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RESEARCH ARTICLE

Wolbachia increases the susceptibility of a parasitoid wasp to hyperparasitism

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ABSTRACT

The success of maternally transmitted endosymbiotic bacteria, such as *Wolbachia*, is directly linked to their host reproduction but in direct conflict with other parasites that kill the host before it reaches reproductive maturity. Therefore, symbionts that have evolved strategies to increase their host's ability to evade lethal parasites may have high penetrance, while detrimental symbionts would be selected against, leading to lower penetrance or extinction from the host population. In a natural population of the parasitoid wasp *Hyposoter horticola* in the Åland Islands (Finland), the *Wolbachia* strain *w*Hho persists at an intermediate prevalence (~50%). Additionally, there is a negative correlation between the prevalence of *Wolbachia* and a hyperparasitoid wasp, *Mesochorus* cf. *stigmaticus*, in the landscape. Using a manipulative field experiment, we addressed the persistence of *Wolbachia* at this intermediate level, and tested whether the observed negative correlation could be due to *Wolbachia* inducing either susceptibility or resistance to parasitism. We show that infection with *Wolbachia* does not influence the ability of the wasp to parasitize its butterfly host, *Melitaea cinxia*, but that hyperparasitism of the wasp increases in the presence of *w*Hho. Consequently, the symbiont is detrimental, and in order to persist in the host population, must also have a positive effect on fitness that outweighs the costly burden of susceptibility to widespread parasitism.

KEY WORDS: Symbiont, Ecological immunity, Host–parasite interaction, Hymenoptera

INTRODUCTION

Heritable endosymbiotic bacteria are extremely widespread among insects, and their presence may have an important impact on their host ecology and evolution. The symbiotic bacterium *Wolbachia* benefits from strategies that increase the number of infected individuals in the host population, especially the number of females that pass the bacterium on to their offspring. There are many mechanisms by which *Wolbachia* enhances its transmission through generations, including parasitic phenotypes that manipulate the host reproductive system by inducing cytoplasmic incompatibility, male-killing, feminization or parthenogenesis (O'Neill et al., 1997). The study of the population dynamics of the *w*Ri strain infecting Californian populations of the fruit fly *Drosophila simulans* (Turelli and Hoffmann, 1991, 1995) provides a classic example of the successful and rapid spread of such manipulative *Wolbachia*.

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Other *Wolbachia* strains are mutualistic, boosting their host fitness by, for example, improving the host's ability to overcome stress due to environmental pressures or poor diet (Zug and Hammerstein, 2015). More recently, *Wolbachia* has attracted wide interest for its ability to increase host resistance to parasite and pathogen infection. Studies have found *Wolbachia*-infected *Drosophila* to be more resistant to viral (Hedges et al., 2008; Teixeira et al., 2008) and bacterial infections (Ye et al., 2013), or parasitoid attacks (Hsiao, 1996) than their *Wolbachia*-free counterparts. The ability to improve host resistance is, however, not pervasive across all host–*Wolbachia*–parasite interactions. Thus, the presence of *Wolbachia* in *D. simulans* did not always improve the flies' resistance to the fungal pathogen *Beauveria bassiana* (Fytrou et al., 2006), diverse viruses (Martinez et al., 2014; Osborne et al., 2009) or bacteria (Wong et al., 2011). Furthermore, although Martinez et al. (2012) found no effect of *w*Ri in flies parasitized by the parasitoid wasp *Leptopilina boulardi*, Fytrou et al. (2006) showed that the same endosymbiotic bacterial strain increased the susceptibility of the flies to the closely related parasitoid wasp *L. heterotoma*. Thus, the role of *Wolbachia* in the susceptibility to other parasites appears extremely variable between host–*Wolbachia*–parasite systems, and may depend on the *Wolbachia* strain and the host genotype or species (Bordenstein et al., 2003; Hornett et al., 2008).

Current research mainly focuses on fly and mosquito (Diptera) host species, because of the utility of *Drosophila* as a model system and the potential for using *Wolbachia* in the control of vector-borne diseases of concern to humans. These studies are mostly laboratory based, with just a few using natural host populations (Skelton et al., 2016; Zele et al., 2014), and only some with parasitoids rather than pathogen infection (Fytrou et al., 2006; Hsiao, 1996; Martinez et al., 2012; Xie et al., 2014). In order to understand the complex role of endosymbionts, such studies should also be conducted under natural conditions, and in a broad range of host taxa. There have been just a few isolated studies of *Wolbachia*–pathogen interactions outside of Diptera (Isopods: Braquart-Varnier et al., 2015; Lepidoptera: Graham et al., 2012; Coleoptera: Hsiao, 1996). Our study is the first exploration of the effect of *Wolbachia* on the relationship between a Hymenoptera host and parasite in a natural population.

We present an analysis of the association of *Wolbachia* with its host, the parasitoid wasp *Hyposoter horticola* (Gravenhorst) (Hymenoptera: Ichneumonidae: Campoplaginae). This wasp is a specialist parasitoid of the Glanville fritillary butterfly, *Melitaea cinxia* (L.) (Lepidoptera: Nymphalidae) (Shaw et al., 2009). The butterfly is widespread across Eurasia. The study area, Åland, is a Finnish archipelago in the Baltic Sea, where the butterfly lives as a classical metapopulation in a 50 by 70 km fragmented landscape (Hanski, 2011). The wasp occupies the entire host metapopulation (Couchoux et al., 2016). About half of the wasp population is infected by a unique *Wolbachia* strain, *w*Hho (Duplouy et al., 2015). It is not clear yet how the bacterium is maintained throughout the

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wasp population. Duplouy et al. (2015) have shown that the transmission rate of the bacterium is high but not perfect. The bacterium has no apparent effect on egg-load, longevity and metabolism of the host wasp, and the sex ratio of the wasp population is not female-skewed. We investigated the potential effect of *w*Hho on the interaction between the wasp *H. horticola* and its specialist hyperparasitoid *Mesochorus* cf. *stigmaticus* (Brischke) (Hymenoptera: Ichneumonidae: Mesochorinae). There is a negative correlation between the prevalence of *w*Hho-infected wasps and the prevalence of hyperparasitism in the landscape in Åland (Duplouy et al., 2015). This pattern of association, if causal, could arise in two ways (Fig. 1). (i) *Wolbachia* may increase host resistance to hyperparasitism, leading to a low density of the specialist hyperparasitoid where *Wolbachia* is common. As the hyperparasitoid is present at some density throughout the landscape, this would suggest that while beneficial with respect to hyperparasitism, the *Wolbachia* infection should have other costs. (ii) *Wolbachia* may decrease host resistance because individuals that are hyperparasitized do not survive to transmit the symbiont. Therefore, the frequency of *Wolbachia*-infected individuals would be low where the hyperparasitoid is common. Under this scenario, *Wolbachia* infection should be beneficial in some way that counterbalances the cost of increased susceptibility to parasitism. We conducted a manipulative field experiment to distinguish between these alternative hypotheses under natural conditions.

MATERIALS AND METHODS

Hyposoter horticola is a specialist solitary egg–larval parasitoid of the Glanville fritillary butterfly, *M. cinxia* (Lei et al., 1997; Shaw et al., 2009). The univoltine host butterfly lays eggs in clutches on host food plants in June. The caterpillars live in gregarious family groups, overwintering in silken nests (Kuussaari et al., 2004). The wasp *H. horticola* parasitizes about a third of the host caterpillars in each host nest in the Åland Islands (Montovan et al., 2015). The hyperparasitoid *M.* cf. *stigmaticus* is a specialist solitary parasitoid of endoparasitoids in *M. cinxia* caterpillars (Shaw et al., 2009). It is present throughout the Åland Islands, parasitizing 20–60% of *H. horticola* in many places (Nair et al., 2016). Extremely rarely it also parasitizes *Cotesia melitaearum*, which is the other specialist endoparasitoid of *M. cinxia* caterpillars (van Nouhuys and Hanski, 2005). In neighboring Estonia, the butterfly and *H. horticola* are present but both *Wolbachia* and the hyperparasitoid are absent (Duplouy et al., 2015; Montovan et al., 2015).

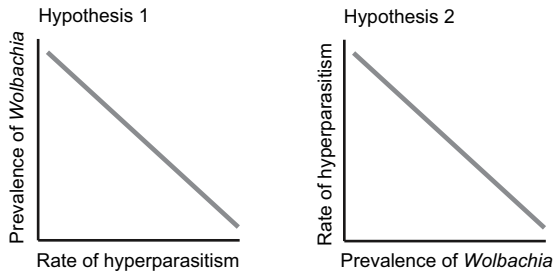

Fig. 1. Negative association of *Wolbachia* presence and rate of hyperparasitism of *Hyposoter horticola* by *Mesochorus* cf. *stigmaticus*. Schematic representation of the two alternative hypotheses tested experimentally in this study for the findings of Duplouy et al. (2015) in Åland, Finland. Hypothesis 1: *Wolbachia* increases resistance to hyperparasitism. Where *Wolbachia* is common, successful hyperparasitism is low, so the hyperparasitoid is rare. Hypothesis 2: hyperparasitism decreases transmission of *Wolbachia*. Where the hyperparasitoid is common, transmission of *Wolbachia* is low, so *Wolbachia* is rare.

For the experiment, we used *H. horticola* reared from host caterpillars that were naturally parasitized in the Åland Islands, Finland, and Saarema, Estonia, in summer 2014. These were collected as parasitized caterpillars, and reared in the laboratory until the parasitoid wasps pupated. Upon reaching adulthood, the wasps were maintained under uniform conditions in individual 100 ml vials in an incubator (12 h:12 h light:dark and 18/10°C day/night temperature) and fed honey water (1:3) daily. Once mature (10 days old), the virgin female wasps were offered ∼10 day old *M. cinxia* clutches from laboratory-reared butterflies originating in the Åland Islands (see Couchoux et al., 2015, for detailed methods of wasp and butterfly rearing and oviposition). Parasitism of each egg cluster was observed. The wasps were unmated because we are unable to make them mate in the laboratory. Because of haplodiploidy, unmated diploid *H. horticola* is a haplodiploid Hymenoptera, so unmated mothers produce haploid male offspring through arrhenotokous parthenogenesis (Normark, 2003). *Wolbachia*-infected females transmit the infection to both sons and daughters (Duplouy et al., 2015). The infection status of individual *H. horticola* females was unknown until after the experiment, but it was assumed that about half the individuals from Åland were infected (Duplouy et al., 2015). After hatching, the parasitized clutches (N=29) were reared until the caterpillars reached the second instar. Several individuals from each clutch were dissected to make sure that the clutch had been successfully parasitized. Groups of 40 caterpillars (large clutches were split) were placed on potted *Veronica spicata* or *Plantago lanceolata*, which are the host plants for *M. cinxia* (Kuussaari et al., 2004), to make 50 nests. After the caterpillars had built a gregarious silken nest on the plants they were placed in *M. cinxia* habitat patches in Åland, where they were exposed to natural hyperparasitism by *M.* cf. *stigmaticus*. The nests (N=45, some natural mortality occurred in the field) were brought back to the lab when they had reached the diapause stage. Caterpillars were then dissected under a microscope to determine which had been parasitized by *H. horticola*. Each parasitoid larva was then dissected to identify individuals that were hyperparasitized by *M.* cf. *stigmaticus*.

The butterfly and the parasitoid wasps are not classified as threatened species in the sampled regions and hence no permits are required for their collection.

Molecular assays

We extracted DNA from the abdomen of each *H. horticola* adult female wasp using a Qiagen DNeasy blood and tissues extraction kit, following the manufacturer's protocol (Qiagen®, USA). The DNA quality was tested by PCR amplification of the mitochondrial *COI* gene (primer pair LCO/HCO; Folmer et al., 1994). The COI amplicons were sequenced to determine the mitotype of each wasp (C or T; Duplouy et al., 2015). Duplouy et al. (2015) showed that despite *w*Hho transmission rates being imperfect in both matrilines, the T-mitotype is more often found associated to *w*Hho-infected wasps, while the C-mitotype is more common in non-infected wasps. The *Wolbachia* infection status of each sample was assessed through the amplification of the *Wolbachia wsp* gene (primer pair 81F/691R; Zhou et al., 1998). Each PCR included both positive and negative controls. Altogether, 25 *H. horticola* wasps were used and screened for this study (six *Wolbachia*-infected and 10 non-infected wasps from Aland Islands, and nine non-infected wasps from Estonia). All infected wasps were of T-mitotype, while non-infected wasps from both Åland and Estonia carried the C-mitotype (Duplouy et al., 2015).

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Statistical models

Statistical analyses were performed using R (http://www.R-project.org/). To test the effects of country of origin and *Wolbachia*-infection status of the parasitoid on the proportion of *M. cinxia* caterpillars parasitized per clutch, we used a cumulative linked model (clm, from 'ordinal' and 'nlme' libraries in R). The proportion of caterpillars parasitized by *H. horticola* was considered categorical with 10 categories ($x \leq 10\%$, $10\% < x \leq 20\%$, $20\% < x \leq 30\%$, $30\% < x \leq 40\%$, $40\% < x \leq 50\%$, $50\% < x \leq 60\%$, $60\% < x \leq 70\%$, $70\% < x \leq 80\%$, $80\% < x \leq 90\%$, $90\% < x \leq 100\%$) to fit the model. We also used a cumulative linked mixed model to test the effects of country of origin and *Wolbachia*-infection status on the proportion of *H. horticola* hyperparasitized by *M.* cf. *stigmaticus*. *Mesochorus* cf. *stigmaticus* tends to hyperparasitize a higher proportion of *H. horticola* larvae when a higher proportion of them are present in a butterfly clutch (Montovan et al., 2015). Because per-nest rate of parasitism varied, we took this into account in the statistical model by first making a linear model of the proportion of *H. horticola* larvae hyperparasitized by *M.* cf. *stigmaticus*, and the proportion *M. cinxia* parasitized by *H. horticola* in the nest. The linear model residuals were then included as categorical data ($x < -50\%$, $< -40\%$, $< -20\%$, $< 0\%$, $< 10\%$, $< 20\%$, $< 30\%$, $< 40\%$, $< 50\%$ and $< 60\%$) in the cumulative linked mixed model. As several nests placed in the field were parasitized by the same *H. horticola* wasp, we also included the ID of the *H. horticola* wasp as a random factor in the model.

RESULTS

Virulence of the parasitoid *H. horticola* in *M. cinxia* caterpillars

Hyposoter horticola from Estonia (without *Wolbachia*) and Åland (individuals with and without *Wolbachia)* parasitized the host egg clusters from Åland at a similar rate (23.9% versus 43.2% of hosts per cluster, d.f.=1, P=0.178; Fig. 2). The *Wolbachia* infection status of the parasitoid *H. horticola* did not affect its parasitism success in *M. cinxia*, as *Wolbachia*-infected and non-infected wasps parasitized the same fraction of caterpillars within *M. cinxia* clutches (32.4% versus 34.3%, d.f.=1, P=0.904; Fig. 2).

Detection of *M. cinxia* caterpillar nests by the hyperparasitoid *M.* cf. *stigmaticus*

Female *M.* cf. *stigmaticus* do not discriminate between *M. cinxia* caterpillar nests parasitized by *Wolbachia*-infected or -free *H. horticola* larvae (P=0.29, Fisher exact test). Of the 45 caterpillar nests placed in the field, 30 were in meadows visited by *M.* cf. *stigmaticus* (at least one larva per meadow was found parasitized by *M.* cf. *stigmaticus*). We found hyperparasitoid larvae in seven of the nests parasitized by *Wolbachia*-infected *H. horticola*, and in 15 of the nests parasitized by *Wolbachia*-free wasps. In contrast, only one nest parasitized by *Wolbachia*-infected *H. horticola* and seven nests parasitized by *Wolbachia*-free wasps remained undetected by *M.* cf. *stigmaticus*. The remaining nests were lost as a result of natural disturbances (e.g. heavy rains or animals).

Hyperparasitism of *H. horticola* by *M.* cf. *stigmaticus*

Wolbachia-free parasitoid larvae from Estonia and from Åland were hyperparasitized by *M.* cf. *stigmaticus* at a similar rate (36.1% versus 41.5%, d.f.=1, P=0.645; Fig. 3). In contrast, a larger proportion of the *H. horticola* larvae from *Wolbachia*-infected wasps were parasitized by the hyperparasitoid *M.* cf. *stigmaticus* (73.9% versus 39.5%, d.f.=1, P=0.0472; Fig. 3). During dissections, we found no evidence of superparasitism, as no *H. horticola* larva had more than one *M.* cf. *stigmaticus* in it. Additionally, all *M.* cf. *stigmaticus* larvae found in *H. horticola* larvae were alive and moving, with no sign of encapsulation at this stage of larval development.

DISCUSSION

A recent study found that the *Wolbachia* strain *w*Hho persists at the intermediate prevalence of 50% in the population of the wasp

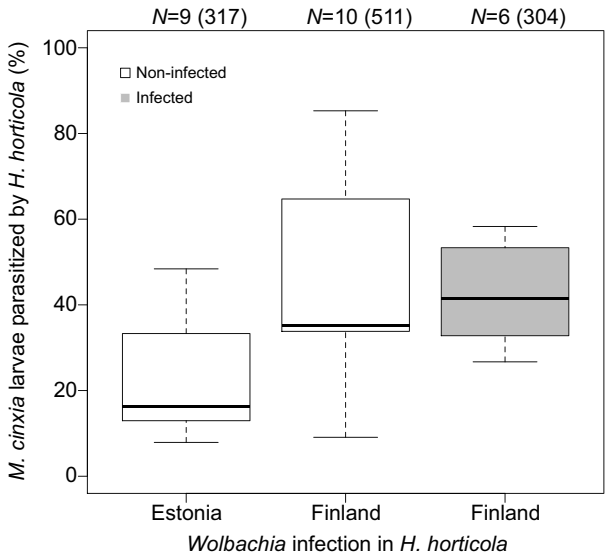

Fig. 2. Proportion of *Melitaea cinxia* caterpillars parasitized by *Wolbachia*-infected and non-infected *H. horticola* larvae in two Baltic countries (Finland and Estonia). The number of clutches of parasitized caterpillars is shown at the top, with the total number of caterpillars dissected in parentheses. There was no significant difference between caterpillar groups (P>0.05).

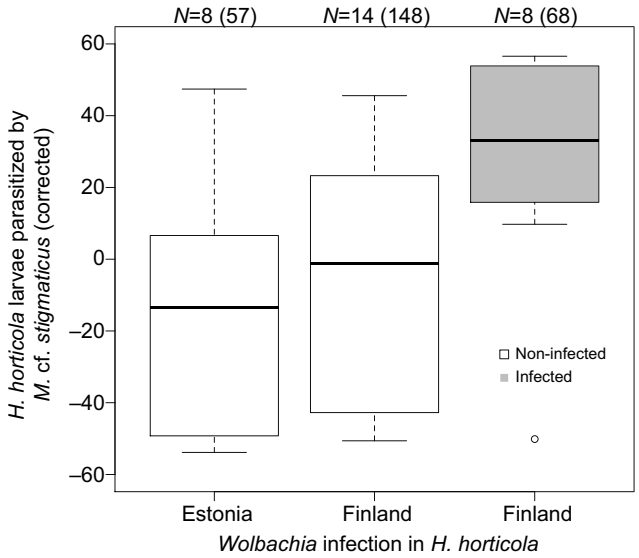

Fig. 3. Proportion of *Wolbachia*-infected and non-infected *H. horticola* larvae parasitized by *M.* cf. *stigmaticus* larvae in the two Baltic countries (Finland and Estonia). Data were corrected for the proportion of *H. horticola* larvae parasitizing the caterpillar groups. The number of groups of parasitized *H. horticola* larvae is shown at the top, with the total number of parasitoid larvae dissected in parentheses. *Wolbachia*-infected larvae were more often parasitized by the hyperparasitoid (P=0.0472).

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H. horticola in the Åland Islands, without impacting the host fecundity, longevity or dispersal (Duplouy et al., 2015). Here, we show that *w*Hho increases wasp susceptibility to hyperparasitism. The specialist hyperparasitoid *M.* cf. *stigmaticus* is present throughout Åland (van Nouhuys and Hanski, 2002). However, there is a negative association of the prevalence of the hyperparasitoid with *Wolbachia* infection in the landscape (Duplouy et al., 2015). The results of our study suggest an increased susceptibility of the *Wolbachia*-infected wasps to hyperparasitism. This could explain the landscape-scale negative association of the two parasites of *H. horticola* (Fig. 1, hypothesis 2). The persistence of *w*Hho in the host population despite the cost that we have identified suggests that there should be a counterbalancing benefit to the infected individuals.

As a maternally inherited endosymbiont, *Wolbachia* can promote its own spread and persistence by enhancing the production of females in its host populations. To this end, the bacterium often has strategies to improve the overall fitness of its insect host. This has been found in some parasitoid wasps; for instance, *Wolbachia* benefits survivorship of the host *Encarsia inaron* (White et al., 2011). *Asobara japonica* wasps infected with *Wolbachia* show more efficient host (*D. melanogaster*)-searching ability (Furihata et al., 2015), while *Wolbachia*-infected *Anagrus sophiae* parasitoid wasps have higher reproductive success than uninfected individuals (Segoli et al., 2013). In another wasp, *Asobara tabida*, the association with *Wolbachia* has evolved into complete mutualism; the bacterium is required for the host to complete oogenesis and reproduction (Dedeine et al., 2001, 2004). However, the presence of *Wolbachia* is not always associated with enhanced host life history traits. For instance, in natural populations of the *Drosophila* parasitoid *Leptopilina heterotoma*, *Wolbachia* infection reduces adult fecundity, survival and mobility (Fleury et al., 2000). Finally, *Wolbachia* may have no association with measured fitness traits, as was previously found for *H. horticola* (Duplouy et al., 2015), but is most likely positively linked to another yet-undefined fitness component(s).

A less direct way for *Wolbachia* to benefit their host fitness is by improving the host resistance to parasites (see Table 1). This is the case for the fruit fly *D. melanogaster*, in which the infectious dose (ID_{50}) for, and the titer of the West Nile Virus (WNV) are high in *Wolbachia*-infected flies, suggesting that *Wolbachia*-infected individuals resist infection by the virus better than non-infected ones (Glaser and Meola, 2010). Such expression of resistance to pathogens is believed to be costly for the host because an individual must maintain a high density of symbionts (Martinez et al., 2015). Thus, the *Wolbachia* strains present in higher density in *D. melanogaster* also shorten the flies' lifespan (Chrostek et al., 2013, 2014). Therefore, if the selection pressure from parasites is weak, there is little chance that the *Wolbachia* strain would spread in its host population (Martinez et al., 2014, 2015). Hence, the presence of *Wolbachia* is not always only beneficial with respect to immunity (Table 1).

We found that wasp larvae from *w*Hho-infected matrilines are more often parasitized by the hyperparasitoid *M.* cf. *stigmaticus* than are larvae from *Wolbachia*-free matrilines (Fig. 3, P=0.0472). Although our results strongly suggest that *Wolbachia* increases susceptibility of *H. horticola* to hyperparasitism, it is possible that *w*Hho-infected and non-infected hosts may differ in ways other than their *Wolbachia* infection status, which could be related to the host susceptibility to parasitism (Ferreira et al., 2014). However, we know at least that *w*Hho infects individuals throughout the well-mixed host population in Åland. It is found in the two mitotypes (less than 1% divergence between matrilines; Duplouy et al., 2015), across the different haplotypes of *H. horticola* (based on 14 microsatellite markers, A.D., unpublished observations), and across the landscape (Duplouy et al., 2015), where different haplotypes occur (Nair et al., 2016).

The mechanistic explanation of *H. horticola* susceptibility to hyperparasitism that is associated with *Wolbachia* infection remains unknown, but there are several possibilities. A foraging hyperparasitoid must first of all find *M. cinxia* caterpillar nests parasitized by *H. horticola.* Herbivory by *M. cinxia* causes the host plant to release volatile odors that lead *H. horticola* to their hosts (Castelo et al., 2010; Pinto-Zevallos et al., 2013). Such volatiles can also be attractive to hyperparasitoids (Zhu et al., 2014). While *Wolbachia* has not yet been found to affect the volatile chemistry of its hosts' food plant, it has been shown to play a crucial role in the manipulation of other aspects of host food plant physiology, inducing the 'green-island' phenotype, allowing a leaf-mining host insect to feed on senescing autumn leaves (Gutzwiller et al., 2015). In our system, the hyperparasitoid detected caterpillar nests parasitized by *w*Hho-infected and non-infected *H. horticola* wasps equally well, suggesting that the bacterium is not involved in manipulation of the volatile plant chemistry.

Once at a nest, a *M.* cf. *stigmaticus* has to find and parasitize *H. horticola* larvae using its ovipositor to probe inside the *M. cinxia* caterpillars (A. Reichgelt, Density-dependent aggregation of hyperparasitoid *Mesochorus stigmaticus*, MSc Thesis, University of Helsinki, 2007). *Drosophila* larvae are able to evade parasitoid wasps by rolling on their side in response to a stimulus such as cuticle piercing by the parasitoid ovipositor (Hwang et al., 2007; Robertson et al., 2013). If *H. horticola* larvae, which can move within the host hemolymph, are similarly evasive, then suppression of that behavior due to the presence of *Wolbachia* could increase their susceptibility to hyperparasitism.

After oviposition, a host may resist parasitism by killing the parasitoid egg or larva (Strand and Pech, 1995). *Wolbachia* induce upregulation of several host immune genes (Bian et al., 2010; Hughes et al., 2011; Kambris et al., 2010, 2009), potentially priming the immune system to respond strongly to pathogens or parasitoids (but see Bourtzis et al., 2000). Alternatively, *Wolbachia* may reduce the fitness of invading pathogens by competing for resources (Martinez et al., 2014; Moreira et al., 2009; Osborne et al., 2009). As we found no evidence of encapsulation of *M.* cf. *stigmaticus*, we suggest that *M.* cf. *stigmaticus* is able to successfully bypass the *H. horticola* immune system, regardless of the *Wolbachia* infection status of the host. The mechanisms of *Wolbachia*-induced protection against parasitism found in arthropods may target only some infection mechanisms, such as those of RNA viruses, but be unable to counteract others, including the virulence mechanisms of the hyperparasitoid *M.* cf. *stigmaticus* (Table 1).

Wolbachia is most well known for its ability to manipulate its host reproductive system in a manner that optimizes its transgenerational transmission (Caspari and Watson, 1959). Turelli and Hoffmann (1991, 1995) documented a rapid spread of the cytoplasmic incompatibility (CI)-inducing *Wolbachia* strain *w*Ri across the Californian populations of *D. simulans*. Indeed, the *w*Ri strain causes uninfected females to be incompatible with *Wolbachia*-infected males, thus increasing the reproductive success of the infected female hosts, whose offspring from matings with both infected and uninfected males are viable. Duplouy et al. (2015) reported that population sex-ratio distortions and female-only broods are not observed for *H. horticola* in the Åland Islands, suggesting that induction of manipulative phenotypes (male-killing,

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Table 1. Diverse studies on the effect of *Wolbachia* on the resistance and susceptibility of several host species to various pathogens

Host	Symbiont	Parasite	References
Protective effect against other pathogens/parasitoids			
Coleoptera: *Hypera postica*	*Wolbachia*	Hymenoptera parasitoid (*Microctonus aethiopoides*)	Hsiao, 1996
Diptera: *Drosophila melanogaster*	*Wolbachia* (*w*MelCS & *w*MelPop)	RNA viruses (DCV, CrPV, FHV)	Hedges et al., 2008
Diptera: *D. melanogaster*	*Wolbachia* (*w*Mel)	RNA viruses (DCV, FHV & NoraV)	Teixeira et al., 2008
Diptera: *Aedes aegypti*	*Wolbachia* (*w*MelPop)	Filarial nematode	Kambris et al., 2009
Diptera: *A. aegypti*	*Wolbachia* (*w*MelPop-CLA)	RNA viruses (dengue & chikungunya) & Avian malaria (*Plasmodium gallinaceum*)	Moreira et al., 2009
Diptera: *Drosophila simulans*	*Wolbachia* (*w*Mel, *w*Au, *w*Ri)	RNA viruses (DCV & FHV)	Osborne et al., 2009
Diptera: *A. aegypti*	*Wolbachia* (*w*AlbB)	RNA virus (dengue)	Bian et al., 2010
Diptera: *D. melanogaster* & *Culex quinquefascatus*	*Wolbachia* (*w*Mel or *w*Pip)	RNA virus (West Nile virus & chikungunya)	Glaser and Meola, 2010
Diptera: *Anopheles gambiae* & *A. aegypti*	*Wolbachia* (*w*MelPop)	Malaria (*Plasmodium berghei*)	Kambris et al., 2010
Diptera: *A. gambiae*	*Wolbachia* (*w*MelPop & *w*AlbB)	Malaria (*Plasmodium falciparum*)	Hughes et al., 2011
Diptera: *A. aegypti*	*Wolbachia* (*w*Mel & *w*MelPop-CLA)	RNA virus (dengue)	Walker et al., 2011
Diptera: *Aedes polynesiensis*	*Wolbachia* (*w*AlbB)	Filarial nematode (*Brugia pahangi*)	Andrews et al., 2012
Diptera: *Aedes albopictus*	*Wolbachia* (*w*Mel)	RNA virus (dengue)	Blagrove et al., 2012
Diptera: *D. simulans*	*Wolbachia* (*w*Au)	Hymenoptera parasitoid with virus (*Leptopilina boulardi* LbFV)	Martinez et al., 2012
Diptera: *A. aegypti*	*Wolbachia* (*w*Mel & *w*MelPop)	RNA viruses (yellow fever & chikungunya)	van den Hurk et al., 2012
Diptera: *Anopheles stephensi*	*Wolbachia* (*w*AlbB)	Malaria (*P. falciparum*)	Bian et al., 2013a
Diptera: *A. polynesiensis*	*Wolbachia* (*w*AlbB)	RNA virus (dengue)	Bian et al., 2013b
Diptera: *A. albopictus*	*Wolbachia* (*w*Mel)	RNA virus (chikungunya)	Blagrove et al., 2013
Diptera: *A. aegypti*	*Wolbachia* (*w*Mel & *w*MelPop-CLA)	Bacteria (*Erwinia carotorovra*, *Burkholderia cepacia*, *Salmonella typhimurium*, *Mycobacterium marinum*)	Ye et al., 2013
Diptera: *D. simulans*	*Wolbachia* (*w*Ma, *w*Stv, *w*Ana, *w*Ha, *w*Pro, *w*Ara, *w*Tro, *w*Au, *w*MelCS, *w*Mel, *w*Yak, *w*Tei)	RNA viruses (DCV &/or FHV)	Martinez et al., 2014
Diptera: *D. melanogaster*	*Wolbachia* (*w*Mel)	Hymenoptera parasitoid (*Leptopilina heterotoma*)	Xie et al., 2014
Isopod: *Armadillidium vulgare* & *Porcellio dilatatus*	*Wolbachia* (*w*Dil & *w*Con)	Bacteria (*Listeria ivanovii*, *S. typhimurium*), pathogenic *Wolbachia*	Braquart-Varnier et al., 2015
No protection against or no increase in susceptibility to other pathogens/parasitoids			
Diptera: *D. simulans*	*Wolbachia* (*w*Ri)	Fungus (*Beauveria bassiana*)	Fytrou et al., 2006
Diptera: *D. melanogaster*	*Wolbachia* (*w*Mel)	DNA virus (IIV-6)	Teixeira et al., 2008
Diptera: *D. simulans*	*Wolbachia* (*w*Ha & *w*No)	RNA viruses (DCV & FHV)	Osborne et al., 2009
Diptera: *D. melanogaster* & *C. quinquefascatus*	*Wolbachia* (*w*Mel or *w*Pip)	RNA virus (La Crosse virus)	Glaser and Meola, 2010
Diptera: *D. simulans* & *D. melanogaster*	*Wolbachia* (*w*Au, *w*Ri, *w*No, *w*Ha & *w*MelCS)	Bacteria (*Pseudomonas aeruginosa*, *Serratia marcescens* & *Erwinia carotovora*)	Wong et al., 2011
Diptera: *Drosophila bifasciata*	*Wolbachia*	RNA viruses (DCV & FHV)	Longdon et al., 2012
Diptera: *D. simulans* & *D. melanogaster*	*Wolbachia* (*w*Ri, *w*Mel, *w*MelPop)	Parasitoid (*L. boulardi* with & without LbFvirus)	Martinez et al., 2012
Diptera: *D. melanogaster*	*Wolbachia* (*w*Mel)	Bacteria (*Listeria monocytogenes, S. typhimurium* & *Providencia rettgeri*)	Rottschaefer and Lazzaro, 2012
Diptera: *A. aegypti*	*Wolbachia* (*w*Mel)	RNA virus (West Nile)	Hussain et al., 2013
Diptera: *D. simulans*	*Wolbachia* (*w*Tri, *w*Sh, *w*Bai, *w*Bic, *w*Inn, *w*Bor, *w*San)	RNA viruses (DCV & FHV)	Martinez et al., 2014
Diptera: *D. melanogaster*	*Wolbachia* (*w*Mel)	Hymenoptera parasitoid (*L. boulardi*)	Xie et al., 2014
Increase in susceptibility to other pathogens/parasitoids			
Diptera: *D. simulans*	*Wolbachia* (*w*Ri)	Hymenoptera parasitoid (*L. heterotoma*)	Fytrou et al., 2006
Lepidoptera: *Spodoptera exempta*	*Wolbachia* (*w*Exe)	Nucleopolydrovirus (baculovirus SpexNPV)	Graham et al., 2012
Diptera: *A. gambiae*	*Wolbachia* (*w*AlbB)	Malaria (*P. berghei*)	Hughes et al., 2012
Diptera: *Aedes fluviatilis*	*Wolbachia* (*w*Flu)	Avian malaria (*P. gallinaceum*)	Baton et al., 2013
Diptera: *A. aegypti*	*Wolbachia* (*w*MelPop)	RNA virus (West Nile)	Hussain et al., 2013
Diptera: *Culex tarsalis*	*Wolbachia* (*w*AlbB)	RNA virus (West Nile)	Dodson et al., 2014
Diptera: *Culex pipiens*	*Wolbachia* (*w*Pip)	Avian malaria (*Plasmodium relictum*)	Zele et al., 2014
Diptera: *Aedes notoscriptus*	*Wolbachia* (*w*Pip)	RNA virus (dengue)	Skelton et al., 2016
Hymenoptera: *Hyposoter horticola*	*Wolbachia* (*w*Hho)	Hymenoptera parasitoid (*Mesochorus* cf. *stigmaticus*)	This study

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thelytokous parthenogenesis or feminization) is not occurring. However, induction of CI is not ruled out, as neither the occurrence nor the absence of incompatibility between *w*Hho-infected males and non-infected females has yet been described in this system. By inducing CI, *w*Hho could overcome the negative effect of the bacterium on its host's susceptibility to hyperparasitism and still maintain an intermediate prevalence (~50%; Duplouy et al., 2015) in the wasp population through a balance of benefits (from CI) and costs (from the increased host susceptibility) to the infected individuals.

Some of the *H. horticola* used in this experiment were from Estonia, just a few hundred kilometers by sea from Åland, where neither the hyperparasitoid wasp nor *Wolbachia* is present. It is possible that both the wasp and the bacterium have not yet arrived here. Should the *w*Hho strain colonize the Estonian population, we would expect the infection to spread rapidly to a high prevalence in the absence of the hyperparasitoid wasp.

Selection due to lethal parasites such as parasitoid wasps can be very strong (Haldane, 1992), so one might expect *Wolbachia* that increase susceptibility to parasites to be rare. However, *Wolbachia* has been found to occur in several Diptera hosts (Table 1). We have shown that it occurs in a natural Hymenoptera host population under strong and consistent attack by a Hymenoptera hyperparasitoid. To date, the mechanisms behind how *Wolbachia* affects the relationship of its host with parasitoids or pathogens remain unclear. However, as the growing literature on diverse host–symbiont–pathogen systems suggests, the interaction is unlikely to be highly specific. In our study system, we saw no evidence of an increase of immune response, nor of any other evading mechanisms due to *Wolbachia*. Thus, the considerable benefit of the *Wolbachia* infection that counterbalances increased susceptibility to parasitism, which is not correlated with fecundity or longevity (Duplouy et al., 2015), must also not be directly related to resistance to parasitism.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
A.D. and S.v.N. designed the research. A.D., M.K. and S.v.N. collected the data. A.D. and S.v.N. analyzed the data and wrote the paper.

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Data availability
Data files are available from the Dryad digital repository: http://dx.doi.org/10.5061/dryad.md880 (van Nouhuys et al., 2016).

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