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***Wolbachia*-induced Apoptosis Associated with Increased Fecundity in *Laodelphax striatellus* (Hemiptera: Delphacidae)**

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Abstract

Wolbachia influence the fitness of their invertebrate hosts. They have effects on reproductive incompatibility and egg production. Although the former are well characterized, the mechanistic basis of the latter is unclear. Here, we investigate whether apoptosis, which has been implicated in fecundity in model insects, influences the interaction between fecundity and *Wolbachia* in the planthopper, *Laodelphax striatellus*. *Wolbachia*-infected females produced about 30% more eggs than uninfected females. We used TUNEL staining to visualize apoptosis. Microscopic observations indicated that the *Wolbachia* strain *wStri* increased the number of ovarioles that contained apoptotic nurse cells in both young and aged adult females. The frequency of apoptosis was much higher in the infected females. The increased fecundity appeared to be due to apoptosis of nurse cells, which provides nutrients to the growing oocytes. In addition, cell apoptosis inhibition by caspase mRNA interference (RNAi) in *Wolbachia* infected *L. striatellus* markedly decreased egg numbers. Together, these data suggest that *wStri* might enhance fecundity by increasing the number of apoptotic cells in the ovaries in a caspase-dependent manner. Our findings establish a link between *Wolbachia*-induced apoptosis and egg production effects mediated by *Wolbachia*, although the way that the endosymbiont influences caspase levels remains to be determined.

Keywords: *Wolbachia*, *Laodelphax striatellus*, apoptosis, TUNEL, caspase, RNAi

Introduction

Wolbachia are intracellular, maternally inherited bacteria that have a wide range of effects on host physiology and reproduction. As well as causing effects on hosts that enhance their spread, like cytoplasmic incompatibility (CI), male-killing, thelytokous parthenogenesis, and feminization (O'Neill *et al.*, 1997), *Wolbachia* can affect host

58 fitness by influencing egg production. There are a range of fecundity effects
59 associated with *Wolbachia*, which include fecundity decreases (Stouthamer & Luck,
60 1993) as well as increases (Fast *et al.*, 2011; Vavre *et al.*, 1999). In fact, *Wolbachia*
61 effects on this trait can vary between closely related species (Stouthamer & Luck,
62 1993; Vavre *et al.*, 1999) and different *Wolbachia* strains in the same species (Toomey
63 *et al.*, 2013). *Wolbachia* effects on egg production can also evolve very rapidly as
64 documented in *Drosophila simulans* where the infection initially decreased fecundity
65 but then evolved to increase it despite an elapsed period of only two decades (Weeks
66 *et al.*, 2007). While *Wolbachia* are dependent on their host for survival, and have
67 developed intimate associations with their hosts, most arthropods can survive and
68 reproduce with or without their *Wolbachia* infections. Nevertheless, there are cases
69 where the host has developed a close dependency on the endosymbiont (Dedine *et al.*,
70 2003).

71 Although *Wolbachia* effects on fecundity were discovered several years ago, the
72 mechanisms involved are still unknown, particularly in cases where the infection
73 causes an increase in fecundity. One way *Wolbachia* might increase fecundity is by
74 influencing apoptosis, a common form of programmed cell death, which regulates cell
75 elimination in multicellular organisms, is needed in several physiological processes
76 and has been implicated in egg production rates. Apoptosis is also a classic feature of
77 female germline development in both vertebrate and invertebrate species
78 (Kamalakaran *et al.*, 2015; Landmann *et al.*, 2011; Mpakou *et al.*, 2011; Mpakou *et*
79 *al.*, 2006; Nezis *et al.*, 2000). Morphological changes associated with apoptosis
80 include chromatin and cytoplasm condensation, chromosomal DNA fragmentation,
81 breaking up of nuclei, and disintegration of the actin cytoskeleton (Mpakou *et al.*,
82 2006). One of the roles of apoptosis in insects involves oogenesis. In the anterior
83 tropharium of the ovariole, a cluster of nurse cells synthesizes all cytoplasmic
84 products stored in the oocyte and apoptosis of these cells is essential for oocyte
85 maturation (Mpakou *et al.*, 2011).

86 The molecular pathways of apoptosis have been examined in several insects
87 (Kumar & Doumanis, 2000; Xu *et al.*, 2009; Bryant *et al.*, 2008; Liu & Clem, 2011).
88 The main effectors in the apoptotic pathway are cysteine proteases called caspases

(Alnemri *et al.*, 1996). Caspases are present as inactive pro-enzyme precursors (initiation caspases) in living cells; however, once they receive an apoptotic signal, they undergo proteolytic processing and are converted into their active forms (effector caspases), which triggers the dismantling of intracellular components. Initiator and effector caspases are the core components in the apoptosis pathway. So far, apoptosis-related caspases have been analyzed in only a few insects (Mpakou *et al.*, 2011; Mpakou *et al.*, 2006; Nezis *et al.*, 2000; Kumar & Doumanis, 2000). In *Drosophila*, seven caspase genes have been identified, including four effector caspases (Dcp-1, Drice, Damm, and Decay) which match caspase-3, the major mammalian effector caspase (Peterson *et al.*, 2003). In *Aedes aegypti*, eleven caspases have been annotated, including two effector caspases like caspase-3 (Bryant *et al.*, 2008).

The small brown planthopper, *Laodelphax striatellus*, is 100% infected with *Wolbachia* strain wStri in China (Zhang *et al.*, 2013). *Wolbachia* are abundant in *L. striatellus* ovarian cells where they are vertically transmitted. *Wolbachia* cause high CI levels in *L. striatellus* (Noda *et al.*, 2001). The influence of *Wolbachia* on host oogenesis and its associated processes is unknown, although a previous paper (Cui *et al.*, 1998) suggests an enhancement of fecundity associated with this infection.

We therefore tested the impact of *Wolbachia* on fecundity, and investigated whether changes in infected ovaries were associated with apoptosis. We identified caspase genes in *L. striatellus*, and inhibited caspase mRNA expression by RNAi to test for impacts on fecundity. Our results demonstrate an increase in fecundity in *Wolbachia*-infected *L. striatellus*, and indicate that caspase-mediated apoptosis plays a role in this process.

Results

Effects of Wolbachia on L. striatellus fitness

Egg production in infected females (137 ± 7 (mean $\pm$ SE) eggs per female) was significantly greater ($P < 0.001$, two-tailed t test) than in uninfected females (103 ± 5 eggs per female) (Fig. 1A). Additionally, the number of offspring that reached maturity was greater for infected females (70 ± 3 per female) than for uninfected

females (58 ± 3) (Fig. 1B). However, *Wolbachia* had no significant effect on either the proportion of offspring that were females (Fig. 1C, generalized linear mixed model: $z = 0.92$, $P = 0.36$) or on the proportion of eggs that hatched (Fig. 1D, generalized linear mixed model: $z = 0.33$, $P = 0.74$). *Wolbachia* infection also did not significantly affect development time (Fig. 1E, Cox proportional hazards mixed model: $z = 1.59$, $P = 0.11$) or adult female longevity (Fig. 1F, Cox proportional hazards mixed model: $z = 0.32$, $P = 0.75$).

Effect of Wolbachia on apoptosis in ovaries

We found that *L. striatellus* ovaries are of the telotrophic meroistic type, with 11–17 short ovarioles in each of the two ovaries (Fig. 2A). The ovarioles have three parts: the terminal filament, tropharium, and vitellarium (Fig. 2B).

TUNEL staining (green fluorescence) revealed no signs of apoptosis in the vitellarium but revealed extensive apoptosis in the nurse cells of the tropharium in both uninfected and infected ovarioles (Figs. 3A–3D). Of 991 ovarioles whole-tropharia that were examined in four-day-old uninfected and infected adult females, 92 (9.3%) were undergoing apoptosis (Figs. 3A, 3B, and 4). The percentage of tropharia that were TUNEL-positive in infected females (11.8%) was somewhat higher than in uninfected females (7.5%) (Fig. 4). These percentages increased proportionately in 10-day-old adults (Fig. 4). As expected, no *Wolbachia* were observed in the ovarioles of uninfected females (Figs. 3A and 3C), and many *Wolbachia* were observed in the ovarioles of infected females (Figs. 3B and 3D). Apoptosis was more prominent in the tropharia of 4-day-old infected females than in uninfected females as shown by TUNEL and FISH staining (Fig. 4).

Though 4-day-old *L. striatellus* females have the strongest fecundity, they nearly lose most of reproductive ability at about 10 days after emergence (Bai *et al.*, 2009; Cui *et al.*, 1998; Noda *et al.*, 2001). At this age, apoptotic nurse cells in the tropharia were present in both uninfected and infected *L. striatellus* ovarioles (Figs. 3C and 3D), but the frequency of apoptosis was significantly higher in infected females ($t = 4.52$, $df = 8$, $P < 0.01$). In the tropharia of both uninfected and infected ovarioles, the posterior region had bright green spots, indicating the presence of apoptotic cells; while the anterior region had weak punctuated green fluorescence (Figs. 3C and 3D,

red arrowheads) but no signs of DAPI, which were considered as TUNEL-negative. These results indicate that the presence of *w*Stri increases the number of nurse cells undergoing apoptosis not only in ovaries of young females but also in ovaries of females near the end of their reproductive period.

Caspase genes in Wolbachia-induced apoptosis

Fragments of four different caspase genes were found in the *L. striatellus* transcriptome (Ju *et al.*, 2017). We used PCR, 5' RACE and 3' RACE methods to obtain the full lengths of the four caspase genes (Extended Data File 1). Two genes (*Lscaspase-1a* MG279146 and *Lscaspase-1c* MG279148) were highly homologous to each other (Fig. 5A left panel) and shared significant sequence similarities with insect caspase-1s (Figs. 5A left panel, 5B). Two long prodomain-carrying caspase genes were also identified. One (*Lscaspase-8* MG279147) shares similar characteristics with the caspase-8 gene in brown planthopper, *Nilaparvata lugens* (Fig. 5A middle panel), which is the closest species to *L. striatellus*. The other (*Lscaspase-Nc* MG279149) has significant sequence identities with the caspase-Nc gene in *N. lugens* that feature a recruitment domain (CARD) (Fig. 5A right panel). A phylogenetic analysis based on the maximum likelihood method suggested that caspase genes in *L. striatellus* were related to similar caspase genes in the other planthopper characterized as well as other insect species (Fig. 5B).

The timing of expression of the four caspases and Vg in uninfected and infected female *L. striatellus* at different times after emergence was examined by RT-qPCR. In 5-day-old females, the expression levels of the four caspase genes and Vg gene were higher in infected females than in uninfected females (Figs. 6A-6E). The particularly high expression levels in infected females on day 5 coincided with a higher *Wolbachia* density on this day, as shown by the increased copy number of *wsp* (Fig. 6F). However expression levels were also high later for some genes, suggesting that there is no simple relationship between expression and *Wolbachia* density, and that there are other ways of triggering caspase/Vg rather than through a density effect in *Wolbachia*-infected *L. striatellus*.

Effect of inhibition of caspases mRNA expression

To confirm that apoptosis has a role in oogenesis, we knocked down *Lscaspase*-specific genes expression using RNAi by microinjecting dsRNA (dscas-) into the *L. striatellus* with dsGFP as a control. Egg production in dscas- females were significantly decreased than in dsGFP females (Fig. 7). The newly emerged adult ovaries targeted mRNA levels of *Lscas-1a*, *Lscas-1c*, *Lscas-8* or *Lscas-Nc* were greatly reduced by approximately 90, 90, 90, and 80%, respectively; while there were no apparent reductions for non-target genes, indicated that the dsRNA mediated silencing was sequence specific (Fig. 8A-8D). Additionally, the percentage of tropharia that were TUNEL-positive in 4-day-old dscas- adult females was lower than in dsGFP females (Fig. 8E). RNAi microinjection had no significant effect on the *Wolbachia* density in dscas- ovaries (Fig. 8F). These results indicate that dscas- significantly reduces egg production in *Wolbachia*-infected *L. striatellus*.

Discussion

The increased fecundity and number of mature offspring produced by infected *L. striatellus* is consistent with findings in other invertebrates, including the fruit flies *D. mauritiana* and *D. simulans* (Weeks *et al.*, 2007; Fast *et al.*, 2011), as well as a previous report for *L. striatellus* (Cui *et al.*, 1998). Other findings also point to an interaction between *Wolbachia* and oogenesis, including the observation that the parasitic wasp *Asobara tabida* cannot produce mature oocytes without the presence of *Wolbachia* (Pannebakker *et al.*, 2007), and the observation that removal of *Wolbachia* from the nematode *Brugia malayi* disrupted embryogenesis and caused widespread intracellular changes in adults (Landmann *et al.*, 2011). The higher egg and offspring production suggests that *Wolbachia* can increase the fitness of *L. striatellus*, reflecting a possible mutualistic association between *Wolbachia* and *L. striatellus*. Because *Wolbachia* are maternally transmitted, the host-bacteria partnership favors infected females. This fitness effect of *Wolbachia* on host reproduction should increase its prevalence in the population, which could contribute to the widespread distribution

and high frequency of *Wolbachia* in *L. striatellus* (Zhang *et al.*, 2013) as in the case of other invertebrates (Hoffmann *et al.*, 2015).

Apoptosis has previously been connected to egg development and can be influenced by ectoparasites and symbionts. Apoptosis of ovarian nurse cells has been implicated in fecundity of the ladybird beetle, *Adalia bipunctata*, and is considered as an essential component of normal female germline development (Mpakou *et al.*, 2011). Extracellular parasitic worms, such as filarial nematodes, schistosomes, and the cestode *Taenia crassiceps*, can induce apoptosis in host cells (Jame & Green, 2004). Bacterial pathogens such as *Neisseria*, *Chlamydia*, and *Legionella pneumophila* can either block or induce apoptosis in host cells, depending on the stage of infection (Faherty & Maurelli, 2008; Lancellotti, 2009). Apoptosis might even have a symbiotic origin (Kroemer, 1997).

In *D. melanogaster*, *Wolbachia* increased the frequency of cyst cell apoptosis in ovarian cells, but it was unclear how apoptosis affected reproduction (Zhukova & Kiseleva, 2012). In the present study, *Wolbachia*-infected *L. striatellus* female ovaries contained apoptotic nurse cells. Thus, *Wolbachia* appears to have the potential through apoptosis of nurse cells to continuously provide nutrition for oogenesis, increasing reproduction in the host.

Although we found evidence that *Wolbachia* triggered apoptosis, the infection can also inhibit programmed cell death (PCD) in the host germarium of some insects (Fast *et al.*, 2011; Pannebakker *et al.*, 2007). In these insects, intracellular bacteria may control host cell death by a different method. Apart from apoptotic PCD, there are two other major types of PCD: lysosomal and cytoplasmic cell death (Bröker *et al.*, 2005; Sperandio *et al.*, 2000). To achieve a rapid and efficient clearance of defunct cells, these programs usually operate synergistically (Mpakou *et al.*, 2011). Perhaps *Wolbachia* in *D. mauritiana* germarium increase apoptosis, while decreasing lysosomal and cytoplasmic cell death at the same time. The role of different types of PCD mechanisms in *Wolbachia*-infected *D. mauritiana* oocyte development and ovary maturation warrants further study, as well as the role of these processes in the dynamic evolutionary interactions between *Wolbachia* which rapidly shifted a fecundity deficit (Hoffmann *et al.*, 1990) into a fecundity advantage (Weeks *et al.*, 2007).

Four caspase homolog genes were identified in *L. striatellus*, including two genes (*Lscaspase-1a* and *Lscaspase-1c*) with similarities to insect caspase-1s. Caspase-1s

proteins are effector death proteases thought to execute apoptosis in insects, which are most closely related to the mammalian apoptotic effectors caspase-3. Two other long prodomain-carrying caspase homolog genes (*Lscaspase-8* and *Lscaspase-Nc*) showed significant sequence similarities with insect caspase-8 and Nedd2-like caspase (Nc), respectively. We speculate that these genes have similar functions with their closest caspase relatives in other insects. *Wolbachia* increased the expression levels of the four caspase genes in 5-day-old *L. striatellus* females. The increased caspase mRNAs and Vg mRNA observed in *Wolbachia*-infected *L. striatellus* also support the hypothesis that *Lscaspase-1a*, *Lscaspase-1c*, *Lscaspase-8*, and *Lscaspase-Nc* are critical in *Wolbachia*-induced apoptosis pathways. Although caspase RNAi in *Wolbachia* infected *L. striatellus* markedly decreased egg numbers, the detailed functions of these *Lscaspase* genes and their interactions in the caspase activation cascades require further elucidation, as does the ways in which the *Wolbachia* directly affect the expression of these genes.

In our study, we found that there was no simple relationship between caspase genes expression and *Wolbachia* density in *Wolbachia*-induced apoptosis. While, no other apoptosis gene was obtained after comparing the apoptosis genes that have been reported with the *L. striatellus* transcriptome sequences. In apoptosis pathway, there may have some apoptosis inhibitors which function together with caspases in *L. striatellus* ovaries cell apoptosis as they do in *Drosophila* (McCall, 2004).

In summary, the present results show that *Wolbachia* wStri increases the fecundity of *L. striatellus*. This effect appears at least partly mediated through caspase genes. The resulting fitness benefits are likely to promote the spread of *Wolbachia* infection and account for its high prevalence, and potentially increase the pest status of *L. striatellus* through population level effects.

Experimental procedures

Insect maintenance and line production

Two different *L. striatellus* lines were used: the naturally infected *Wolbachia* wStri line, which originated from Nanjing, Jiangsu, China, and a tetracycline-cured counterpart. A tetracycline-cured uninfected line was generated by antibiotic treatment of infected insects. Tetracycline hydrochloride solution (0.1%) was added to rice seedlings that were fed to *Wolbachia*-infected *L. striatellus*. The treatment lasted two consecutive generations until

Wolbachia was not detected by diagnostic PCR. The cured *L. striatellus* were maintained for over 10 generations on antibiotic-free rice seedlings in a laboratory to eliminate any effects of residual tetracycline (Ju *et al.*, 2017). To ensure a similar nuclear background, all uninfected insects treated by tetracycline and infected insects were offspring of one infected pair of *L. striatellus* (Extended Data Fig. 1).

Fitness effects of Wolbachia on L. striatellus

To analyze the effect of *Wolbachia* on *L. striatellus*, infected and uninfected lines were scored for fecundity, adult longevity, development time, egg hatch rate, sex ratio, and number of mature offspring. All insects used in the experiments were checked to confirm that intracellular bacteria other than *Wolbachia* were not present using an approach previously described (Koukou *et al.*, 2006; Negri *et al.*, 2006).

For reproduction, 20 newly emerged uninfected and infected *L. striatellus* pairs were randomly selected, and each pair was introduced into a glass beaker (7-cm diameter × 14-cm height) with antibiotic-free rice seedlings. Seedlings were changed every 24 h, and eggs were counted by gently stripping the sheaths under a binocular stereomicroscope. Eggs were counted until the female died, and newly emerged males were supplemented if the original male died before the experiment ended. Then, fecundity and adult longevity were recorded.

At the same time, 20 eggs were randomly selected from each of the 20 uninfected and infected *L. striatellus* pairs. The eggs developed into adults in individual glass tubes with rice seedlings, and their emergence was checked daily until all nymphs completed development. The developmental time of a randomly selected individual of the F₁ generation was recorded. The eggs that were not selected for observation were left to develop. When the *L. striatellus* F₁ generation was visible as nymphs, the survival of juveniles was calculated based on the numbers of eggs. All nymphs were kept on rice seedlings until they developed into adults. Then, the sex ratio and number of F₁ mature offspring were recorded. The experiments were undertaken at 25.0°C and 60% relative humidity, with a 16: 8 h light: dark photoperiod. All experiments that measures fitness traits were replicated four times.

To test the effect of *Wolbachia* on *L. striatellus*, data were analyzed with different tests. Female adult longevity and development time were analyzed using a Cox proportional hazard model in R (version 2.3.0) (Ahmed *et al.*, 2015). We analyzed both the fecundity and amount of mature offspring using a two-tailed *t* test in IBM Statistics SPSS (version 20.0). Both egg hatch rate and sex ratio were treated as ratios and analyzed using generalized linear mixed models with a binomial error structure model. The model parameters were estimated by maximum likelihood in R (version 2.3.0) (Ahmed *et al.*, 2015).

FISH

Wolbachia was detected in ovarioles by fluorescence in situ hybridization (FISH) as described by Gold *et al.* (1994). Specific oligonucleotide probes were designed against the 16S rDNA of *Wolbachia*. Two *Wolbachia* probes labeled with rhodamine red at the 5' end were used: *W*₁, 5'-AATCCGGCCGACCGACCC-3', and *W*₂, 5'-CTTCTGTGAGTACCGT CATTATC-3'. Ovaries from 4- and 10-day-old adult female *L. striatellus* were dissected in phosphate buffered saline (PBS) and separated into individual ovarioles. The ovarioles were fixed in 4% formaldehyde solution plus 0.1% Triton X-100 (Sigma) for 15 min, washed twice in PBS for 5 min each, permeabilized with 20 µg/ml DNase-free proteinase K (Roche Applied Sciences) in 10 mM Tris-HCl pH 7.5 for 10 min at 37°C, preincubated at 50°C in 0.1% SDS, 5 × SSC, 20 mM Tris-HCl, 50% formamide, 250 mg/ml Salmon sperm DNA, and 0.5 × Denhardt's solution for 1 h, incubated in 10 ng of each probe for 6 h, rinsed in a 2 × SSC wash buffer with 0.1% SDS and 20 mM Tris-HCl twice for 15 min each at 55°C, washed twice in 1 × SSC wash buffer for 15 min each, washed in PBS three times for 10 min each, washed in wash solution for 15 min and double labeled for apoptotic cells by hybridization.

TUNEL assay

TUNEL preferentially labels apoptotic cells relatively late in the apoptotic process (Landmann *et al.*, 2010) and facilitates independent identification of apoptotic cells in the ovaries. The TUNEL reaction and subsequent steps were performed using the Dead EndTM Fluorometric TUNEL System kit (Promega) according to the manufacturer's protocol. Fluorescein-labeled dUTP was used to detect the fragmented genomic DNA at 37°C for 1 h in the dark. Nuclei that contained fragmented DNA and/or denatured cytoplasm of the cells stained green. After hybridization, the ovarioles were washed in PBS and mounted on a glass slide with the DAPI-containing mounting Vectashield (Vector Laboratories). Finally, samples were observed under a Zeiss LSM-710 confocal laser scanning microscope.

To quantify the TUNEL-labeled apoptosis in the tropharium of ovarioles, representative confocal Z stacks were imaged at 0.5 µm intervals. For each ovarioles 30 µm thick sections of the tropharium were obtained and projected onto single images. Cells stained with TUNEL and DAPI fluorescences were considered as apoptotic cells. Due to ovaries under staining were difficult to keep integrity, TUNEL-positive tropharia were counted and expressed as a percentage of the examined tropharia. The frequency of apoptotic tropharia was compared between the uninfected and infected females using a two-tailed *t* test in IBM Statistics SPSS

(version 20.0).

Identification of caspase genes

The fragment of caspase genes were searched against the *L. striatellus* transcriptome sequences as developed in a previous study (Huang *et al.*, 2015). The full lengths of the four caspase genes were obtained by 5' RACE and 3' RACE methods with the SMARTer® RACE 5'/3' Kit (Clontech laboratories) following the manufacturer's instructions (Extended Data File 1). *L. striatellus* caspase genes were aligned with the best-matched homologs of other insect species using ClustalX with multiple alignment parameters.

A phylogenetic tree was constructed by the maximum likelihood (ML) method using proMega 6.06. Caspase gene sequences were aligned using MUSCLE algorithm; large gaps and ambiguous sites were deleted manually. ML analyses were performed using the GTRGAMMAI model in raxmlGUI 1.3. Clade support was assessed using a nonparametric bootstrap with 1000 replicates.

RNA interference (RNAi)

L. striatellus caspase-specific nucleotide sequences were cloned into the pGEM-T Easy vector (Promega) individually. *Aequorea victoria* green fluorescent protein (GFP) was used as a control. The specific primers used to generate DNA templates are shown in Extended Data Table 1. dsRNA was synthesized, as previously described (Huang *et al.*, 2015).

Second instar nymphs of infected *L. striatellus* were used in the RNAi microinjection. Each nymph was anesthetized using carbon dioxide. Approximately 250 ng of dscas- or dsGFP was microinjected into the thorax of each nymph using the FemtoJet Microinjection System (Eppendorf). Microinjected nymphs were reared under laboratory conditions. Relative silencing efficiency of the ovaries of newly emerged adults was calculated by RT-qPCR. To confirm the effect of dscaspase on *L. striatellus* females in reproductive period, ovaries from 4-day-old dscas- adult females were dissected to observe TUNEL-positive tropharia under confocal laser scanning microscope and detect *Wolbachia* density by qPCR.

RT-qPCR and qPCR

Nearly no egg was laid within 72 h after *L. striatellus* female eclosion (Cui *et al.*, 1998). While Vg, the precursor of egg yolk protein associated with oviposition capacity, began to be absorbed into ovaries within 12 h after female emergence (Dong *et al.*, 2011). We detected relative caspase mRNA and Vg mRNA levels in ovaries of *L. striatellus* female that emerged at the first day and every other day (1, 3, 5, 7, 9, 11 days after emergence) by RT-qPCR with the SYBR Primex Ex Tap™ Kit (Takara Biotechnology) following the manufacturer's instructions. Total RNA was extracted using TRIZOL reagent (Takara) and converted to cDNAs using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara), according to the manufacturer's instructions. RT-qPCR was performed in 20µl reactions containing 10µl SYBR Primex Ex Tap, 2 µl of diluted cDNA template, 0.4 µl of each primer, 0.4 µl ROX Reference Dye II, 6.8 µl ddH₂O, on a 7500 real-time system (Applied Biosystems) using the following procedures: denaturation at 95 °C for 30 s, followed by 40 cycles of amplification (95 °C for 5 s and 60 °C for 34 s). Primers are shown in Extended Data Table 2. The dissociation curve analysis was analyzed for both primer pairs, and all experimental samples had a single sharp peak at the amplicon's melting temperature. The relative expression levels of caspase and Vg genes were calculated according to the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001) and the actin gene of *L. striatellus* was used as a housekeeping gene. All the RT-qPCR experiments were repeated three times. All the data were presented as relative mRNA expression (mean ± SD).

Wolbachia density in *L. striatellus* that emerged at different times was characterized by qPCR as described previously (Lu *et al.*, 2012). *Wolbachia* genome copy number was determined by qPCR with primers specific for the surface protein (*wsp*) of *w*Stri (Extended Data Table 2). The copy number of the *Wolbachia wsp* gene was normalized to that of the host gene actin.

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Declarations of interest:

None.

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Figure legends

Figure 1. Impact of *Wolbachia* on *L. striatellus* fitness. A: female adult fecundity, B: mature offspring number, C: proportion of offspring females, D: egg hatch rate, E: development time, and F: female adult longevity. Box and whiskers represent data quartiles and ranges, respectively. (** $P < 0.01$, *** $P < 0.001$, NS = not significant)

Figure 2. *L. striatellus* telotrophic meroistic ovary. A: Each of the two *L. striatellus* ovaries are composed of 11–17 short ovarioles that open into the bulbous end of the lateral oviducts. B: Previtellogenic ovariole stained with DAPI for DNA (blue). The *L. striatellus* ovariole is composed of a terminal filament on the tip, followed by the egg tube. The tropharium is at the tip of egg tube, which is composed of nurse cells and a trophic core, the vitellarium, just below the tropharium, in which oogenesis is completed. ET: egg tube, LO: lateral oviduct, MO: median oviduct, CP: copulatory pouch, SG: spermathecal gland, TF: terminal filament, Tr: tropharium, Vt: vitellarium.

Figure 3. Visualization of TUNEL- and DAPI-stained *L. striatellus* tropharium cells. Morphology of the structure and organization of the developing tropharia (Tr) from 4-day-old *Wolbachia*-uninfected (A) and -infected (B) female *L. striatellus* stained with TUNEL (green) and DAPI (blue) clearly demonstrates that the TUNEL-labeled cell nuclei lost their normal morphology and were undergoing apoptosis. In aged tropharia (Tr) from 10-day-old *Wolbachia*-uninfected (C) and -infected (D) female *L. striatellus*, apoptosis-specific TUNEL staining also showed copious apoptotic cell nuclei. A large majority of nurse cell nuclei in all examined tropharia (Tr) exhibited a positive and strong staining

pattern. The anterior region of the aged tropharium had punctuated TUNEL fluorescences (red arrowheads) (no DAPI signs), which were considered as TUNEL-negative. Red represents *Wolbachia*, blue represents *L. striatellus* DNA, and green represents TUNEL-labeled apoptotic cells.

Figure 4. TUNEL quantification. For each *Wolbachia*-uninfected and -infected sample, TUNEL-positive tropharia were counted and expressed as a percentage of the examined tropharia. The total number of examined tropharia is indicated in black; bars show the average percentage per experiment $\pm$ SEM.

Figure 5. Caspase genes in *L. striatellus*. (A) Sequence alignment of caspase domains of the deduced *L. striatellus* caspase proteins. The ClustalX program was used for alignments. The active site histidine, and cysteine residues are boxed. The cleavage sites that generate the large (p20), small (p10) subunit and CARD domain are marked by arrows. Black and gray shades in amino acids indicate the conserved and type-conserved residues, respectively. Left panel: caspases-1; Middle panel: caspases-8; Right panel: caspases-Nc. (B) Phylogenetic analysis used maximum likelihood method with 1000 bootstrap replications. Four caspase genes (arrowheads) belong to the family of cysteine proteases. The caspases fall into three major clusters, caspases-1s, caspases-Ncs and caspases-8s.

Figure 6. Transcript levels of caspases and Vg in ovaries of uninfected and infected *L. striatellus* females at different times after emergence. (A) caspase-1a; (B) caspase-1c; (C) caspase-8; (D) caspase-Nc; (E) Vg; (F) *wsp*. The genome copies of *Wolbachia wsp* were measure by qPCR. The copy number of *wsp* and mRNA content of caspase genes and Vg gene are expressed as fold change relative to 1-day-old females. Bars show the average fold change per experiment $\pm$ SD. * represents a significant difference in transcript levels of caspases and Vg between W- and W+ females on the same day, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 7. Impact of dscas- on *L. striatellus* fecundity. The egg production of dscaspase (dscas-1a, dscas-1c, dscas-8, and dscas-Nc) *L. striatellus* was examined. 20 newly emerged dscas- and dsGFP *L. striatellus* pairs were randomly selected, and each pair was introduced into a glass beaker with rice seedlings. Seedlings were changed every 24 h, and eggs were counted until the female died. Newly emerged males were supplemented if the original male died before the experiment ended; bar show the average eggs per female $\pm$ SD.

Figure 8. The silencing of *Lscaspase* genes expression in *L. striatellus*. Second instar nymphs were microinjected with each *dscaspase* (*dscas-1a*, *dscas-1c*, *dscas-8*, and *dscas-Nc*) individually. Relative silencing efficiency of the newly emerged adult ovaries was calculated by RT-qPCR. Low *Lscaspase* mRNA was detected in *dscas*- treated ovaries. (A) *dscas-1a*; (B) *dscas-1c*; (C) *dscas-8*; (D) *dscas-Nc*. Bars show the average relative transcript level per experiment $\pm$ SD. (E) TUNEL-positive tropharia in 4-day-old *dscas*- adult female ovaries were counted and expressed as a percentage of the examined tropharia. The total number of examined tropharia is indicated in black; bars show the average percentage per experiment $\pm$ SEM. (F) The genome copies of *Wolbachia wsp* in 4-day-old *dscas*- adult female ovaries were measured by qPCR. The copy numbers of *wsp* are expressed as fold change relative to dsGFP females. Bars show the average fold change per experiment $\pm$ SD.

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