D. D., Majerus, M. E. N. & Walker, L. E. — 1993. The importance of cytoplasmic male killing elements in natural populations of the two spot ladybird, *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae). — *Biol. J. Linn. Soc.*, 49, 195-202.

Hurst, G. D. D., Purvis, E. L., Sloggett, J. J. & Majerus, M. E. N. — 1994. The effect of infection with male-killing *Rickettsia* on the demography of female *Adalia bipunctata* L. (two spot ladybird). — *Heredity*, 73, 309-316.

Hurst, G. D. D., Walker, L. E. & Majerus, M. E. N. — 1997. Bacterial infections of hemocytes associated with the maternally inherited male-killing trait in British populations of the two spot ladybird, *Adalia bipunctata*. — *J. Invert. Path.*, 68, 286-292.

Hurst, L. D. — 1991. The incidences and evolution of cytoplasmic male killers. — *Proc. R. Soc. Lond. B*, 244, 91-99.

Johnstone, R. A. & Hurst, G. D. D. — 1996. Maternally inherited male-killing microorganisms may confound interpretation of mtDNA variation in insects. — *Biol. J. Linn. Soc.*, 58, 453-470.

Lus, Y. Y. — 1947a. Some aspects of the population increase in *Adalia bipunctata* 2. The strains without males. — *Dokl. Akad. Nauk SSSR*, 57, 951-954.

Lus, Y. Y. — 1947b. Some rules of reproduction in populations of *Adalia bipunctata*: heterozygosity of lethal alleles in populations. — *Dokl. Akad. Nauk SSSR*, 57, 825-828.

Majerus, M. E. N. — 1994. Ladybirds (No. 81 New Naturalist Series). — *Harper Collins*, London.

Matsuka, M., Hashi, H. & Okada, I. — 1975. Abnormal sex-ratio found in the lady beetle, *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae). — *Appl. Ent. Zool.*, 10, 84-89.

Niijima, K. — 1983. Experimental transfer of abnormal sex ratio in two ladybird species. — *Bull. Fac. Agric. Tamagawa Univ.*, 23, 11- 17.

Niijima, K. & Nakajima, K. — 1981. Abnormal sex ratio in *Menochilius sexmaculatus* (Fabricius). *Bull. Fac. Agric. Tamagawa Univ.*, 21, 59-67.

Shull, H. F. — 1948. An all-female strain of lady beetles with reversion to normal sex ratios. — *Am. Nat.*, 82, 241-251.

Werren, J. H., Hurst, G. D. D., Zhang, W., Breeuwer, J. A. J., Stouthamer, R. & Majerus, M. E. N. — 1994. Rickettsial relative associated with male killing in the ladybird beetle (*Adalia bipunctata*). — *J. Bacteriol.*, 176, 388-394.

Wratten, S. D. — 1973. The effectiveness of the coccinellid beetle, *Adalia bipunctata* (L.) as a predator of the lime aphid, *Eucallipterua tiliae* L. — *J. Anim. Ecol.*, 42, 785-802.

Wratten, S. D. — 1976. Searching by *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae) and escape behaviour of its aphid and ciccadellid prey on lime (*Tilia* x *vulgaris* Hayne). — *Ecol. Entomol.*, 1, 139-142.

Zakharov, I. A., Hurst, G. D. D., Majerus, M. E. N. & Chersheva, N. — 1996. Male-killing in the St. Petersburg population of *Adalia bipunctata* is not caused by a *Rickettsia*. — *Russ. J. Genet.*, In press.