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SHORT COMMUNICATION

Spiroplasma infection causes either early or late male killing in *Drosophila*, depending on maternal host age

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Abstract Symbiont-induced male-killing phenotypes have been found in a variety of insects. Conventionally, these phenotypes have been divided into two categories according to the timing of action: early male killing at embryonic stages and late male killing at late larval stages. In *Drosophila* species, endosymbiotic bacteria of the genus *Spiroplasma* have been known to cause early male killing. Here, we report that a spiroplasma strain normally causing early male killing also induces late male killing depending on the maternal host age: male-specific mortality of larvae and pupae was more frequently observed in the offspring of young females. As the lowest spiroplasma density and occasional male production were also associated with newly emerged females, we proposed the density-dependent hypothesis for the expression of early and late male-killing phenotypes. Our finding suggested that (1) early and late male-killing phenotypes can be caused by the same symbiont and probably by the same mechanism; (2) late male killing may occur as an attenuated expression of early male killing; (3) expression of early and late male-killing phenotypes may be dependent on the symbiont density, and thus, could potentially be affected by the host immunity and regulation; and (4) early male killing and late male killing could be alternative strategies adopted by microbial reproductive manipulators.

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Introduction

Maternally inherited female-biased sex ratios have been found in a variety of arthropods, the majority of which are due to male killing caused by microbial endosymbionts. Conventionally, these phenotypes have been classified into two categories: "early male killing" wherein embryos or young larvae are killed, and "late male killing" whereby mature larvae are killed (Hurst 1991, 1993; Hurst and Majerus 1993).

Early male killing is caused by diverse endosymbiotic bacteria including *Spiroplasma*, *Rickettsia*, *Wolbachia*, and others (O'Neill et al. 1997; Bourtzis and Miller 2003). It has been suggested that the early male killing can be adaptive for the maternally inherited symbionts via the following mechanisms: (1) resource reallocation from killed males to females; (2) inbreeding avoidance of females with their brothers; and (3) reduced cannibalism of females (Hurst 1993; Hurst and Majerus 1993).

On the other hand, the late male killing, known only from mosquitoes, is caused by unicellular eukaryotes of the phylum Microsporidia (Hurst 1991; Dunn and Smith 2001). While the early male killers are vertically transmitted solely through matrilines, the late male killers have horizontal route of transmission in addition to vertical one. Hence, the microsporidians gain the maximal benefit from the late male killing: male hosts are selectively killed because they do not contribute to vertical transmission, and the timing of killing is the last larval stage when the number of released microsporidian cells for horizontal transmission is maximal (Hurst 1991; Dunn and Smith 2001).

The classification of male-killing phenotypes is based not only on the timing of action, but also on the evolutionary strategies adopted by the symbionts and the microbial agents responsible for the phenotypes. Thus, it has been assumed that the early and late male-killing phenotypes are fundamentally different phenomena whose underlying mechanisms are also entirely different (O'Neill et al. 1997; Bourtzis and Miller 2003).

In fruit flies of the genus *Drosophila*, *Spiroplasma paulsonii*, and allied endosymbionts cause typical early male killing, where male eggs of infected mothers die during embryogenesis and do not hatch (Counce and Poulson 1962; Anbutsu and Fukatsu 2003). Meanwhile, it was reported that interspecific transfer of the spiroplasmas sometimes resulted in attenuated level, reduced stability, and delayed stage of male-killing expression in recipient flies (Sakaguchi and Poulson 1963; Counce and Poulson 1966). Here, we report that a spiroplasma strain normally causing early male killing also induces late male killing depending on maternal age of the host insect *Drosophila melanogaster*.

Materials and methods

Material The male-killing *Spiroplasma* sp. strain NSRO was artificially transfected into the wild-type Oregon-R strain of *D. melanogaster* as described previously (Anbutsu and Fukatsu 2003). The infected flies were reared on a standard cornmeal agar medium at 25°C under a long-day regimen (16 h of light–8 h of dark). Because the spiroplasma-infected flies produce all-female broods, males were supplied from an uninfected Oregon-R strain for maintenance of the fly stock. It has been confirmed by polymerase chain reaction (PCR) that the Oregon-R strain used in this study is free from *Wolbachia* infection.

Sampling The spiroplasma-infected females were allowed to mate with males immediately after emergence, and their eggs were collected. For examination of pupal and adult mortality, eight females were allowed to lay eggs, the eggs were collected 0–4, 4–6, 6–10, 10–14 days after emergence, and emerged adults and dead pupae were counted and morphologically sexed. For examination of larval mortality, 40 females were allowed to lay eggs. The eggs were collected 1–2, 4–5, and 8–9 days after emergence. The larvae and pupae were harvested 4 and 7 days after egg collection and were counted, staged, and sexed by using male-specific PCR.

DNA extraction and PCR Insects were individually squashed in 100 μl of sodium–boric acid (SB) buffer (10 mM Tris–HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 25 mM NaCl) containing 2 mg of Proteinase K and were incubated at 55°C for 30 min followed by 94°C for 5 min. The supernatant was subjected to PCR detection. To check the quality of extracted DNA, the *Attacin A* gene of the host insect was amplified by using the primers AttA241F (5′-CCGGAAA CACTCAAAGTGGTC-3′) and AttA381R (5′-TGAAT AAATTGGCATGGGCC-3′). Symbiont detection was performed by using the primers SpoulF (5′-GCTTAACTCC AGTTCGCC-3′) and SpoulR (5′-CCTGTCTCAATGTT AACCTC-3′) targeting the 16S rRNA gene of the spiroplasma. While the symbiont was detected from all the pupae of both sexes, the symbiont detection failed in some (~20%) of the larvae. These negative larvae were all first or second instar and contained both males and females. Considering that these young larvae are very small in size and infection density of the spiroplasma is the lowest at young larval stages (Anbutsu and Fukatsu 2003), we suppose that most of these samples were actually infected but negative due to the sensitivity of the detection method.

Sexing For sexing of adult insects that died before emergence, pupal cases were opened by fine forceps and their male-specific morphological traits (i.e., sex combs in forelegs and claspers in external genitalia) were examined. For sexing of larvae and young pupae, we adopted male-specific PCR targeting a Y-linked gene of *D. melanogaster kl-5* (Gepner and Hays 1993) by using the primers kl5-1 (5′-GCTATAAACTTTAACGCAGTC-3′) and kl5-2 (5′-GCAAGCAATATGCTCTC-3′).

Results

The spiroplasma-infected fly strain generally expressed complete male killing. Incomplete expression was observed only in young mothers (data not shown, but see Fig. 3 in Anbutsu and Fukatsu 2003). Anbutsu and Fukatsu (2003) examined adult insects that emerged in the rearing bottles. However, inspection of pupae attaching on the wall of the bottles led to an unexpected discovery. Specifically, offspring of young mothers died in the pupal stage. Dissection of the dead pupae revealed that most of them were male insects.

Our observation of adult insects confirmed the progressive expression of complete male killing according to maternal age (Fig. 1a). In addition to healthy adult insects, we also counted frail adult insects that died soon after emergence and dead adult insects in pupal cases. Across all maternal ages, few females died at pupal and young adult stages (Fig. 1b). In contrast, male offspring of young

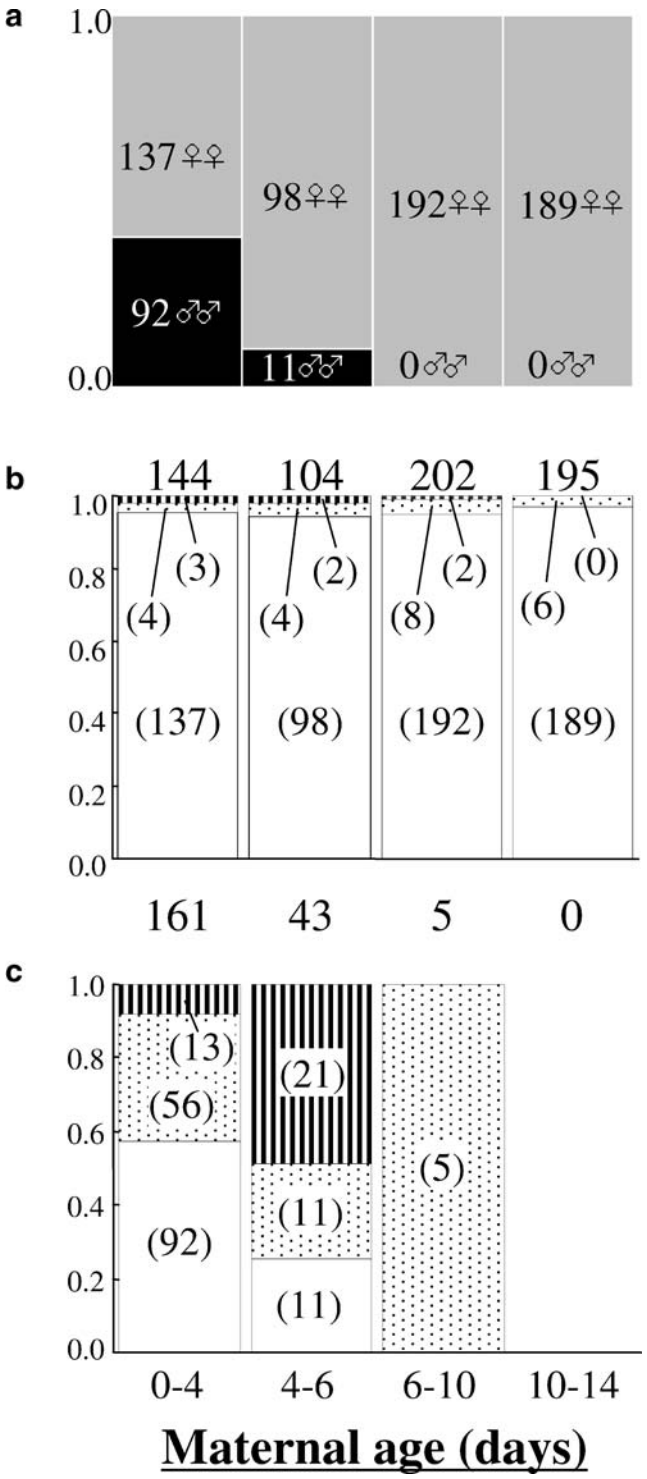

Fig. 1 Mortality of the *Spiroplasma*-infected *D. melanogaster* at pupal and young adult stages. **a** Adult offspring sex ratios of females of different ages. Females are *grey* and males are *black*. **b** Proportions of offsprings that were healthy adult females (*open*), frail adult females that died soon after emergence (*dotted*), and dead adult females in pupal cases (*striped*). **c** Proportions of healthy adult males (*open*), frail adult males that died soon after emergence (*dotted*), and dead adult males in pupal cases (*striped*). Sample sizes are shown on the *columns*

mothers exhibited high mortalities (Fig. 1c). These results indicated that specifically in the offspring of young mothers, the spiroplasma infection caused a late male-killing phenotype at pupal stage.

Next, we examined whether similar male-specific mortality is observed in larvae. At day 4, overall sex ratios were not biased. However, the sex ratios in different larval/pupal stages consistently exhibited skewed patterns: males were predominant at younger stages while females were more common at older stages (Fig. 2a). In the seventh day cohort, sex ratios of larvae and pupae were strongly female-biased. Here, too, the sex ratios of the immature stages appeared to exhibit a similar or even more exaggerated pattern. Of the few young larvae that remained, most of them were males, while the great majority of the pupae were females (Fig. 2). Adult insects that emerged from these broods also exhibited the progressive expression of all-female progeny depending on the maternal age (Fig. 2c). Although inherent difference of developmental times between male and female, if existed, might contribute the difference in sex ratio between stages, the overall results strongly suggested that the male-killing phenotype is also expressed during the host larval development.

Discussion

We concluded that the early male-killing spiroplasma also causes late male killing at larval and pupal stages of the host insect. It was documented that in the offspring of spiroplasma-infected females, male embryos are selectively killed before gastrulation, and thus, male eggs do not hatch (Counce and Poulson 1962). Meanwhile, several studies have reported that the expression of male killing is often incomplete in the offspring of young mothers despite the spiroplasma infection (Counce and Poulson 1966; Anbutsu and Fukatsu 2003). The gap between the complete early male killing and the leaky male production was bridged by the late male-killing phenomenon.

Infection density of the spiroplasma is the lowest in newly emerged insects and becomes higher as they get older, and only the newly emerged females produce some male offspring (Counce and Poulson 1966; Anbutsu and Fukatsu 2003). It appears plausible, although speculative, that infection density of the spiroplasma in maternal body is correlated not only with the occurrence/absence of male-killing expression (Anbutsu and Fukatsu 2003) but also with the timing of male-killing expression. Fig. 3 shows a schematic illustration of the density-dependent hypothesis for the expression of early and late male-killing phenotypes caused by the spiroplasma. To verify

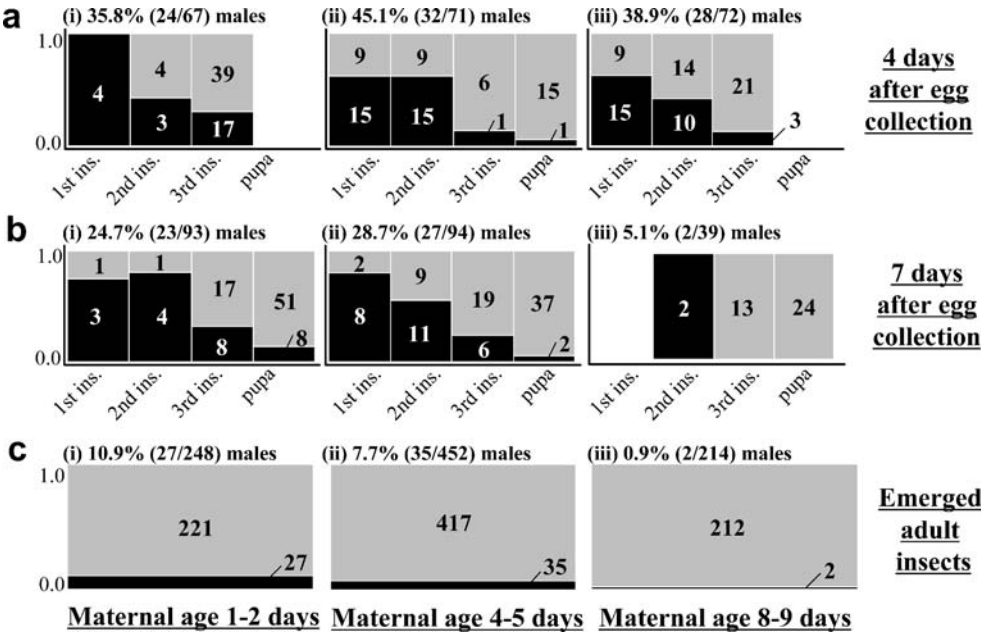

Fig. 2 Sex ratios of the *Spiroplasma*-infected *D. melanogaster* at larval and young pupal stages. **a** Sex ratios of living larvae and pupae produced by mothers at different ages, inspected 4 days after egg collection. **b** Sex ratios of living larvae and pupae produced by mothers of different ages, inspected 7 days after egg collection. **c** Sex ratios of adult insects emerged from the respective broods. *Gray columns* and *black columns* indicate females and males, respectively. Sample sizes are shown on the *columns*

the hypothesis, of course, further experimental studies, particularly quantitative PCR analyses of the infection densities in young females, are needed.

On the basis of the different timing of action and the evolutionary logic, the distinction between early and late male killing has been widely accepted (Hurst 1991; Hurst and Majerus 1993; O'Neill et al. 1997; Bourtzis and Miller 2003). However, we suggest that the dichotomized categories could be merged into a physiological, ecological and evolutionary continuum. If the same symbionts can express different levels of early and late male-killing phenotypes, the traits will be differentially selected for under different ecological contexts. For example, early male-killing variants will be selected for when newborns suffer a severe resource competition, while late male-killing variants will be favored when horizontal transfer of the symbiont is facilitated. Then, the early and late male-killing phenotypes can be alternative strategies adoptable by the symbiont, which enables an evolutionary change of the reproductive strategy in response to the ecological necessity. If the early and late male-killing phenotypes are dependent on the symbiont density as shown in Fig. 3, the timing of male killing can be influenced by the host immunity and regulation.

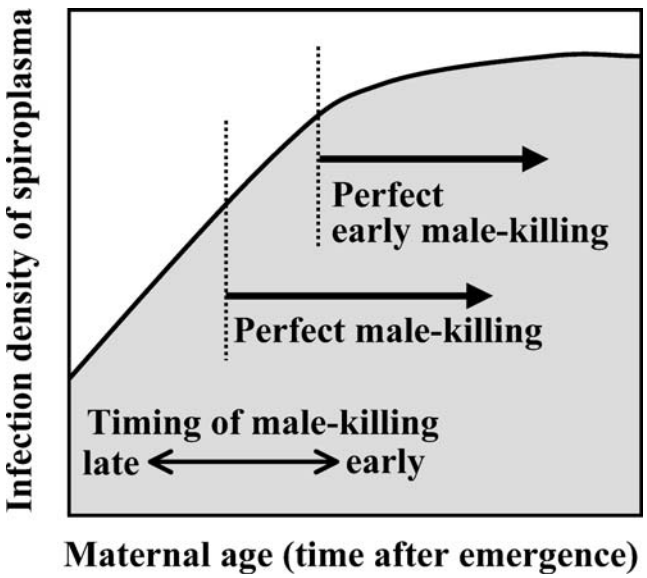

Fig. 3 Schematic illustration of the density-dependent hypothesis for the expression of early and late male-killing phenotypes caused by the spiroplasma

In conclusion, we demonstrated that the early male-killer *Spiroplasma* also causes a late male-killing phenotype according to the maternal age of the *Drosophila* host, which led to the following concepts: (1) early male killing and late male killing can be caused by the same symbiont and probably by the same mechanism; (2) late male killing can occur as an attenuated expression of early male killing; (3) early and late male-killing phenotypes might be dependent on the symbiont density, and thus, could potentially be affected by the host immunity and regulation; and (4) early male killing and late male killing could be regarded as alternative strategies adoptable by microbial reproductive manipulators. These concepts would promote our empirical and theoretical approaches to the diverse symbiont-induced male-killing phenomena.

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