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Spiroplasma and host immunity: activation of humoral immune responses increases endosymbiont load and susceptibility to certain Gram-negative bacterial pathogens in *Drosophila melanogaster*

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Summary

***Spiroplasma poulsonii* and its relatives are facultative, vertically transmitted endosymbionts harboured by several *Drosophila* species. Their long-term survival requires not only evasion of host immunity, but also that *Spiroplasma* does not have a net detrimental effect on host fitness. These requirements provide the central framework for interactions between host and endosymbiont. We use *Drosophila melaogaster* as a model to unravel aspects of the mechanistic basis of endosymbiont–host immune interactions. Here we show that *Spiroplasma* does not activate an immune response in *Drosophila* and is not susceptible to either the cellular or humoral arms of the *Drosophila* immune system. We gain unexpected insight into host factors that can promote *Spiroplasma* growth by showing that activation of Toll and Imd immune pathways actually increases *Sprioplasma* titre. *Spiroplasma*-mediated protection is not observed for variety of fungal and bacterial pathogens and *Spiroplasma* actually increases susceptibility of *Drosophila* to certain Gram-negative pathogens. Finally, we show that the growth of endosymbiotic *Spiroplasma* is apparently self-regulated, as suggested by the unhindered proliferation of non-endosymbiotic *Spiroplasma citri* in fly haemolymph.**

Introduction

Most insect species harbour heritable endosymbiotic bacteria. In some cases, for example aphids and *Buchnera,* an endosymbiont is required for normal host development (Moran and Baumann, 2000). The majority of heritable endosymbionts are facultative, and although not required for development, they still have important implications for the biology of their hosts (Hurst and Hutchence, 2010). In some cases, facultative endosymbionts increase their own fitness by manipulating host reproduction (Oneill *et al*., 1992; Rousset *et al*., 1992; Stouthamer *et al*., 1993). Several recent studies have shown that facultative endosymbionts can confer their hosts with resistance to parasites (Jaenike *et al*., 2010; Xie *et al*., 2010).

The most widespread and widely studied facultative endosymbiont is *Wolbachia,* which is harboured by between 20% and 70% of all insect species (Hilgenboecker *et al*., 2008). *Wolbachia* confers hosts with resistance to viral pathogens (Hedges *et al*., 2008; Teixeira *et al*., 2008). *Wolbachia* also causes reproductive manipulations, such as cytoplasmic incompatibility (Yen and Barr, 1973), which very effectively drives *Wolbachia* through a host population (Turelli and Hoffmann, 1991). These features have led to the proposition of *Wolbachia*-based strategies to lower competence of disease vectors (Bourtzis, 2008). Much of the research in this field has focused on *Wolbachia*, but there is a growing appreciation for other common facultative endosymbionts, such as *Spiroplasma.*

Spiroplasma are members of the Mollicutes, a wall-less eubacterial group related to the Gram-positive lineage, which are very widespread and is likely to be present in over 5% of all insect species (Duron *et al*., 2008). It was recently shown that *Spiroplasma* can protect its host from infection (Jaenike *et al*., 2010; Xie *et al*., 2010) and might therefore also prove to be a useful tool for the control of vector borne disease. *Drosophila melanogaster*, one of the most commonly used model organisms, naturally harbours both *Spiroplasma* (Montenegro *et al*., 2005) and *Wolbachia* (Bourtzis *et al*., 1994).

We used the *Drosophila–Spiroplasma* system to further characterize the mechanistic basis of interaction between host and endosymbiont and specifically we focused on the link between *Spiroplasma* and host immunity. Endosymbiotic *Spiroplasma* associated with *Drosophila* and other insect species are largely unable to survive or replicate outside of their hosts and although horizontal transmission between hosts can occur (Haselkorn *et al*., 2009),

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it is rare. Colonization of new hosts occurs almost entirely by vertical transmission from mother to offspring. To persist in the longer term, *Spiroplasma* needs to confer a relative fitness advantage to the matriline that is harbouring them. This can be achieved by 'male killing', where *Spiroplasma* are selectively pathogenic to males, usually at the embryonic stage (Hurst and Jiggins, 2000). When there is competition between siblings for resources, 'male killing' increases infected female fitness at the expense of non-transmitting infected males (Hurst and Majerus, 1993). Despite some advances (Veneti *et al*., 2005), the actual mechanisms used by *Spiroplasma* to selectively kill male embryos remain unknown. The strain of *Spiroplasma* that naturally infects *D. melanogaster,* MSRO (*melanogaster* sex ratio organism), causes male killing (Montenegro *et al*., 2005). Since the MSRO *Spiroplasma* was only recently discovered, most of the work on endosymbiotic *Spiroplasma* and the mechanism of male killing has focused on NSRO (*nebulosa* sex ratio organism), which was isolated from *Drosophila nebulosa* and transferred to *D. melanogaster* (Counce and Poulson, 1961). There are also *Spiroplasma* strains that are harboured by *Drosophila* but do not cause male killing, as is the case for a *Spiroplasma* strain associated with *Drosophila hydei* (SPHY) (Ota *et al*., 1979). In recent studies, SPHY and another non-male killing *Spiroplasma* strain harboured by *Drosophila neotestacea* were found to confer their native hosts with resistance to the parasitoid wasp *Leptopilina heterotoma* (Xie *et al*., 2010) and the nematode *Howardula aoronymphium* (Jaenike *et al*., 2010) respectively.

The insect immune system is comprised of a combination of cellular and humoral responses, in addition to physical barriers, which together afford formidable protection against infection by a variety of invading microorganisms. Landmarks of the *Drosophila* immune response to systemic infection are the phagocytosis by macrophage-like haemocytes called plasmatocytes, activation of the melanization reaction and the synthesis, in the fat body, of several antimicrobial peptides that have distinct specificity (Lemaitre and Hoffmann, 2007). These peptides are secreted into the haemolymph, where they directly kill invading microorganisms. Genetic analyses show that the Toll and Imd pathways regulate antimicrobial peptide genes (Lemaitre *et al*., 1996; Lemaitre and Hoffmann, 2007). The Toll pathway is triggered by the proteolytic cleavage of the Toll ligand, *Spätzle* (*Spz*), and leads to activation of the Rel proteins *DIF* and *Dorsal*. The Toll pathway is mainly activated by Gram-positive bacteria and fungi and largely controls the expression of antimicrobial peptides active against fungi (e.g. *Drosomycin*). In contrast, the Imd pathway mainly responds to Gram-negative bacterial infection and controls antibacterial peptide genes (e.g. *Diptericin*) via the activation of the Rel protein *Relish* (Lemaitre and Hoffmann, 2007). Studies have shown that in *Drosophila*, *Spiroplasma* do not induce the expression of antimicrobial peptide genes (Hurst *et al*., 2003; Anbutsu and Fukatsu, 2010) or other immunity-related genes (Hutchence *et al*., 2011). It has also been reported that activation of the fly immune system can significantly decrease *Spiroplasma* titres (Hurst *et al*., 2003; Anbutsu and Fukatsu, 2010).

In this study, we analysed the interaction between MSRO *Spiroplasma* (hitherto mainly referred to as *Spiroplasma*) and the *Drosophila* immune system. Since MSRO *Spiroplasma* has not been studied in detail, we first sought to establish the kinetics of *Spiroplasma* growth variation over its host's life cycle. We then monitored the effects of attenuating or activating host immunity on *Spiroplasma* titre. To determine the implications of harbouring *Spiroplasma* on the host, we investigated *Spiroplasma's* effects on host immune responses and the outcome of infection by other bacterial pathogens on *Spiroplasma*-harbouring hosts. Finally, we show that a non-coevolved and cultivable *Spiroplasma*, *S. citri*, is able to grow in the haemolymph of *D. melanogaster,* causing fly death.

Results

Spiroplasma *titre and growth rate depends on developmental stage and fly age*

In previous studies, the *Spiroplasma Drosophila* interaction was examined using a strain of *Spiroplasma* isolated from *D. nebulosa* (NSRO) and transferred to *D. melanogaster* by microinjection of *Spiroplasma* laden haemolymph (Counce and Poulson, 1961). NSRO *Spiroplasma* titres were found to increase moderately over larval development, followed by an exponential increase in titres after fly eclosion and finally a decrease in titres after flies are aged beyond 3 weeks (Anbutsu and Fukatsu, 2003). We decided to study a recently isolated strain of *Spiroplasma* which naturally infects *D. melanogaster,* MSRO (Montenegro *et al*., 2005). NSRO and MSRO *Spiroplasma* strains are closely related to each other (Haselkorn *et al*., 2009) but may still have important differences reflecting co-evolution with their native hosts. Our *Spiroplasma*-harbouring wild-type (Oregon-R) stocks were initially established by microinjection of *Spiroplasma* laden haemolymph into Oregon-R flies. Before investigating the relationship between MSRO *Spiroplasma* and the *Drosophila* immune response, we sought to quantify the growth kinetics of MSRO over the *Drosophila* life cycle in Oregon-R flies. We used quantitative PCR to determine the range of *Spiroplasma* infection titres and the growth rate of *Spiroplasma* over *Drosophila* development (Fig. 1). Absolute *Spiroplasma*

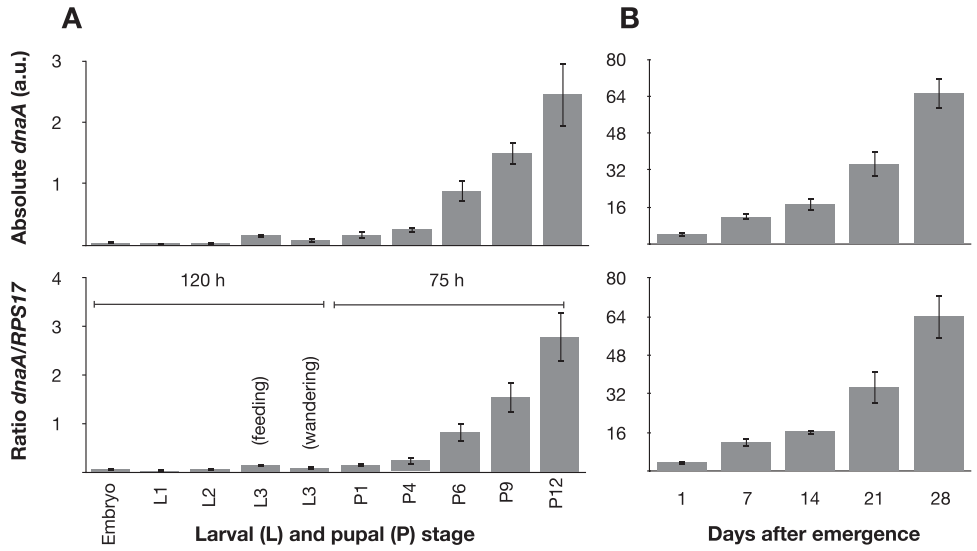

Fig. 1. *Spiroplasma* titres over (A) larval and pupal development and (B) ageing of Oregon-R flies. Pupal stages and duration according to Bainbridge and Bownes (1981). This experiment was repeated four times. Growth trends were highly consistent but data were not pooled between experiments due to variability in *Spiroplasma* titre.

dnaA gene values represent a measure of *Spiroplasma* titres that is independent of the host gene *RPS17* copy number, which could be expected to vary over fly development. We decided to display absolute *dnaA* values for experiments where host cell number might not be constant. Overall, we never observed any significant qualitative differences between estimates of *Spiroplasma* titre based on absolute *dnaA* or *dnaA* relative to *RPS17*. *Spiroplasma* titres and growth rate were low over larval development. *Spiroplasma* growth drastically increases between pupal stage 4 and 6. *Spiroplasma* growth increased from a doubling time of 117 h over larval development to a doubling time of 20 h over metamorphosis (P1–P12). Exponential growth of *Spiroplasma* continued over the life of adult flies, but doubling times increased to around 170 h. *Spiroplasma* were also be observed by microscopy of adult haemolymph samples (Fig. 2) using SYTO-9 (Invitrogen), a nucleic acid stain. SYTO-9 stains all nucleic acid containing entities in the haemolymph samples, but due to their distinctive helical morphology *Spiroplasma* cells are easily identifiable (Fig. 2C). We performed counts of *Spiroplasma* in haemolymph samples to verify the trends in *Spiroplasma* growth over *Drosophila* development.

Phagocytosis, Toll and Imd-mediated immune responses do not play a major role in controlling titre of Spiroplasma

To determine whether the host immune system plays an active role in controlling *Spiroplasma* titres, we measured *Spiroplasma* in flies with impaired immune function. We performed these experiments with 7-day-old flies. We crossed mutant genotypes and genetic constructs into our previously characterized *Spiroplasma*-harbouring Oregon-R strain, which been maintained for 15 genera-

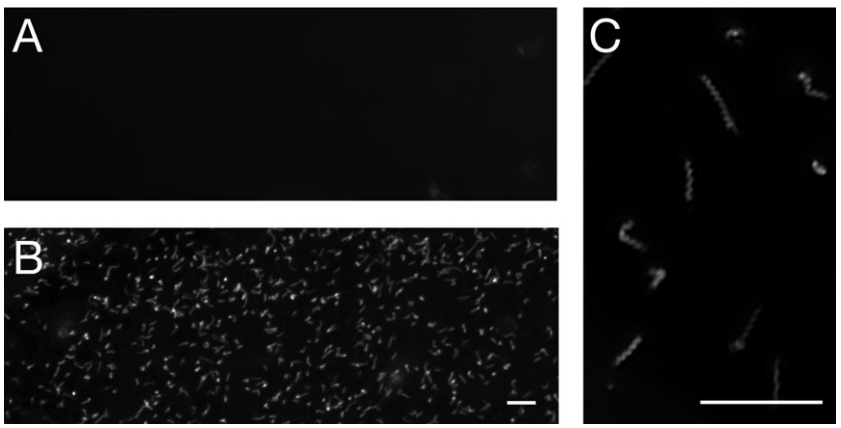

Fig. 2. Visualization of *Spiroplasma* in fly haemolymph. Haemolymph was extracted from 7-day-old flies and subjected to fluorescence microscopy after treatment with the DNA stain SYTO9 (Invitrogen). Haemolymph from Oregon-R flies that do not harbour *Spiroplasma* is shown as a control in (A). (B) and (C) are haemolymph from Oregon-R flies harbouring *Spiroplasma*. Scale bars represent 10 μm.

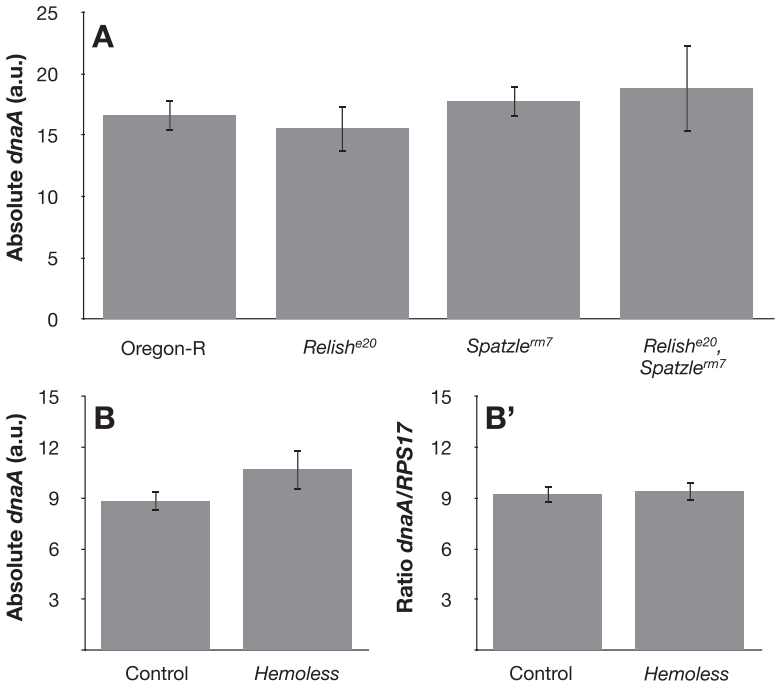

Fig. 3. A. *Spiroplasma* titre (absolute *dnaA*) in flies with attenuated Toll and Imd pathways. B. *Spiroplasma* titre in flies devoid of haemocytes, B depicts absolute *dnaA* values and B′ values of *dnaA* over *RPS17*. *Hemoless* genotype: *UAS hid, UAS Reaper/+; hml(Δ)-Gal4/+, UAS-GFP/+*. Control genotype: *hml(Δ)-Gal4/+, UAS-GFP/+*.

tions to allow *Spiroplasma* titres to stabilize. Since maternal conditions are likely to affect *Spiroplasma* titres in progeny (Fukatsu *et al*., 2007), we ensured that prior to crossing all females were 7 days old and maintained under identical conditions. Even with these precautions, we observed that *Spiroplasma* levels in Oregon-R were not always very stable between repeated experiments. *Spiroplasma* titres were examined in *Drosophila* lines carrying null mutations of *Relish* (*rel*^{E20}) affecting the Imd pathway or *Spätzle* (*spz*^{rm7}) affecting the Toll pathway as well as a combination of both (*rel*^{E20},*spz*^{rm7}) (Fig. 3A). We did not observe any significant difference between *Spiroplasma* titre in Oregon-R, *Relish* (*rel*^{E20}), *Spätzle* (*spz*^{rm7}), *Spätzle* (*rel*^{E20},*spz*^{rm7}). We conclude that neither Toll nor Imd pathways play a major role in controlling global-level *Spiroplasma* titres.

In adult *Drosophila*, the cellular response is largely mediated by plasmatocytes, which are professional phagocytes that patrol the haemolymph and engulf invaders (Meister and Lagueux, 2003). Since *Spiroplasma* are found mainly in the haemolymph we sought to determine the nature of any interaction between *Spiroplasma* and plasmatocytes. We used the GAL-4/UAS system to generate flies lacking haemocytes (*Hemoless*). Specifically, we induced the expression of the pro-apoptotic genes, *hid* and *reaper,* using the haemocyte-specific *haemolectin* [*hml*(Δ)-*Gal4*] driver in a manner similar to that described in Charroux and Royet (2009). Although it is still possible that *Spiroplasma* interacts with haemocytes, we found that near complete elimination of haemocytes does not significantly affect *Spiroplasma* titre (Fig. 3B and C).

Activation of a systemic immune response leads to an increase in Spiroplasma *titre*

Previous studies have shown that the ectopic activation of an immune response resulted in a decrease in NSRO *Spiroplasma* titre (Hurst *et al*., 2003; Anbutsu and Fukatsu, 2010). This observation prompted us to monitor the impact of Toll and Imd immune activation on MSRO *Spiroplasma* titres. The Toll and Imd pathway immune responses were first induced by subjecting flies to septic injury with the Gram-positive bacterium *Micrococcus luteus* (inducer of the Toll pathway) or the Gram-negative bacterium *Erwinia carotovora carotovora strain 15* (*Ecc15*) (inducer of the Imd pathway). We then used qPCR to measure the effect of this activation on *Spiroplasma* titre (Fig. 4). To our surprise, we observed that bacterial infection and clean injury stimulate *Spiroplasma* growth, with the effect being more marked after infection with *Ecc15*. Next, we sought to determine if a similar effect was observed when the Toll or Imd pathway was activated without the presence of microorganisms. We found that flies carrying the *Toll*^{10b} mutation had higher levels of *Spiroplasma* (Fig. 4B), which contrasts with the results obtained by others for NSRO *Spiroplasma* (Hurst *et al*., 2003; Anbutsu and Fukatsu, 2010). We activated the Imd pathway using the ubiquitous heat-shock activated *HS-GAL4* (*Heat Shock-GAL4*)

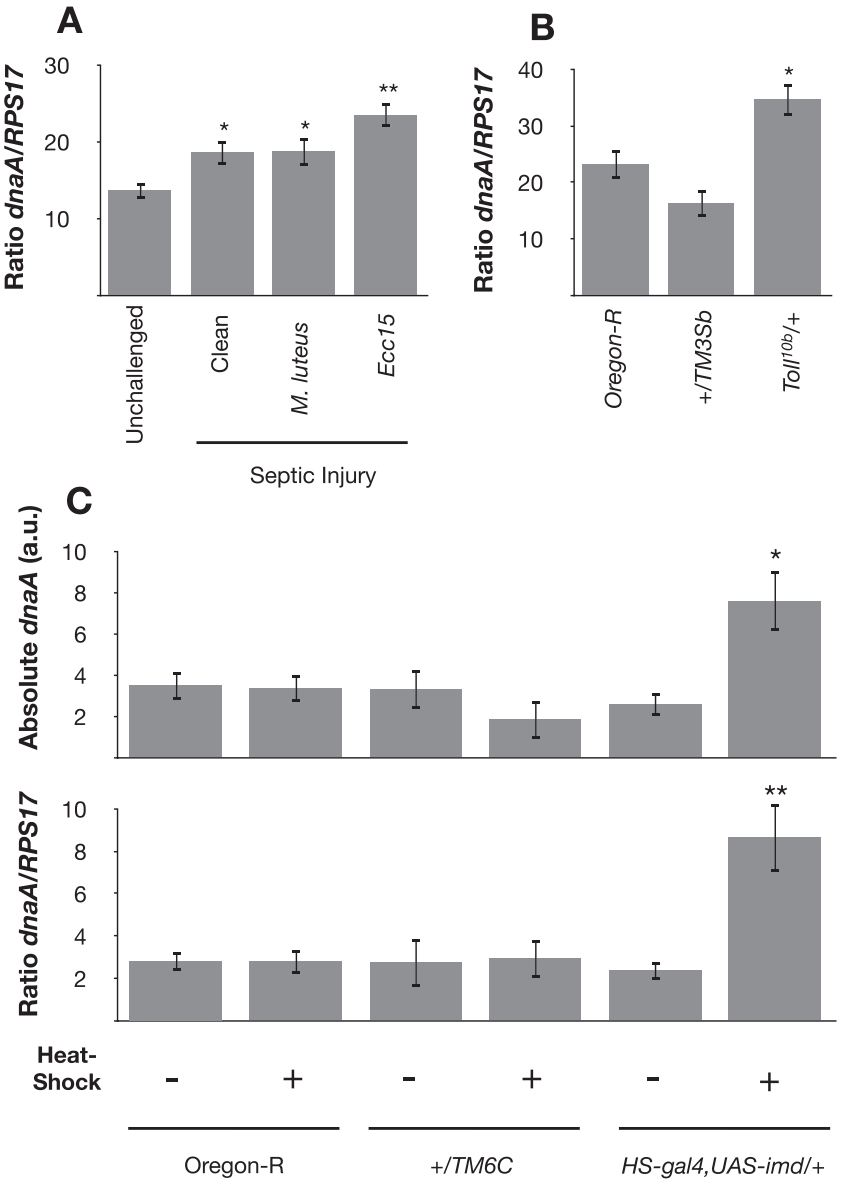

Fig. 4. *Spiroplasma* titre after ectopic activation of immune responses.
A. *Spiroplasma* titres after septic injury of flies. Flies were collected for DNA extraction at 3.5 days post infection, when they were 7 days old.
B. *Spiroplasma* titre in flies with constitutive Toll-pathway activation. *+/TM3Sb* are an additional control that are siblings of *+/Toll*^{10b} but without the mutant *Toll*^{10b} allele.
C. *Spiroplasma* titres after ectopic Imd pathway activation. To rule out the effect of the heat shock on *Spiroplasma* titres, controls were subject to identical heat-shock conditions. *+/TM6C* flies are an additional set of controls that are the siblings of *HS-gal4,UAS-imd/+* flies.

to drive the expression of the *Imd* gene under the control of an upstream activating sequence (UAS) (Georgel *et al*., 2001). Inducing the expression of Imd resulted in a strong increase in *Spiroplasma* titres (Fig. 4C). Ectopic activation of the Imd pathway is known to induce apoptosis (Georgel *et al*., 2001), which could impact the host gene *RPS17* copy number. However, as shown in Fig. 4C, we did not observe a difference between absolute and relative quantification of *dnaA*. The strong effect on *Spiroplasma* titres was no longer observed in a mutant of *Dredd* (Leulier *et al*., 2000), which is a downstream component of the Imd pathway (data not shown). We still observed higher *Spiroplasma* titres upon overexpression of the Imd pathway in mutants of another downstream component of the Imd pathway, *dTAK1* (Vidal *et al*., 2001) (data not shown).

Spiroplasma *does not affect the Toll and Imd pathways but reduces the resistance of flies to bacterial infection*

We next investigated whether the presence of *Spiroplasma* could affect the Toll and Imd pathways. Figure 5 shows that the expression levels under unchallenged conditions of *Drosomycin* and *Diptericin*, which are read-outs of Toll and Imd pathway activation, respectively, were not significantly different between flies harbouring *Spiroplasma* and those that did not. We also found that harbouring *Spiroplasma* does not significantly impact the expression levels of *Diptericin* or *Drosomycin* following

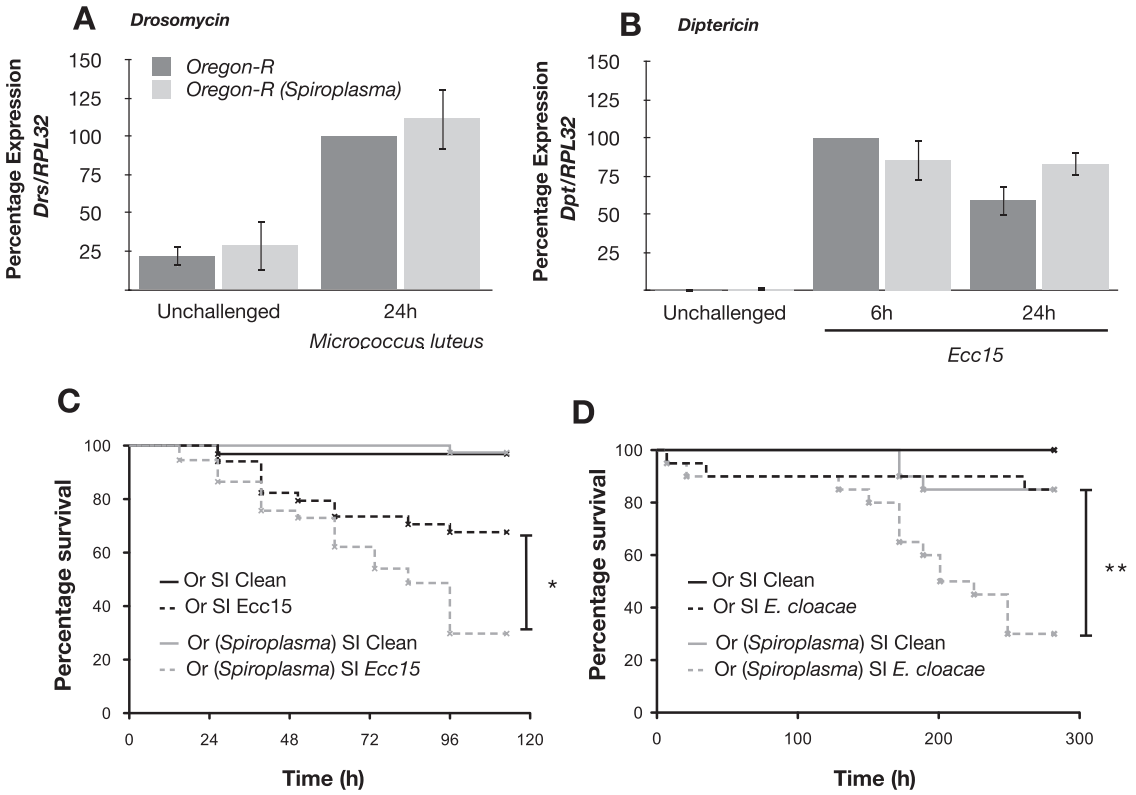

Fig. 5. A and B. Level of *Drosomycin* (A) and *Diptericin* (B) expression in *Spiroplasma*-harbouring flies. Expression levels are shown relative to the expression of an endogenous control, RPL32. Ratios have also been normalized, with *Drosomycin* expression 24 h after *M. luteus* and *Diptericin* expression 6 h after *Ecc15* infection representing 100% expression. Error bars represent standard deviation from mean of two independent experiments.
C and D. Survival of flies after septic injury. The asterisks (*, **) indicate that *Spiroplasma*-harbouring flies died significantly faster after septic injury with either Ecc15 or *E. cloacae* than did control flies with a clean septic injury.

septic injury with the Gram-positive bacterium *M. luteus* (Fig. 5A) or the Gram-negative bacterium *Ecc15* (Fig. 5B).

We next determined whether harbouring *Spiroplasma* could affect host resistance to a variety of well-characterized *Drosophila* microbial pathogens.

Figure 5C and D shows that flies harbouring *Spiroplasma* died significantly faster after septic injury with either *Ecc15* or *Enterobacter cloacae* than control flies without *Spiroplasma*. In contrast, the presence of *Spiroplasma* did not affect the resistance of flies infected with the Gram-positive bacterium *Enterococcus faecalis* or the fungal pathogen *Beauveria bassiana* (data not shown).

We conclude that *Spiroplasma* does not affect the production of antimicrobial peptides, which is consistent with previous studies (Hurst *et al*., 2003; Anbutsu and Fukatsu, 2010). The absence of a host immune response to *Spiroplasma* is not due to suppression of the immune system but due to the fact that *Spiroplasma* cells are not detected by flies. However, *Spiroplasma* do affect the resistance of flies to infection by certain bacterial pathogens.

Injection of S. citri *strain GII-3 kills flies*

Spiroplasma citri is a cultivable species of *Spiroplasma,* this bacterium is vectored by leafhoppers and infects the phloem of a variety of plant species (Bove *et al*., 2003). To gain further insight in the interaction between mollicutes and *Drosophila*, we monitored the survival and antimicrobial peptide expression levels of flies after microinjection of *S. citri* strain GII-3. We show that the injection of *S. citri* GII-3 does not activate a systemic immune response above the level observed with a phosphate-buffered saline (PBS) injection (Fig. 6A and B). Interestingly, we found that *S. citri* GII-3 grows well in the haemolymph of wild-type flies eventually leading to death of infected flies (Fig. 6C). Five days post injection, *S. citri* GII-3 could be observed by fluorescence microscopy at a very high density in the haemolymph (data not shown). Flies lacking a functional Toll pathway or devoid of haemocytes experienced the same survival rates as wild-type flies upon infection with *S. citri* GII-3 while *Relish* (Imd-impaired) flies had slightly lower survival rates. One to two days before dying flies appeared to be severely debilitated, unable to

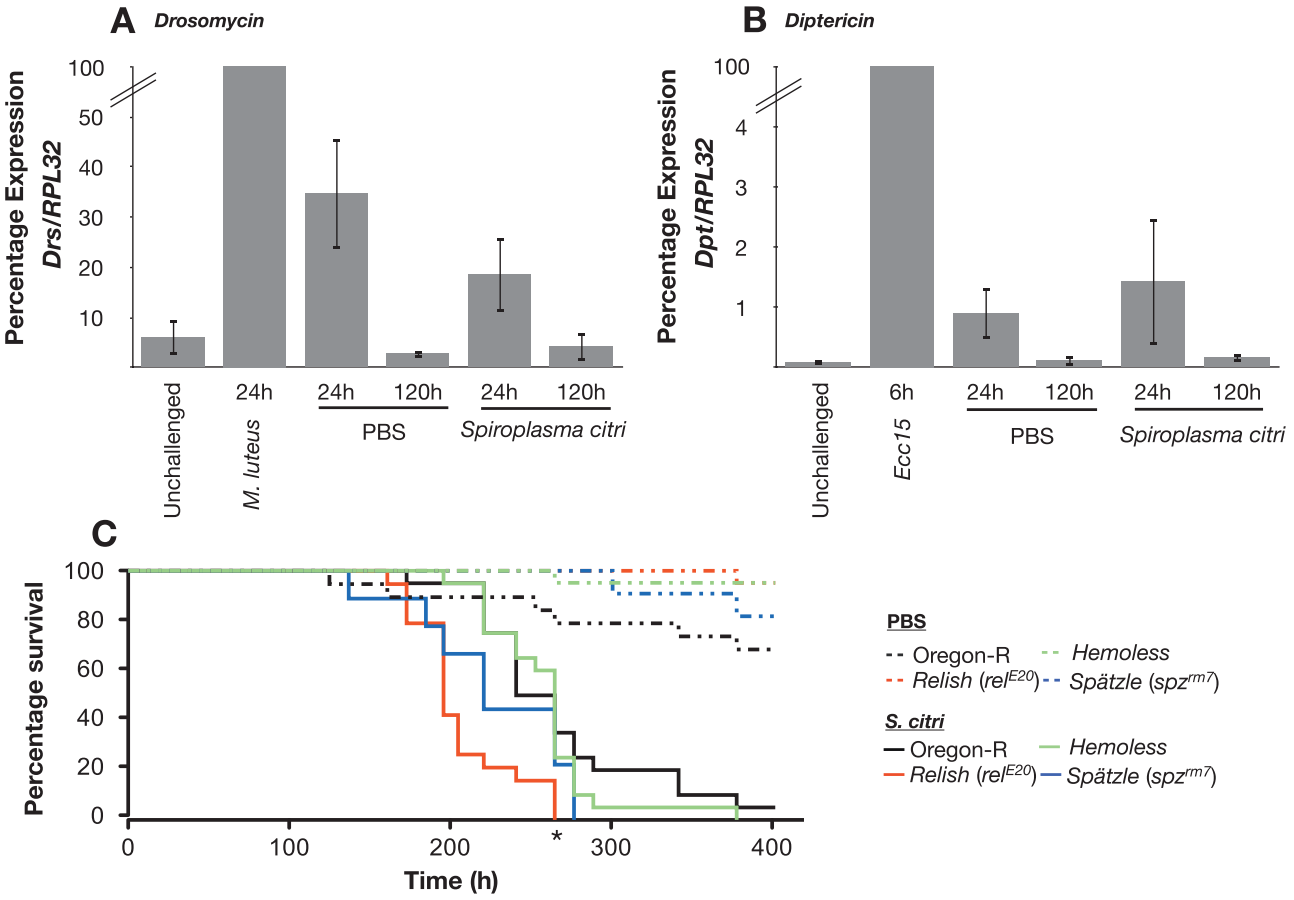

Fig. 6. A and B. Level of (A) *Drosomycin* and (B) *Diptericin* in whole flies after microinjection of *S. citri* GII-3**.** Expression levels are shown relative to the expression of an endogenous control, RPL32. Ratios have also been normalized, with *Drosomycin* expression 24 h after *M. luteus* and *Diptericin* expression 6 h after *Ecc15* infection representing 100% expression. Error bars represent standard deviation from mean of two independent experiments.
C. Survival of flies of after microinjection of *S. citri* GII-3. The asterisk (*) indicates that *Relish* (*rel*^{E20}) were killed significantly faster by *S. citri* GII-3 than Oregon-R. The log-rank test was used to determine statistical significance. Controls have been subjected to microinjection of PBS.

climb the sides of the vial and in some cases exhibiting uncoordinated shacking consistent with damage to their nervous system.

Collectively, these results indicate that uncontrolled and pathogenic growth of *Spiroplasma* can occur in *Drosophila* and that the major arms of the immune system are not an effective means of controlling this type of bacteria.

Discussion

Previous studies used quantitative PCR to assess *Spiroplasma* titre over development (Anbutsu and Fukatsu, 2003; 2006). These studies focused on a *Spiroplasma* NSRO strain that had been introduced into a novel host, *D. melanogaster*. We assessed titres of the native endosymbiont of *D. melanogaster,* MSRO *Spiroplasma*. We found an overall trend of low titres in larvae and much higher titres in adult flies that was generally similar to NSRO (Anbutsu and Fukatsu, 2003; 2006). The growth rate of both NSRO (Anbutsu and Fukatsu, 2003; 2006) and MSRO *Spiroplasma* increases during metamorphosis. Maintaining lower *Spiroplasma* titres over larval development might be advantageous because it could decrease the host incurred cost of harbouring *Spiroplasma*. *Drosophila* fitness depends on rapid larval development, which could be delayed if *Spiroplasma* titres were high in larvae. High *Spiroplasma* titres are relevant for increasing transmission fidelity, but since transmission is only important in adults, maintaining high titres prior to insect sexual maturity is unlikely to be beneficial. By collecting pupal samples at staged intervals we were able to identify the interval between pupal stage 4 and 6 as the period at which *Spiroplasma* growth rate increases dramatically. This increase in *Spiroplasma* growth rate appears to coincide with the initiation of apoptotic cell death in salivary glands, with occurs approximately 15 s after puparium formation (Jiang *et al*., 1997). By calculating the *Spiroplasma* doubling time over segments of

Drosophila development we show that growth rate is markedly higher during metamorphosis (20 h) than during larval development (117 h) or adult ageing (170 h). We also noted that the peak doubling time of 20 h we observed during fly metamorphosis is similar to the 19.2 h that was observed for NSRO *Spiroplasma* growth in cell-free media after adaptation from insect cell culture (Hackett *et al*., 1986). This value might represent an upper limit to the growth rate of *Spiroplasma*. In contrast to the profile of NSRO *Spiroplasma* titre (Anbutsu and Fukatsu, 2003), we did not observe any decline in the growth rate of MSRO *Spiroplasma* in flies after having been aged 3 weeks.

We found that the *Drosophila* cellular response and humoral immune responses in their basal condition do not play a major role in controlling MSRO *Spiroplasma* titre at a global level. While it is still possible that immune responses are active against *Spiroplasma* at a tissue-specific level, we did not find any evidence in support of this.

Activation of the Toll pathway results in an increase in MSRO *Spiroplasma* titre. This effect differs from the reported decrease in *Spiroplasma* NSRO strain titre (Hurst *et al*., 2003). We did observe that relative to Oregon-R controls NSRO and SPHY strain titres were lower in flies with a constitutively active Toll pathway (see Fig. S1). However, *TM3Sb* controls, which were siblings of the *Toll^{10b}* flies that received the balancer chromosome *TM3Sb* instead of the *Toll^{10b}* mutation, also had lower titres of *Spiroplasma*, suggesting that the genetic background of the mutant strain most likely caused this effect. The same background effect most likely resulted in an underestimation of the Toll-mediated increase in MSRO *Spiroplasma* titres, when compared with Oregon-R controls. It is intriguing that the Toll-mediated increase in *Spiroplasma* only affects the MSRO strain. Since the NSRO and SPHY *Spiroplasma* are native to other *Drosophila* species, this difference could relate to co-evolution between *Spiroplasma* and its host.

Our findings regarding the activation of a systemic immune response by MSRO *Spiroplasma* are similar to previous reports of NSRO *Spiroplasma* (Hurst *et al*., 2003; Anbutsu and Fukatsu, 2010), with neither strain causing activation. This might be because cell wall-less *Spiroplasma* lack the molecular motifs such as peptidoglycan and β-1,3 glucan that are the major elicitors of the Toll and Imd pathways (Leulier *et al*., 2003; Gottar *et al*., 2006). In support of this, we also show that infection of flies with *S. citri* strain GII-3 does not activate a systemic immune response. Studies of another endosymbiont, *Wolbachia*, have generally shown that it also does not induce global-level immune system activation (Bourtzis *et al*., 2000). There are however some notable exceptions: *Wolbachia* has been shown to induce the upregulation of reactive oxygen species (ROS) genes in an *Aedes albopictus* cell line (Brennan *et al*., 2008). An over-proliferating strain of *Wolbachia,* wMelPop (Min and Benzer, 1997), induces expression of a variety of immunity-related genes after transfection into *Aedes aegypti* (Kambris *et al*., 2009; Moreira *et al*., 2009) and *Anopheles gambiae* (Kambris *et al*., 2010).

Ectopic activation of the Imd pathway results in an increase in MSRO, SPHY and NSRO *Spiroplasma* titre (Fig. S2). That none of the *Spiroplasma* strains experienced a decrease in titre after Imd pathway activation indicates that *Spiroplasma* are not susceptible to Imd pathway mediated immune response. Since *Spiroplasma* are relatives of Gram-positive bacteria, they could be expected to be more susceptible to a Toll-mediated response than an Imd-mediated response (Tzou *et al*., 2002). The very significant rise in *Spiroplasma* titres after Imd pathway activation might be the result of *Spiroplasma* using antimicrobial peptides in the haemolymph as a nutrition source. On the other hand, over-activation of the Imd pathway has been found to induce apoptosis (Georgel *et al*., 2001). Apoptotic signals themselves or factors released by cells undergoing apoptosis could be contributing to the increase in *Spiroplasma* observed after *HS-GAL4,UAS-Imd*-induced Imd pathway activation. In agreement with this hypothesis, we found Imd pathway activation in *Dredd* mutants did not dramatically increase *Spiroplasma* titre. The caspase *Dredd* is a downstream component of the Imd pathway that, in contrast to *dTAK1,* is required for Imd-induced lethality (Georgel *et al*., 2001; Leulier *et al*., 2002). The Imd pathway-related increase in *Spiroplasma* titre affects all tested *Spiroplasma* strains, contrasting with the strain-specific nature of Toll pathway-related effects on *Spiroplasma* (Figs S1 and S2). Using bacteria to induce Toll and Imd systemic immune responses had an effect on MSRO *Spiroplasma* titres that was similar, albeit less pronounced, to Toll and Imd activation by either the *Toll^{10b}* mutation or *Heat Shock-GAL4,UAS-Imd* respectively.

We report that *S. citri* GII-3 is highly pathogenic and able to proliferate inside *Drosophila*, suggesting that the *Drosophila* immune system is not able to control *S. citri* GII-3 growth. Taken together with our results for endosymbiotic *Spiroplasma*, this suggests that the *Drosophila* haemolymph is a very favourable environment for the growth of *Spiroplasma*. In contrast to *S. citri* GII-3, endosymbiotic *Spiroplasma* do not over-proliferate and cause pathogenesis. It therefore seems likely that endosymbiotic *Spiroplasma* have a growth regulating mechanism to limit damage to their host. The basis of such a mechanism is not known, but could be related to the changes in *Spiroplasma* growth rate that we observed over *Drosophila* development.

We showed that flies had increased susceptibility to the Gram-negative bacterial pathogens *Ecc15* and *E. cloacae* when they harboured MSRO *Spiroplasma.* Since we also showed that activation of systemic immune responses increase *Spiroplasma* titre, it may be that higher loads of *Spiroplasma* are affecting the ability of flies to recover from bacterial infections. Natural selection should favour endosymbionts that increase resistance to pathogens. This leads us to speculate that the decreased resistance is an unintentional consequence something else *Spiroplasma* does. The effect of *Spiroplasma* on host resistance might even be an immunity-related compromise; for example, a mechanism that increases resistance to one parasite or pathogen [e.g. parasitoid wasps (Xie *et al.*, 2010)] could be simultaneously decreasing resistance to others (e.g. bacteria).

Experimental procedures

Fly stocks and handling

We used an Oregon-R stock had been cured of *Wolbachia* by antibiotic treatment and then maintained in the lab for over 30 generations. For our experiments we introduced three different strains of *Spiroplasma* into Oregon-R by microinjection of 9 nl of undiluted haemolymph, using a Nanoject microinjector (Drummond Scientific). This enabled us to establish genetically identical lines of Oregon-R that harboured MSRO, NSRO and a non-male-killing strain of *Spiroplasma* from *D. hydei* (SPHY). All stocks were maintained at 25°C in yeasted tubes containing corn-meal fly medium (Romeo and Lemaitre, 2008). *Spiroplasma*-harbouring Oregon-R females were collected and aged as virgins for 7 days before crosses. For attenuation of immune responses, we used stocks carrying null mutations of *Relish* (rel^{E20}) (Hedengren *et al.*, 1999) or *Spätzle* (spz^{rm7}) (Lemaitre *et al.*, 1996) or a stock carrying both (rel^{E20},spz^{rm7}). The stocks *hml*(Δ)*-Gal4),UAS-GFP* and *yw,UAS-Hid,UAS-Reaper* were used to generate flies lacking haemocytes. To activate the Toll and Imd pathways, we crossed Oregon-R with $Toll^{10b}$*/TM3Sb* or *HS-GAL4,UAS-Imd/TM6C* males, respectively, and collected offspring lacking the *Sb*. For Imd activation flies with *HS-GAL4,UAS-Imd* were heat shocked three times at 37°C for 30 min each time, over a 7-day period (at days 3, 4 and 6). The null mutant stocks *y,w,dTAK1*^{1} and *y,w,Dredd*^{D44} are described in Vidal *et al.* (2001) and Leulier *et al.* (2000) respectively. Flies were aged for 7 days at 25°C prior to DNA extraction.

Imaging

To observe *Spiroplasma* in fly haemolymph, flies were dissected on microscope slides in 5 μl of PBS containing 0.02 mM SYTO9 (Invitrogen). Slides were then mounted and observed on a Zeiss Axioimager Z1. Images were captured with an Axiocam MRn camera and Axiovision software.

Collection of samples to measure Spiroplasma *titre over development*

We placed roughly 100 mated 7-day-old Oregon-R (MSRO) females in standard fly bottles containing standard *Drosophila* medium and let them lay eggs for 12 h. The progeny from these crosses were used for our analysis of *Spiroplasma* titre over development. Each sample consisted of five individuals collected and pooled for DNA extraction, except for embryo, L1 and L2 larvae where 25, 20 and 15 individuals, respectively, were collected and pooled. After emergence adults were placed in yeasted *Drosophila* vials with corn-meal fly medium and flipped every 3 days. Pupal stages were determined based on Bainbridge and Bownes (1981). Flies developed at 25°C.

DNA extraction

Once collected, *Drosophila* samples were stored at –80°C prior to DNA extraction. DNA was extracted from the flies using the Puregene DNA purification kit (Qiagen, Valencia, CA). DNA was extracted from five flies using three times the suggested reagents for a single fly. DNA was hydrated in 240 μl of DNA hydration solution and then further diluted in DNA hydration solution at a ratio of 1:4. Isolated DNA was stored at 4°C until screening.

Quantitative PCR

Spiroplasma titre was determined using a Roche Lightcycler (LC) 2.0 detection system. Each 10 μl of qPCR reaction included 5 μl of FastStart DNA Master Sybr Green, 1.2 μl of 25 mM $MgCl_2$, 2.05 μl of H_2O, 1 μl of DNA template and 0.5 μl each of the forward and reverse primers. *Spiroplasma dnaA* gene primers used were DnaA109F 5′-TTAAGAGCAGTTTCAAAATCGGG-3′ and DnaA246R 5′-TGAAAAAAACAAACAAATTGTTATTACTTC-3′ from Anbutsu and Fukatsu (2003). Primers for the host *RPS17* gene were Dmel.rps17F 5′-CACTCCCAGGTGCGTGGTAT-3′ and Dmel.rps17R 5′-GGAGACGGCCGGGACGTAGT-3′ from Osborne *et al.* (2009). Reactions were done using a Roche Lightcycler with the following conditions: one cycle of 95°C for 10 min, followed by 40 cycles of 95°C 5 s, 56°C 5 s, 72°C 5 s followed by one cycle of 72°C for 10 min. Dilution series were used to calculate the efficiency of PCR amplification for both *RPS17* and *dnaA* DNA extracted from flies without *Spiroplasma* was used as a negative control. Absolute *dnaA* values (shown in arbitrary units) represent the ratio of *dnaA* for an individual sample and the average of the *RPS17* values for all samples in the experiment.

RT-qPCR

Diptericin, *Drosomycin* and *rpL32* mRNA quantification was carried out as previously described in Romeo and Lemaitre (2008). All data are the ratio of the expression level of the mRNA of interest to that of the invariant *rpL32.* Each sample consisted of RNA extracted from 15 flies.

Bacterial stocks

Bacterial stocks were kept frozen in 15% DMSO and subsequently cultured on LB-Agar plates and in LB medium or in the case of *S. citri* SP4 medium (Tully *et al.*, 1977). *Ecc15* has been previously described (Basset *et al.*, 2000). Cultures of *Ecc15* and *E. cloacae* were grown overnight at 29°C and used as pellets of $OD_{600} = 200$, pellets were not washed prior to use. *E. faecalis* was grown overnight at 37°C and used as a pellet of $OD_{600} = 15$.

S. citri strain GII3 (Vignault *et al.*, 1980) was grown at 32°C and used as a low-concentration pellet.

Infection and survival experiments

Septic injuries were performed by pricking adults in the thorax with a thin needle dipped into a bacterial pellet (Romeo and Lemaitre, 2008). In the case of *B. bassiana* natural infection was performed by placing anaesthetized flies on a Petri dish containing sporulating fungus and shaking until flies were covered with spores (Romeo and Lemaitre, 2008). *S. citri* infection was performed by microinjection of 9 nl bacterial pellet resuspended in PBS, using a nanoject microinjector (Drummond Scientific). Infected flies were then maintained at 29°C in yeast-free tubes. For each pathogen tested three independent repeats of 20 flies per genotype were subjected to septic injury. Survival was scored every at least every 24 h and flies were transferred to new tubes every 3 days. Samples for *Spiroplasma* titre analysis were taken 3.5 days after septic injury. For antimicrobial peptide expression analysis, flies were collected 6 and 24 h post septic injury. All flies were 4 days old at the beginning of experiments.

Statistics

Each experiment was repeated independently a minimum of three times (unless otherwise indicated). A minimum of four replicate crosses were examined for each genotype in each repeat of an experiment, error bars represent the standard error of the mean of replicate crosses of one representative experiment (unless otherwise indicated). Statistical significance was calculated using a standard two-tailed *t*-test and considered significant if *P*-values were lower than 0.05. Asterisks indicate the level of significance, $*P < 0.05$, $**P < 0.01$.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. NSRO (A) and SPHY (B) *Spiroplasma* titres do not increase in flies with constitutive Toll-pathway activation. *+/TM3Sb* are an additional control that are siblings of $Toll^{10b}$/+ but without the mutant $Toll^{10b}$ allele.
Fig. S2. NSRO (A) and SPHY (B) *Spiroplasma* titres increase after ectopic Imd pathway activation. Oregon-R and *+/TM6C* controls subject to identical heat shock, which did not affect their *Spiroplasma* titre and therefore have been omitted from this figure for clarity.