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Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts

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ABSTRACT

Wolbachia are the most abundant bacterial endosymbionts among arthropods. Although maternally inherited, they do not conform to the widespread view that vertical transmission inevitably selects for beneficial symbionts. Instead, *Wolbachia* are notorious for their reproductive parasitism which, although lowering host fitness, ensures their spread. However, even for reproductive parasites it can pay to enhance host fitness. Indeed, there is a recent upsurge of reports on *Wolbachia*-associated fitness benefits. Therefore, the question arises how such instances of mutualism are related to the phenotypes of reproductive parasitism. Here, we review the evidence of *Wolbachia* mutualisms in arthropods, including both facultative and obligate relationships, and critically assess their biological relevance. Although many studies report anti-pathogenic effects of *Wolbachia*, few actually prove these effects to be relevant to field conditions. We further show that *Wolbachia* frequently have beneficial and detrimental effects at the same time, and that reproductive manipulations and obligate mutualisms may share common mechanisms. These findings undermine the idea of a clear-cut distinction between *Wolbachia* mutualism and parasitism. In general, both facultative and obligate mutualisms can have a strong, and sometimes unforeseen, impact on the ecology and evolution of *Wolbachia* and their arthropod hosts. Acknowledging this mutualistic potential might be the key to a better understanding of some unresolved issues in the study of *Wolbachia*–host interactions.

Key words: *Wolbachia*, arthropods, mutualism, fitness benefits, host protection, pathogen interference, dependence, compensatory evolution, tolerance, reproductive parasitism.

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I. INTRODUCTION

Symbiosis – the living together of unlike organisms – has long been acknowledged to be of fundamental importance in the history of life (De Bary, 1879; Douglas, 2010). Endosymbiosis relates to the situation in which symbionts, usually microbes, reside within the cells of their hosts. Bacterial endosymbionts are tremendously abundant among invertebrates, particularly among arthropods (Zchori-Fein & Bourtzis, 2011). Their effects on host fitness span the whole range from mutualism (beneficial) to parasitism (harmful). Symbiont transmission modes are likewise diverse, ranging from vertical (heritable) to horizontal (infectious), and there is a general view that horizontal transmission selects for parasitism, whereas vertically transmitted endosymbionts should evolve towards mutualism because their evolutionary fate is closely linked to that of their hosts.

Wolbachia are endosymbiotic bacteria that live within cells of arthropods and filarial nematodes (in the latter, they form stable mutualistic associations which are beyond the scope of this article; see Fenn & Blaxter, 2007 for a review). They have been estimated to infect a large proportion of all arthropod species and probably are the most abundant intracellular symbionts on earth (Hilgenboecker *et al.*, 2008; Zug & Hammerstein, 2012). Beyond that, *Wolbachia* fascinate evolutionary biologists because they fundamentally violate the view that heritable symbionts must be mutualists: although *Wolbachia* are predominantly transmitted vertically, they harmfully manipulate the reproduction of arthropod hosts to their own benefit, often causing a substantial decrease in host fitness. These reproductive manipulations (or reproductive phenotypes) include cytoplasmic incompatibility, killing or feminization of genetic males, and induction of thelytokous parthenogenesis (Werren, Baldo & Clark, 2008). The adaptive rationale behind such reproductive parasitism is the fact that vertical transmission of *Wolbachia* occurs exclusively through the female germline. Since all reproductive manipulations directly or indirectly increase the proportion of infected females, *Wolbachia* are thus able to spread through populations without being mutualists.

Although there is no need for *Wolbachia* in arthropods to become mutualistic, it still pays for them to evolve traits that increase host fitness. A mutant strain that, in addition to manipulating host reproduction, confers some fitness benefit to the host is at an advantage over non-mutualistic strains (Turelli, 1994). Hence, even reproductive parasites are in principle selected to enhance host fitness. Indeed, recent years have seen a growing body of evidence suggesting that *Wolbachia* can have positive effects on the fitness of arthropod hosts and thus behave as mutualists, both of the facultative and obligate type (Fig. 1) (see Table 1 for definitions; for an early account of this topic, see Dedeine *et al.*, 2003). The fact that such fitness benefits can occur in the presence or absence of a reproductive manipulation prompts the question of how both effects are related to each other. In other words, are *Wolbachia* in arthropod hosts parasitic, mutualistic, or both? Moreover, considering potential benefits of *Wolbachia* infection might be helpful in elucidating several other outstanding issues. For example, how can *Wolbachia* persist in novel host species, although they initially often perform poorly in new hosts? Why has host resistance to *Wolbachia* been found only so rarely, given that selection would act on hosts to suppress reproductive parasites (Koehncke *et al.*, 2009)? And can *Wolbachia* become ultimate mutualists (see Table 1), so that the host performs better than it would ever have done without the bacteria (De Mazancourt, Loreau & Dieckmann, 2005)?

In this review, we gather evidence of *Wolbachia* mutualisms in arthropods and thus outline possible answers to these questions. After reviewing why heritable symbionts do not necessarily evolve into mutualists, we briefly sketch the well-known reproductive manipulations induced by *Wolbachia*. We then describe phenotypes of facultative mutualism and conditions that are favourable for its emergence, with a special emphasis on *Wolbachia*-mediated protection. Next, we provide evidence of obligate mutualism induced by *Wolbachia* in arthropod hosts and discuss how different forms of dependence may have evolved. To this end, we present three case studies on the evolution of dependence in order to highlight common features as well as differences between them. Finally, we sketch possible evolutionary fates of *Wolbachia*–arthropod mutualisms and outline directions for future research.

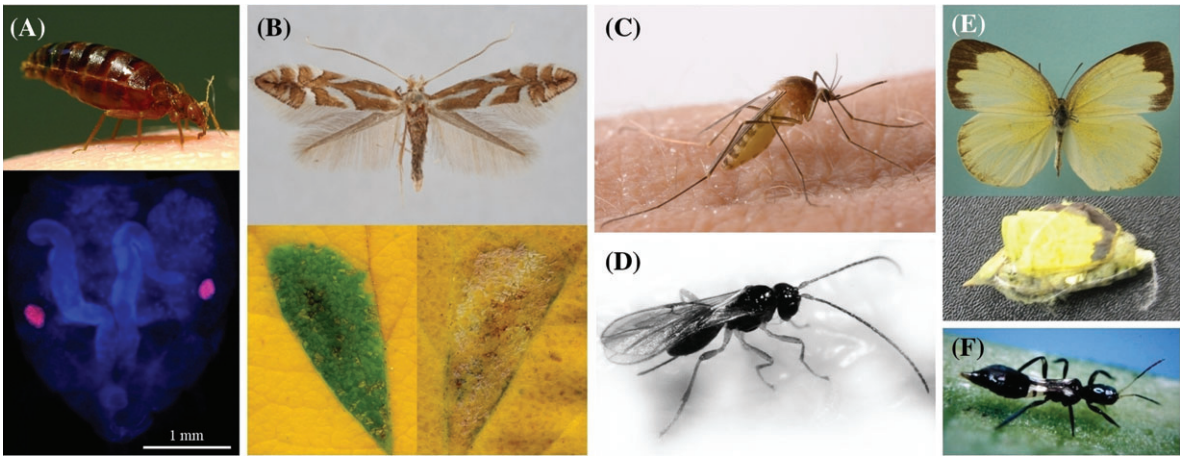

Fig. 1. *Wolbachia* mutualisms in arthropod hosts. (A) In the bedbug *Cimex lectularius* (top), *Wolbachia* provide essential B vitamins and are housed in specialized organs, the bacteriomes (bottom, magenta spots). © Dr. Richard Naylor, cimexstore.co.uk (top), Takahiro Hosokawa (bottom). (B) The leaf miner *Phyllonorycter blancardella* (top) relies on *Wolbachia* to cope with nutritional constraints in senescent leaves. Infected larvae are able to induce so-called 'green-islands' (bottom left), whereas cured larvae are not (bottom right). © Bert Gustafsson (top), David Giron (bottom). (C) The mosquito *Culex pipiens* is naturally infected with *Wolbachia* and the pathogen *Plasmodium relictum*. *Wolbachia* protects its host against *Plasmodium*-induced mortality. © Hans M. Smid, bugsinthepicture.com. (D) The parasitic wasp *Asobara tabida* depends on *Wolbachia* for oogenesis. © Kees Hofker. (E) The butterfly *Eurema hecabe* (top) is infected with feminizing *Wolbachia*. After larval antibiotic treatment, many adults show an intersexual phenotype, fail to escape from the pupal case and die (bottom). It is possible, though, that intersexual defects, rather than the lack of *Wolbachia*, are the cause of death. © Daisuke Kageyama. (F) In *Franklinothrips vespiformis*, *Wolbachia*-induced parthenogenesis has led to the complete loss of sexual function, making the symbiont an obligate mutualist for daughter production. © Entocare, Wageningen NL.

Table 1. Definitions of mutualism-related terms used in this review

Term	Definition
Mutualism	A symbiotic relationship in which both partners (host and symbiont) benefit
Parasitism	A symbiotic relationship in which one partner benefits at the expense of the other
Facultative mutualism[a]	A mutualistic relationship in which the symbiont is not necessary for successful host development or reproduction, but if it is present, the host enjoys some benefit from it
Obligate mutualism[a]	A mutualistic relationship in which the symbiont is required for host reproduction or survival
Proximate mutualism	A mutualistic relationship in which symbiont removal results in a decreased performance of the host. Proximate mutualisms can be the result of either ultimate mutualism or evolved dependence
Ultimate mutualism[b]	A mutualistic relationship in which the host could never have performed as well without the symbiont, i.e. the host gains some 'real' benefit from the interaction. In practice, detecting ultimate mutualisms is difficult because two different host genotypes must be compared: an infected host that is adapted to the presence of its symbiont must perform better than an uninfected conspecific that is adapted to the symbiont's absence
Evolved dependence[b]	A mutualistic relationship in which the host has lost the ability to perform well in the absence of its symbiont. Evolution of dependence is a precursor to obligate mutualism
'Jekyll and Hyde' infection	A symbiotic relationship in which a reproductive parasite simultaneously acts as a mutualist
'Stand-alone benefit' infection	A symbiotic relationship in which symbiont-associated benefits occur without any reproductive manipulation

[a]Both in facultative and obligate mutualisms, the endosymbiont benefits because it cannot survive outside of the host cell.
[b]For a more detailed discussion on ultimate mutualism and evolved dependence, see De Mazancourt *et al.* (2005).

II. SYMBIONT TRANSMISSION MODE AND THE EVOLUTION OF MUTUALISM

It has long been acknowledged that the way in which symbionts are transmitted plays a crucial role in determining whether parasitism or mutualism will evolve. In the conventional view, horizontal transmission favours parasitism (Anderson & May, 1982), whereas vertically transmitted symbionts will evolve towards mutualism because their survival depends on that of their hosts (Fine, 1975; Ewald, 1987; Yamamura, 1993; Lipsitch *et al.*, 1995). For the same reason, vertical transmission is thought to select for stable co-evolutionary relationships between symbiont and host. This is nicely illustrated by heritable bacterial symbionts such as *Buchnera* in aphids and *Wigglesworthia* in tsetse flies. These endosymbionts provide their hosts with essential

nutrients and are housed in a specialized host organ, the bacteriome. In such cases, strict vertical transmission has inextricably linked the evolutionary fates of symbiont and host and has thus led to the evolution of mutualism.

However, vertical transmission by no means guarantees benevolence. Selfish genetic elements (SGEs), including reproductive parasites such as *Wolbachia*, pose a major challenge to the conventional hypothesis. SGEs manipulate the genetic system of their hosts in order to favour their own transmission (see Werren, 2011 for a review). Because these manipulations are sufficient to ensure their spread, SGEs can afford to decrease host fitness even though they are predominantly transmitted vertically. The strategy of SGEs, particularly the reproductive parasitism of *Wolbachia* and other heritable symbionts, thus represents an alternative route to persist in hosts, without evolving towards mutualism. This alternative has often been neglected in the face of the long-lasting notion that vertical transmission necessarily selects for stable mutualistic associations (Werren & O'Neill, 1997).

Several arguments can be raised to reconcile the view that vertical transmission leads to mutualistic interactions with the existence of SGEs such as *Wolbachia*. Obviously, *Wolbachia* can indeed evolve into mutualists, as discussed herein. In the bedbug, for example, *Wolbachia* reside in a bacteriome and supply the host with B vitamins (Hosokawa *et al.*, 2010; Fig. 1A). Secondly, there is broad phylogenetic evidence for recurrent horizontal transmission of *Wolbachia* on evolutionary timescales (Zug, Koehncke & Hammerstein, 2012). Horizontal transmission is likely to be a major reason why *Wolbachia* have not evolved more frequently to mutualists in arthropods (Dedeine *et al.*, 2003). Lastly, it has been argued that SGEs are consistent with the conventional hypothesis if symbiont transmission is measured from the perspective of host genes instead of host organisms. In this gene-centered view of symbiont transmission, host sexual reproduction can be regarded as horizontal transmission of SGEs which allows them to become virulent (Smith, 2007).

III. THE CANONICAL VIEW: *WOLBACHIA* AS REPRODUCTIVE PARASITES

Wolbachia have evolved intriguing ways to interfere with key reproductive processes of their arthropod hosts (see Werren *et al.*, 2008 for a review). All these reproductive manipulations enhance the proportion of infected females and thus benefit the maternally inherited *Wolbachia*. In the case of cytoplasmic incompatibility (CI), offspring from uninfected females suffer high mortality rates when fathered by infected males. By contrast, infected females can mate successfully with both infected and uninfected males. CI thus benefits infected females and favours the spread of *Wolbachia* through host populations. The other reproductive phenotypes (male-killing, feminization, and induction of thelytokous parthenogenesis) all distort the offspring sex ratio of infected mothers towards females and thus directly increase the proportion of infected females. In so doing, these manipulations can have a huge impact on host sex determination (Cordaux, Bouchon & Grève, 2011). Male-killing *Wolbachia* kill a large proportion of a female's male offspring. This phenotype is advantageous to the bacteria when surviving (and infected) daughters benefit from the death of their brothers through some form of fitness compensation, for example resource reallocation. In the feminization phenotype, infected but non-transmitting male embryos develop as females, which do transmit the infection. Induction of thelytokous parthenogenesis has so far been found only in haplodiploid host taxa. Here, *Wolbachia* induce unfertilized eggs, which would normally develop into haploid males (arrhenotoky), to develop into diploid females (thelytoky), thus again increasing the percentage of transmitting hosts. In sum, regardless of how *Wolbachia* manipulate host reproduction, they do so in order to enhance their own transmission and therefore are commonly referred to as reproductive parasites and serve as textbook examples of SGEs (Werren, 2011; see Section II).

Inducing a reproductive phenotype is sufficient to drive *Wolbachia* through populations, even if infection decreases host fitness. Both for highly prevalent CI- and parthenogenesis-inducing *Wolbachia*, infection has been found to be associated with reduced fecundity of female hosts (Hoffmann, Turelli & Harshman, 1990; Stouthamer & Luck, 1993; Perrot-Minnot *et al.*, 2002). Likewise, male-killing *Wolbachia* can spread to high prevalence although they strongly reduce the fitness of infected females by killing half of their offspring (Jiggins *et al.*, 2002). Feminizing *Wolbachia* are widespread among isopod hosts although they impose a fitness cost on infected neo-females (i.e. feminized males) in that males prefer genetic females over neo-females, which have lower mating rates and receive less sperm (Moreau *et al.*, 2001). Moreover, sex-ratio-distorting phenotypes reduce the fitness of infected females since, in populations with a female-biased sex ratio, it is costly to produce less offspring of the rare, male sex. Thus, although reproductive manipulations can be associated with severe fitness costs, they are the main driver of *Wolbachia* through host populations.

IV. FROM PARASITISM TO MUTUALISM

In contrast to *Wolbachia*'s well-established role as a reproductive parasite, recent years have witnessed rapid accumulation of evidence for *Wolbachia* conferring some fitness benefits to their arthropod hosts. Originally, the idea of fitness-enhancing *Wolbachia* was launched by recurrent findings showing that the infection can be prevalent within a population even though reproductive manipulation is low or absent (Giordano, O'Neill & Robertson, 1995; Hoffmann, Clancy & Duncan, 1996; Hoffmann, Hercus & Dagher, 1998; Perrot-Minnot *et al.*, 2002; Charlat, Le Chat & Merçot, 2003; Bouwma & Shoemaker, 2011). Theory suggests that, in such cases, *Wolbachia* should increase host fitness in order to be maintained. Turelli (1994) showed that selection on CI-inducing *Wolbachia* favours variants that increase the relative fecundity of infected females, even if these variants

reduce the strength of CI. Under different conditions, however, selection on fecundity-enhancing strains is likely to preserve CI. Thus, once selection for increasing fecundity is operating, *Wolbachia* might either continue to manipulate host reproduction (case I), or not (case II). In case I, *Wolbachia* simultaneously act as a beneficial symbiont and as a reproductive parasite – a situation called 'Jekyll and Hyde' infection (Jiggins & Hurst, 2011; see Table 1). Beneficial effects of CI-inducing *Wolbachia* facilitate their invasion and spread in host populations (Dobson, Marsland & Rattanadechakul, 2002; Fenton *et al.*, 2011), making 'Jekyll and Hyde' infections good candidates for particularly successful *Wolbachia* strains. Moreover, such infections blur the distinction between mutualistic and parasitic *Wolbachia* (Herre *et al.*, 1999; Sachs, Essenberg & Turcotte, 2011*a*).

In case II, *Wolbachia*-associated benefits occur without reproductive manipulations. These 'stand-alone benefit' infections are likely to exhibit larger net benefits than 'Jekyll and Hyde' infections and are perhaps the best candidates for ultimate mutualisms (see Table 1), although it is difficult to prove that a given relationship actually reflects an ultimate mutualism (De Mazancourt *et al.*, 2005). Although speculative, the ability to induce a reproductive phenotype might only be hidden behind the beneficial trait and might suddenly become visible, for example after a host shift. Such a hiding effect has not yet been demonstrated for beneficial *Wolbachia* traits, but it has been shown that the ability to induce one reproductive manipulation can be hidden by another (Hornett *et al.*, 2008). Therefore, it is possible that 'stand-alone benefit' infections might easily turn into 'Jekyll and Hyde' infections.

By showing that, under certain circumstances, reproductive parasites are selected to become increasingly benign, the analysis by Turelli (1994) provides theoretical evidence for the notion that mutualistic *Wolbachia* evolved from parasitic ancestors (transition 1 in Fig. 2). This view is supported by more general studies on the origins of bacterial mutualism (Ewald, 1987; Sachs, Skophammer & Regus, 2011*b*). Accordingly, transitions from parasitism to mutualism have been found in several *Wolbachia*–arthropod associations (Vavre, Girin & Boulétreau, 1999; Fry, Palmer & Rand, 2004; Weeks *et al.*, 2007). Among the several phenotypes of reproductive parasitism, CI is probably the best candidate for a hypothetical starting point for a transition from parasitism to mutualism. In contrast to the sex-ratio-distorting phenotypes, CI causes selection on females to improve bacterial transmission because *Wolbachia*-free females suffer from incompatibility with infected males (Koehncke *et al.*, 2009).

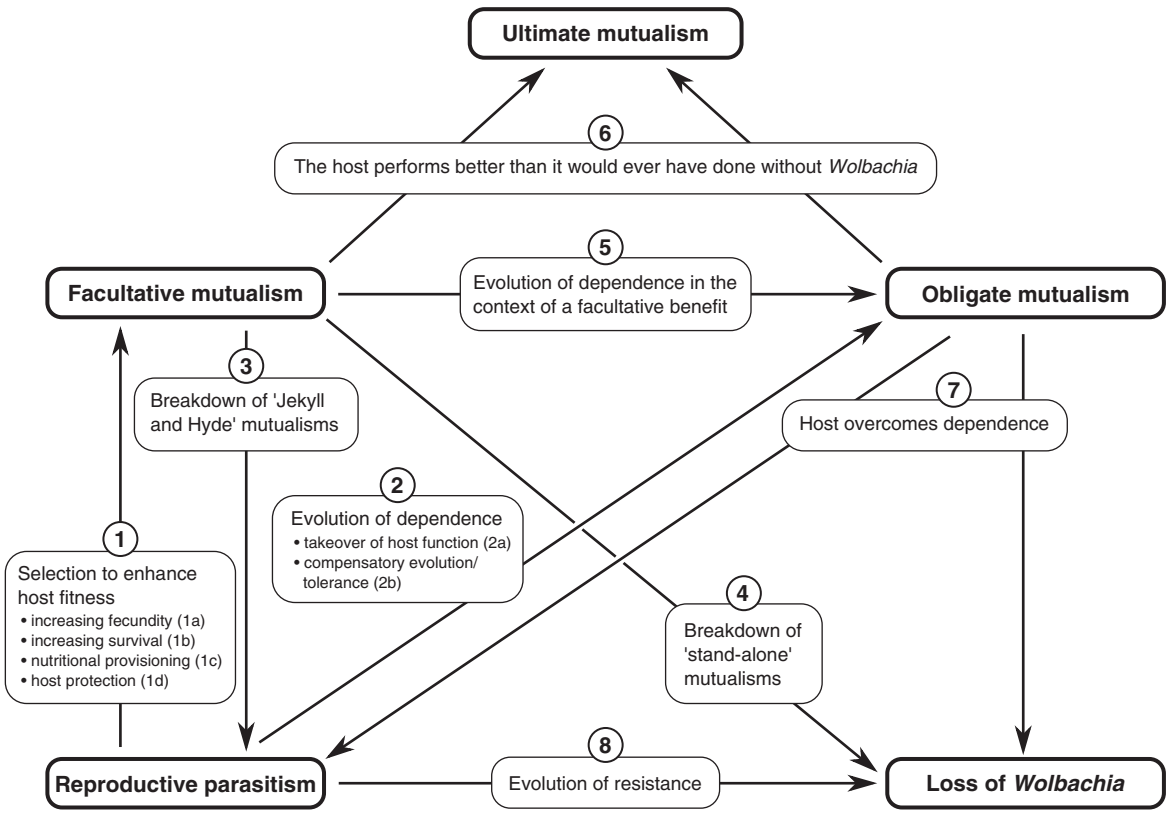

Fig. 2. A schematic overview of the possible transitions between different symbiotic relationships of *Wolbachia* and arthropod hosts. Each transition is depicted by an arrow and explained by the overlying numbered box (box 6 and box 7 belong to two transitions each). The numbers correspond with the descriptions of the transitions in the text. 'Jekyll and Hyde' mutualisms are those which occur together with a reproductive manipulation, whereas 'stand-alone' mutualisms do not. Note that the overview is non-exhaustive and also makes no statements on how likely each transition is. See text for further details.

This selection for high vertical transmission is likely to have two effects: firstly, it favours fixation of CI-inducing *Wolbachia* within populations; once near fixation, CI does little damage because most individuals are infected and thus protected from the phenotype. Secondly, high vertical transmission enables the host to adapt to the presence of *Wolbachia*. The fact that both effects promote the evolution of mutualism makes CI the most likely parasitic ancestor of a mutualistic phenotype (Dedeine *et al.*, 2003; Engelstädter & Hurst, 2009).

V. *WOLBACHIA* AS FACULTATIVE MUTUALISTS

(1) Overview

In this section, we provide an overview of beneficial *Wolbachia* phenotypes that are facultative from the host's point of view, i.e. although hosts benefit from infection, they do not depend on *Wolbachia* for survival or fecundity. Therefore, infected individuals can be cured of infection by antibiotic treatment or introgression crosses (but see Section VII for some shortcomings of antibiotic treatment as a method to identify *Wolbachia* effects). A straightforward way to examine *Wolbachia*-induced fitness effects is to compare survival or fecundity rates of infected *versus* uninfected females. Due to maternal inheritance of *Wolbachia*, there is no selection to increase male fitness (although there are a few cases known in which *Wolbachia* enhance male fertility: Wade & Chang, 1995; Hariri, Werren & Wilkinson, 1998). By comparing the performance of infected *versus* uninfected females (detection of proximate mutualisms; see Table 1), facultative fitness benefits due to *Wolbachia* infection have been found in many arthropod host species, often measured as a direct increase in fecundity or longevity (see Table 2; cases 1a and 1b in Fig. 2). Many of these fitness effects have been measured in the laboratory, but a recent study suggests that *Wolbachia* also increase lifetime reproductive success in the field (Segoli *et al.*, 2013). Frequently, infection exhibits the 'Jekyll and Hyde' type in which *Wolbachia* induce a reproductive phenotype and simultaneously confer some fitness benefit. For example, CI-inducing *Wolbachia* have been found to increase female survival in *Aedes albopictus* (Dobson, Rattanadechakul & Marsland, 2004), and *Drosophila innubila* females infected by male-killing *Wolbachia* produce significantly more daughters than do uninfected females (Unckless & Jaenike, 2012). These examples illustrate that the clear-cut distinction between parasitic and mutualistic *Wolbachia* is not always possible. In some cases, however, *Wolbachia* increase host fitness without any evident reproductive phenotype ('stand-alone benefit' infection): in the parasitoid wasp *Trichogramma bourarachae*, for example, the only known *Wolbachia* phenotype consists of an increase in fecundity (Vavre *et al.*, 1999; in most *Trichogramma* species, by contrast, *Wolbachia* induce thelytokous parthenogenesis, see Huigens & Stouthamer, 2003).

Facultative benefits, both of the 'Jekyll and Hyde' and 'stand-alone' type, could help to explain an unresolved issue concerning the spread of *Wolbachia*: on the one hand, the bacteria infect a major proportion of arthropod species worldwide (Zug & Hammerstein, 2012). Horizontal transmission into new host species is likely to be a key factor in shaping this pandemic (Zug *et al.*, 2012). On the other hand, *Wolbachia* commonly perform poorly after transmission into new hosts. Moreover, reproductive parasitism alone is often insufficient to ensure successful invasion into novel host populations. In the case of CI, for example, there exists a threshold infection frequency below which *Wolbachia* become extinct. Modelling shows that providing a fitness benefit greatly facilitates the invasion and spread of CI-inducing *Wolbachia* in novel hosts, e.g. by removing the invasion threshold (Fenton *et al.*, 2011). A recent experimental study suggests that beneficial effects might facilitate *Wolbachia* invasion even if the reproductive phenotype is lost after transmission to the new host. After transfer of a male-killing *Wolbachia* strain from *Drosophila innubila* to *D. simulans*, the recipient host did not suffer from any reproductive manipulation, but instead showed increased longevity. Such immediate beneficial effects could provide the necessary condition for *Wolbachia* to spread from low initial frequencies in novel host species, independently of any reproductive manipulation (Veneti *et al.*, 2012). Note, however, that after *Wolbachia* have overcome the initial obstacles to invasion by providing a fitness benefit, the beneficial effect might attenuate over time (e.g. in the case of host protection; see Section V.3). In the absence of benefits, the bacteria would have to make use of reproductive parasitism to be maintained in the population. Nevertheless, even such temporary beneficial effects are probably important facilitators of *Wolbachia* invasion into new hosts.

Most studies that analysed *Wolbachia* effects on host fecundity or longevity did not investigate possible mechanisms underlying these effects. Recent work on *Wolbachia*'s role in the female ovaries of *Drosophila mauritiana* might be informative in this respect. Strikingly, *Wolbachia*-infected females produce about four times more eggs than uninfected females (Fast *et al.*, 2011). In *Drosophila*, egg chambers are produced in the germarium, the anterior part of each ovariole that contains the germline stem cells. *Wolbachia* infection in *D. mauritiana* leads to increased mitotic activity of germline stem cells and to decreased apoptosis in the germarium. The combination of both effects results in the fourfold increase in egg production (Fast *et al.*, 2011). Although it is questionable whether such a huge fecundity effect is still beneficial to the host, *Wolbachia* could make use of these mechanisms to a lesser extent in order to enhance host fecundity in a beneficial way. Moreover, in *Drosophila melanogaster*, *Wolbachia* infection influences the expression level of *chico* (Zheng *et al.*, 2011), a gene that is involved in lifespan regulation (Clancy *et al.*, 2001). This could indicate a possible mechanistic basis for *Wolbachia*'s positive effect on longevity in *Drosophila* spp. (Fry & Rand, 2002; Fry *et al.*, 2004).

It is well known that the particular manifestation of mutualistic interactions is often context-dependent (Bronstein, 1994). Accordingly, *Wolbachia*-associated facultative benefits

Table 2. *Wolbachia*-induced facultative fitness benefits

Fitness benefit Host species	Reproductive manipulation?[a]	Notes	References
Increased fecundity			
ACARI			
Tetranychus truncatus	CI	Perhaps due to double infection with *Wolbachia* and *Cardinium*	Zhao *et al.* (2013*b*)
INSECTA			
Diptera			
Aedes albopictus	CI		Dobson *et al.* (2002, 2004)
Drosophila innubila	MK		Unckless & Jaenike (2012)
Drosophila mauritiana	?	Due to increased mitotic activity of germline stem cells and decreased apoptosis	Fast *et al.* (2011)
Drosophila melanogaster	—		Fry *et al.* (2004)
Drosophila simulans	CI		Weeks *et al.* (2007)
Hemiptera			
Nilaparvata lugens	—		Zhang *et al.* (2010)
Hymenoptera			
Nasonia vitripennis	CI	Probably due to host genetic background; see Bordenstein & Werren (2000)	Stolk & Stouthamer (1996)
Trichogramma bourarachae	—		Girin & Boulétreau (1995) and Vavre *et al.* (1999)
Trichogramma oleae	PI		Silva (1999)
Trichogramma pretiosum	PI		Grenier *et al.* (2002)
Psocoptera			
Liposcelis tricolor	?		Dong *et al.* (2007)
Increased survival/longevity			
ACARI			
Tetranychus phaselus	—	Perhaps due to the interplay between multiple *Wolbachia* and *Cardinium* strains	Zhao *et al.* (2013*a*)
INSECTA			
Diptera			
Aedes albopictus	CI		Dobson *et al.* (2002, 2004) and Gavotte *et al.* (2010)
Aedes polynesiensis	CI		Brelsfoard & Dobson (2011)
Culex quinquefasciatus	CI	Only in blood-fed females	Almeida *et al.* (2011)
Drosophila melanogaster	—		Fry & Rand (2002) and Fry *et al.* (2004)
Drosophila melanogaster	?		Alexandrov *et al.* (2007) and Toivonen *et al.* (2007)
Hemiptera			
Bemisia tabaci	?		Xue *et al.* (2012)
Psocoptera			
Liposcelis tricolor	?		Dong *et al.* (2007)
Nutritional provisioning			
INSECTA			
Coleoptera			
Diabrotica virgifera virgifera	CI	Due to down-regulation of defence genes in maize host plant; but see Robert *et al.* (2013)	Barr *et al.* (2010)
Diptera			
Drosophila innubila	MK	In low-nutrient environment	Unckless & Jaenike (2012)
Drosophila melanogaster	CI	In low or high iron environment	Brownlie *et al.* (2009)
Lepidoptera			
Phyllonorycter blancardella	?	Due to cytokinin-mediated induction of 'green-island' phenotype	Kaiser *et al.* (2010)

[a]CI, cytoplasmic incompatibility; MK, male-killing; PI, parthenogenesis induction; ?, unknown/not reported; —, not detected.

are likely to depend on the environmental conditions experienced by the host. For example, female *Aedes albopictus* larvae that are infected with *Wolbachia* experience higher survivorship under low larval densities, but not under high densities (Gavotte *et al.*, 2010). Additional conditions under which *Wolbachia*-associated benefits appear to be particularly valuable are the presence of pathogens (see Sections V.2 and V.3) and nutritional stress (see Table 2; case 1c in Fig. 2). When exposed to low-nutrient food, infected *D. melanogaster* and *Drosophila innubila* females laid significantly more eggs than uninfected females (Brownlie *et al.*, 2009; Unckless & Jaenike, 2012). A notable case of *Wolbachia*-induced nutritional provisioning was observed in the leaf miner *Phyllonorycter blancardella*. In autumn, *Ph. blancardella*

larvae induce 'green islands' in otherwise senescent leaves (Fig. 1B). These photosynthetically active patches present a nutrient-rich microenvironment to feeding larvae. Interestingly, larvae lost their ability to induce green islands when their mothers were cured of *Wolbachia*, leading to high mortality rates (Kaiser *et al.*, 2010). Probably, *Wolbachia* impact green island formation by manipulating cytokinin levels in the plant, possibly by directly synthesizing the phytohormone. If it could be shown that *Ph. blancardella* on its own (i.e. without *Wolbachia*) has never been able to induce green islands, this would represent a good example of an ultimate mutualism. It has also been suggested that *Wolbachia* may manipulate plant physiology in order to help its herbivorous insect host to cope with plant defence mechanisms. Larvae of the western corn rootworm, *Diabrotica virgifera virgifera*, feed on maize root tissues. A recent microarray study reported that *Wolbachia*-infected larvae induce a down-regulation of maize defence genes compared to their antibiotic-treated counterpart (Barr *et al.*, 2010). However, a follow-up study could not find any evidence of this effect (Robert *et al.*, 2013). Lastly, it is noteworthy that *Wolbachia* might also act as a nutritional mutualist in fungus-growing ants. Workers of the leaf-cutting ant *Acromyrmex octospinosus* cultivate their fungus garden by feeding it with freshly cut leaves and manuring it with faecal droplets. Surprisingly, *Wolbachia* occur extracellularly in the workers' gut lumen and faecal droplets (Andersen *et al.*, 2012). It is tempting to speculate that *Wolbachia* might contribute to the nutritional function of the faecal droplets in the ant–fungus symbiosis. Taken together, these examples illustrate the role of mutualistic symbionts as 'hidden players' in insect–plant interactions (Frago, Dicke & Godfray, 2012), but also show that *Wolbachia*'s role in such interactions needs further investigation.

(2) Protection against pathogens: the evidence

The presence of natural enemies is another situation that might reveal possible host benefits provided by *Wolbachia* (case 1d in Fig. 2). During the last few years, numerous studies have reported that *Wolbachia* infection has an anti-pathogenic effect in the host, for example against several RNA viruses, different *Plasmodium* species, fungi, bacteria, and nematodes. Antiviral effects, in particular, have been observed frequently and across different *Wolbachia* strains, multiple hosts, and diverse viral families (see Table 3 and references therein). Some of these studies have aroused great interest, not least because *Wolbachia*'s anti-pathogenic potential might be used as an effective means to control insect-borne human diseases (Kambris *et al.*, 2009; Moreira *et al.*, 2009; Iturbe-Ormaetxe, Walker & O'Neill, 2011; Blagrove *et al.*, 2012; Mousson *et al.*, 2012). The recent upsurge in reports on that topic is also in line with a generally increasing interest in symbiont-mediated protection among arthropod hosts (for reviews, see Haine, 2008; Brownlie & Johnson, 2009). Modelling predicts that host protection will evolve in vertically transmitted parasites when they compete with horizontally transmitted pathogens in the same host; in this case, host protection can maintain otherwise costly symbionts within host populations (Lively *et al.*, 2005; Jones, White & Boots, 2007, 2011; Fenton *et al.*, 2011).

At this point, we put forward a clarification of terminology by distinguishing between 'anti-pathogenic effect' (or 'pathogen interference') on the one hand and 'protection' on the other hand. Symbiont-mediated protection can result from a reduction in pathogen load (resistance), from an increased ability of the host to compensate for negative effects of the pathogen (tolerance), or from a combination of both mechanisms. We define 'protection' as an increase in host fitness as a result of increased resistance and/or tolerance in the presence of pathogens. By contrast, the term 'anti-pathogenic effect' ('interference') is meant to include all cases of increased resistance/tolerance, regardless of whether a corresponding fitness benefit has been demonstrated. While many studies observed an anti-pathogenic effect of *Wolbachia* (mostly based on increased resistance), only some of them have tested for a fitness effect (see Table 3). In light of other potential drawbacks (see Section V.3), there remain only a few reports that make a convincing case for *Wolbachia*-mediated host protection (e.g. Hedges *et al.*, 2008; Teixeira, Ferreira & Ashburner, 2008; Osborne *et al.*, 2009; Zélé *et al.*, 2012; see also Fig. 1C).

The molecular mechanisms underlying *Wolbachia*-associated anti-pathogenic effects are still unclear. Antiviral activity seems to be more frequent than antibacterial activity, indicating that the underlying mechanisms are independent (Wong *et al.*, 2011; Rottschaefer & Lazzaro, 2012). Moreover, no effect against a DNA virus has been found so far (Teixeira *et al.*, 2008; Unckless, 2011; Graham *et al.*, 2012), pointing to another way of how to elucidate the mechanisms of pathogen interference. For a discussion on possible mechanisms underlying *Wolbachia*'s antiviral effects, see also Merkling & van Rij (2013), Rainey *et al.* (2014) and Sinkins (2013). In general, there is good evidence that *Wolbachia* density is correlated to the strength of anti-pathogenic activity (Osborne *et al.*, 2009, 2012; Frentiu *et al.*, 2010; Lu *et al.*, 2012). Consistent with this finding, two major (mutually non-exclusive) hypotheses have been proposed to explain the mechanism of *Wolbachia*-induced pathogen interference. On the one hand, interference may be due to the possibility that both *Wolbachia* and pathogens compete for limited host resources (Moreira *et al.*, 2009; Osborne *et al.*, 2009, 2012; Frentiu *et al.*, 2010; Wong *et al.*, 2011; Lu *et al.*, 2012). On the other hand, several studies suggest that *Wolbachia* upregulate the host immune response, particularly genes involved in the Toll and the Immune Deficiency (IMD) pathway, and that such immune upregulation underlies anti-pathogenic effects (Xi *et al.*, 2008; Moreira *et al.*, 2009; Kambris *et al.*, 2009, 2010; Bian *et al.*, 2010; Pan *et al.*, 2012). However, all of these studies analysed *Wolbachia* effects in hosts that are either naturally uninfected or infected with a different strain. By contrast, *Wolbachia*-induced anti-pathogenic effects in naturally infected hosts are not associated with immune activation, indicating that upregulation of immune genes (or at least of those in the Toll and IMD pathway) is not required for host protection in the field (Wong *et al.*, 2011; Rancès *et al.*, 2012, 2013) (see Section V.3).

Table 3. *Wolbachia*-induced anti-pathogenic effects (pathogen interference)

Host/vector species	Reproductive manipulation?[a]	Pathogen	Natural infection?		Fitness effect tested?[b]		*w*MelPop?	References
			Wolbachia	Pathogen	*Wolbachia*	Pathogen		
INSECTA								
Diptera								
Aedes aegypti	CI	Nematode *Brugia pahangi*	No[c]	Yes	No	No	Yes	Kambris *et al.* (2009)
	CI	Bacterium *Burkholderia cepacia*	No[c]	?	Yes	Yes	Yes	Ye *et al.* (2013)
	CI	Chikungunya virus	No[c]	Yes	No	No	Yes[e]	Moreira *et al.* (2009) and Van den Hurk *et al.* (2012)
	CI	Dengue virus	No[c]	Yes	Yes[d]	Yes[d]	Yes[e]	Moreira *et al.* (2009), Bian *et al.* (2010), Walker *et al.* (2011)
	CI	Bacterium *Erwinia carotovora*	No[c]	?	Yes	Yes[f]	Yes[e]	Kambris *et al.* (2009) and Ye *et al.* (2013)
	CI	Bacterium *Mycobacterium marinum*	No[c]	No	Yes	Yes	Yes	Ye *et al.* (2013)
	CI	*Plasmodium gallinaceum*	No[c]	No	No	No	Yes	Moreira *et al.* (2009)
	CI	Bacterium *Salmonella typhimurium*	No[c]	No	Yes	Yes	Yes[e]	Ye *et al.* (2013)
	CI	Yellow fever virus	No[c]	Yes	No	No	Yes	Van den Hurk *et al.* (2012)
Aedes albopictus	CI	Chikungunya virus	No[g]	Yes	No	No	No	Blagrove *et al.* (2013)
	CI	Dengue virus	No[g]	Yes	No	No	No	Blagrove *et al.* (2012)
	CI	Dengue virus	Yes	Yes	Yes[h]	No	No	Mousson *et al.* (2012)
Aedes polynesiensis	CI	Nematode *Brugia pahangi*	No[g]	Yes	Yes[i]	Yes	No	Andrews *et al.* (2012)
Anopheles gambiae	?[j]	*Plasmodium berghei*	No[c]	No	No	No	Yes	Kambris *et al.* (2010)
	?[j]	*Plasmodium falciparum*	No[c]	Yes	Yes[i]	Yes[h]	Yes[e]	Hughes *et al.* (2011)
Anopheles stephensi	CI	*Plasmodium falciparum*	No[c]	Yes	No	No	No	Bian *et al.* (2013)
Culex pipiens	CI	*Plasmodium relictum*	Yes	Yes	Yes	Yes	No	Zélé *et al.* (2012)
Culex quinquefasciatus	CI	West Nile virus	Yes	Yes	No	No	No	Glaser & Meola (2010)
Drosophila innubila	MK	Flock house virus	Yes	No	Yes	Yes	No	Unckless & Jaenike (2012)
Drosophila melanogaster	?	Fungus *Beauveria bassiana*	Yes	Yes	Yes	No	No	Panteleev *et al.* (2007)
	CI	Chikungunya virus	Yes	No	No	No	No	Glaser & Meola (2010)
	CI	Cricket paralysis virus	Yes	Yes	Yes	No	No	Hedges *et al.* (2008)
	CI	Dengue virus	Yes	No	No	Yes[h]	Yes[e]	Rancès *et al.* (2012)
	CI	Drosophila C virus	Yes	Yes	Yes	Yes[k]	Yes[e]	Hedges *et al.* (2008) and Teixeira *et al.* (2008)
	CI	Flock house virus	Yes	No	Yes	No	No	Hedges *et al.* (2008) and Teixeira *et al.* (2008)
	CI	Nora virus	Yes	Yes	Yes	No	No	Teixeira *et al.* (2008)
	CI	West Nile virus	Yes	No	No	No	No	Glaser & Meola (2010)
Drosophila simulans	CI	Drosophila C virus	Yes	Yes	Yes	Yes	No	Osborne *et al.* (2009)
	CI	Flock house virus	Yes	No	Yes	Yes	No	Osborne *et al.* (2009)
Hemiptera								
Cimex lectularius	?	Opportunistic bacteria that are transferred during traumatic insemination	Yes	Yes	?	?	No	(L. L. Heaton & M. T. Siva-Jothy, unpublished data)

The column 'Natural infection?' indicates whether the host/vector is naturally infected with *Wolbachia* or the pathogen. The column 'Fitness effect tested?' indicates whether any fitness effects of *Wolbachia* or pathogen infection were tested. The column '*w*MelPop?' indicates whether the laboratory *Wolbachia* strain *w*MelPop was used. Grey shading indicates characters that are not suited for an assessment of *Wolbachia*'s protective potential in the field. For more information see main text.

[a]CI, cytoplasmic incompatibility; MK, male-killing; ?, unknown/not reported.

[b]If yes, then a positive *Wolbachia* effect/negative pathogen effect was found, unless noted otherwise.

[c]Host naturally uninfected.

[d]Only Bian *et al.* (2010) tested for a fitness effect; *Wolbachia* effect was slightly positive, but there was no significant pathogen effect.

[e]Not in all experiments.

[f]Only Ye *et al.* (2013) tested for a fitness effect; pathogen effect was negative.

[g]Cured of its native *Wolbachia* and then transfected with a non-native strain.

[h]No significant effect.

[i]Negative effect.

[j]Only transient somatic infections have been established.

[k]Only Teixeira *et al.* (2008) tested for a fitness effect; pathogen effect was negative.

(3) Protection against pathogens: a critique

There are some caveats to the experimental findings of *Wolbachia*-associated anti-pathogenic effects and the conclusions that can be drawn from them. Primarily, these caveats relate to the question of whether pathogen interference does occur in nature and, if yes, whether it is associated with a fitness benefit to the host. In other words, does an anti-pathogenic effect actually represent a case of host protection? To answer this question, it is crucial to have a closer look at the ménage à trois between host, pathogen, and *Wolbachia*. With regard to the *Wolbachia*–host relationship, one should ask whether the arthropod species under study is naturally infected

with *Wolbachia*, and whether the anti-pathogenic effect is associated with an increase in host fitness. Likewise, one should examine the studied host–pathogen relationship: is it actually found in nature, and is pathogen presence associated with a decrease in host fitness? We consider each issue in turn.

(*a*) *Does the* Wolbachia*–host relationship exist in nature?*

Firstly, several studies that found *Wolbachia*-induced anti-pathogenic effects used the virulent *Wolbachia* strain *w*MelPop (see Table 3). This strain was detected in a laboratory strain of *Drosophila melanogaster* and possibly does not exist in nature. It is therefore unclear what these findings tell us about naturally existing symbioses. Secondly, almost all experiments were done using laboratory host strains or even cell lines (Frentiu *et al.*, 2010; Lu *et al.*, 2012). These strains are highly adapted to laboratory conditions which are more benign than those in the field. Again, it is unclear what we can learn about *Wolbachia*-mediated host protection in natural environments. Lastly, and most importantly, a number of studies found pathogen interference in hosts that are naturally uninfected with *Wolbachia* and were only transfected with the symbiont, e.g. the mosquitoes *Aedes aegypti* and *Anopheles gambiae* (Moreira *et al.*, 2009; Kambris *et al.*, 2009, 2010; Bian *et al.*, 2010; Hughes *et al.*, 2011; Walker *et al.*, 2011). Other reports on anti-pathogenic effects involve hosts that had been cured of their native *Wolbachia* and that were then transfected with a non-native strain (Blagrove *et al.*, 2012, 2013) (see Table 3).

(*b*) *Why does the distinction between natural and artificial* Wolbachia *infections matter?*

Transfection of *Wolbachia* into naturally uninfected hosts (or into hosts naturally infected with a different *Wolbachia* strain) is likely to be the cause of immune upregulation and thus of the anti-pathogenic effects in these artificially created *Wolbachia*–host associations. By contrast, in many co-evolved associations *Wolbachia* infection is not associated with immune upregulation (Bourtzis, Pettigrew & O'Neill, 2000; Wong *et al.*, 2011; Rancès *et al.*, 2012) and also has no anti-pathogenic effect, but rather is neutral or even 'pro-pathogenic' (see Table 4). We also note that even

Table 4. Naturally occurring *Wolbachia*–host associations in which infection has either no anti-pathogenic effect or even a deleterious ('pro-pathogenic') effect in the presence of pathogens. This neutral/negative effect was proven by comparing pathogen load or host fitness (survival) in the presence *versus* absence of *Wolbachia*

Host/vector species	Pathogen	References
INSECTA		
Diptera		
Aedes aegypti	Chikungunya virus	Van den Hurk *et al.* (2012)
	Yellow fever virus	Van den Hurk *et al.* (2012)
Aedes albopictus	Chikungunya virus	Mousson *et al.* (2010)
	Dengue virus	Bian *et al.* (2010)
Aedes fluviatilis	*Plasmodium gallinaceum*[a]	Baton *et al.* (2013)
Aedes pseudoscutellaris	Nematode *Brugia pahangi*	Dutton & Sinkins (2005)
Armigeres subalbatus	Japanese encephalitis virus	Tsai *et al.* (2006)
Drosophila bifasciata	Drosophila C virus	Longdon *et al.* (2012)
	Flock house virus[a]	Longdon *et al.* (2012)
Drosophila innubila	*Drosophila innubila* Nudivirus	Unckless (2011)
Drosophila melanogaster	Bacterium *Burkholderia cepacia*	Ye *et al.* (2013)
	Bacterium *Erwinia carotovora*	Wong *et al.* (2011)
	Insect iridescent virus 6[a]	Teixeira *et al.* (2008)
	La Crosse virus[a]	Glaser & Meola (2010)
	Bacterium *Listeria monocytogenes*	Rottschaefer & Lazzaro (2012)
	Bacterium *Mycobacterium marinum*	Ye *et al.* (2013)
	Bacterium *Providencia rettgeri*	Rottschaefer & Lazzaro (2012)
	Bacterium *Pseudomonas aeruginosa*	Wong *et al.* (2011)
	Bacterium *Salmonella typhimurium*	Rottschaefer & Lazzaro (2012)
	Bacterium *Serratia marcescens*	Wong *et al.* (2011)
Drosophila neotestacea	Nematode *Howardula aoronymphium*	Jaenike *et al.* (2010)
Drosophila simulans	Fungus *Beauveria bassiana*	Fytrou *et al.* (2006)
	Drosophila C virus	Osborne *et al.* (2009)
	Bacterium *Erwinia carotovora*	Wong *et al.* (2011)
	Flock house virus[a]	Osborne *et al.* (2009)
	Parasitoid *Leptopilina heterotoma*	Fytrou *et al.* (2006)
	Bacterium *Pseudomonas aeruginosa*	Wong *et al.* (2011)
	Bacterium *Serratia marcescens*	Wong *et al.* (2011)
Lepidoptera		
Spodoptera exempta	*Spodoptera exempta* nucleopolyhedrovirus	Graham *et al.* (2012)

[a]No natural pathogen.

an artificial *Wolbachia* infection can be pro-pathogenic, e.g. by increasing pathogen load (Hughes *et al.*, 2012). Nevertheless, the overall trend of the findings is that a strong immune response and concomitant pathogen interference are frequent in artificial, but rare in natural *Wolbachia*–host associations. A possible conclusion is that anti-pathogenic effects are present only in newly infected hosts and will attenuate through co-evolution between host and symbiont (Vavre & Charlat, 2012). Therefore, *Wolbachia*-induced pathogen interference (and associated host protection) might only be a temporary phenomenon. However, even a temporary anti-pathogenic effect in naturally uninfected hosts might be of biological relevance: it could boost *Wolbachia* from very low initial frequencies and thus facilitate invasion into novel host populations (Fenton *et al.*, 2011; see Section V.1).

(*c*) *Is* Wolbachia *infection associated with a fitness benefit?*

In order to demonstrate that *Wolbachia* actually protects its host against a pathogen, the anti-pathogenic effect must be shown to confer a fitness benefit (e.g. increased survival). In many studies, however, the impact of pathogen interference on host fitness was not analysed at all (see Table 3). Furthermore, some of the studies that did test for fitness effects could not find a positive effect (Mousson *et al.*, 2012) or even found a negative one (Hughes *et al.*, 2011; Andrews *et al.*, 2012). In conclusion, many analysed *Wolbachia*–host associations are not suited to prove the symbiont's ability to protect its host against pathogens. Lastly, high-density *Wolbachia* infections, which are often associated with strong anti-pathogenic effects (see Section V.2), might shorten host lifespan. Therefore, even if *Wolbachia* infection protects against pathogens, this benefit might be counteracted by the cost of shortened lifespan, possibly causing selection to favour lower levels of protection (Chrostek *et al.*, 2013).

(*d*) *Does the host-pathogen relationship exist in nature?*

The second big task in assessing *Wolbachia*'s protective potential is to scrutinize the relationship between host and pathogen. Not all host–pathogen relationships that were examined in the laboratory are actually found in the field. For example, *Wolbachia*-associated effects against the Flock House virus (FHV) were examined in three different *Drosophila* species (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Osborne *et al.*, 2009; Unckless & Jaenike, 2012), although FHV is not a natural pathogen of *Drosophila*, but was isolated from a coleopteran (Scotti, Dearing & Mossop, 1983). Likewise, *Wolbachia*-induced anti-pathogenic effects were observed in mosquito–*Plasmodium* combinations that are not found in nature (Moreira *et al.*, 2009; Kambris *et al.*, 2010). There are more examples of unnatural host–pathogen relationships (see Table 3). Tripartite interactions between *Wolbachia*, its host and an unnatural pathogen are probably not well suited to evaluate *Wolbachia*'s protective abilities.

(*e*) *Is pathogen infection associated with a fitness cost?*

A last crucial point is to demonstrate a pathogen-induced fitness cost to the host (usually increased mortality). To do so, one has to compare survival rates of pathogen-challenged and unchallenged hosts. Despite this simplicity, only some studies in Table 3 used this approach to confirm a pathogen-related fitness cost (Teixeira *et al.*, 2008; Osborne *et al.*, 2009; Andrews *et al.*, 2012; Unckless & Jaenike, 2012; Zélé *et al.*, 2012). Nevertheless, this check is important because not all symbionts commonly referred to as pathogens necessarily reduce host fitness. For example, Teixeira *et al.* (2008) found a *Wolbachia*-induced anti-pathogenic effect against the *Drosophila* Nora virus. However, this virus causes infections that are essentially symptom-free (Habayeb *et al.*, 2009). Even in an artificially created host–pathogen association, there was no significant pathogen effect on host fitness (Rancès *et al.*, 2012). Moreover, it is possible that a fitness cost is only due to the experimental mode of pathogen transmission. In its natural host *Drosophila melanogaster*, for example, Drosophila C virus (DCV) is transmitted by feeding and shows varying pathogenicity (Thomas-Orillard, Jeune & Cusset, 1995; Hedges & Johnson, 2008). However, when injected into adult flies, DCV turns out to be highly pathogenic, with flies dying within several days after injection. Accordingly, microarray studies that analysed the antiviral response of *D. melanogaster* revealed that only few genes are induced after oral ingestion of DCV, whereas a broad response is triggered after DCV injection (Roxström-Lindquist, Terenius & Faye, 2004; Dostert *et al.*, 2005). Therefore, experiments involving the injection of pathogens might be biased towards higher fitness costs than those that are found in natural host–pathogen relationships. This might be a serious problem because injection of pathogens into adult hosts is a standard transfection procedure and was used in all studies listed in Table 3 that consider *Drosophila*–virus relationships. Last we note that a particular strain of DCV (termed DCV_C) even has a beneficial effect on its *Drosophila* host: although DCV_C enhances pre-adult mortality, it increases fecundity and longevity in adult females and might thus confer a net fitness benefit to the host (Thomas-Orillard, 1990; Gomariz-Zilber & Thomas-Orillard, 1993). In this case, an antiviral effect by *Wolbachia* would probably be disadvantageous to the host.

Up to this point, we have considered relationships between a pathogen and its principal host (such as DCV and Nora virus in *Drosophila*). However, the question of whether pathogens induce a fitness cost is particularly controversial in cases where an arthropod species acts as vector of a pathogen, not as its principal host. Indeed, many studies listed in Table 3 consider relationships between pathogens and their arthropod vectors, for example mosquito–*Plasmodium* systems or mosquito-borne viruses such as chikungunya and dengue. The degree of pathogen virulence in arthropod vectors is still under debate. Two meta-analyses suggest that, overall, arthropod-borne pathogens reduce the survival of their vectors (Ferguson & Read, 2002; Lambrechts & Scott, 2009). Nevertheless, there are exceptions: For two mosquito–*Plasmodium* combinations in Table 3 (*Aedes*

Table 5. Evolved host dependencies upon *Wolbachia*

Defects in aposymbiotic females Host species	Reproductive manipulation?[a]	Notes	References
Female sterility (oogenesis defects)			
COLLEMBOLA			
Folsomia candida	PI	*Via* facilitation of parthenogenesis; see Hafer & Pike (2010)	Pike & Kingcombe (2009) and Timmermans & Ellers (2009)
INSECTA			
Coleoptera			
Coccotrypes dactyliperda	?	Caused by *Wolbachia* or *Rickettsia* (or both)	Zchori-Fein *et al.* (2006)
Lissorhoptrus oryzophilus	?		Chen *et al.* (2012)
Otiorhynchus sulcatus	?		Son *et al.* (2008)
Diptera			
Drosophila paulistorum	CI		Miller *et al.* (2010)
Exorista sorbillans	?		Puttaraju & Prakash (2009)
Hemiptera			
Cimex lectularius	?	*Via* supply of B vitamins	Hosokawa *et al.* (2010)
Hymenoptera			
Asobara tabida	—		Dedeine *et al.* (2001)
Lethality			
INSECTA			
Lepidoptera			
Eurema hecabe	FE	Lethality may also be explained by intersexual defects	Narita *et al.* (2007)
Ostrinia furnacalis	MK		Sakamoto *et al.* (2007)
Ostrinia scapulalis	MK		Kageyama & Traut (2004)

[a]CI, cytoplasmic incompatibility; FE, feminization; MK, male-killing; PI, parthenogenesis induction; ?, unknown/not reported; —, not detected.

aegypti–*P. gallinaceum* and *Anopheles gambiae*–*P. falciparum*), Ferguson & Read (2002) found no reduction in vector survival (see also Hughes *et al.*, 2011). Similarly, there are cases where chikungunya and dengue infection had no influence on vector survival (Bian *et al.*, 2010; Mousson *et al.*, 2010). Therefore, the fact that pathogens do not necessarily impose a fitness cost holds true both for hosts and vectors. However, if there is no fitness cost of pathogen infection, any anti-pathogenic effect induced by *Wolbachia* will probably not be beneficial to the host.

Taken together, we have shown that many reports on *Wolbachia*-associated anti-pathogenic effects fail to prove naturally occurring host protection. While *Wolbachia*-induced pathogen interference is a promising field of research, given its far-reaching implications for disease control, we feel that there is a need to examine more rigorously its significance in the field. We do not claim that *Wolbachia*-induced protection is unimportant in nature; rather, our survey shows that the evidence is limited so far. Future research might easily change the picture.

VI. *WOLBACHIA* AS OBLIGATE MUTUALISTS

(1) Overview

It is becoming increasingly clear that several arthropod species cannot survive or reproduce when their *Wolbachia* symbionts are removed (see Table 5). In such cases of evolved dependence (transition 2 in Fig. 2), hosts have adapted to the presence of *Wolbachia* (De Mazancourt *et al.*, 2005). For example, the latter might have evolved to provide some vital component of a host developmental or reproductive process. Subsequent relaxed selection on host genes for this trait would allow for the accumulation of mutations in these genes. Once the host has lost the ability to provide the vital function on its own, *Wolbachia* could permanently take over control of the corresponding process. Such sheltering of deleterious mutations has been observed in *Drosophila melanogaster* where *Wolbachia* infection suppresses sterility in *Sex-lethal* (*Sxl*) mutants and lethality of *chico* mutants, respectively (Starr & Cline, 2002; Clark *et al.*, 2005). Similarly, infection also rescues female fertility in *bag of marbles* (*bam*) mutants (H. A. Flores, personal communication). Since all genes are involved in *D. melanogaster* oogenesis (*Sxl*, in addition, is the master regulator of sex determination in this species), these observations indicate that dependence on *Wolbachia* might frequently be associated with the ability of the symbiont to interfere with key host reproductive processes, such as oogenesis and sex determination (see Sections VI.2–VI.4).

The takeover of some host function by *Wolbachia* is likely to be typical of the initial steps towards obligate mutualism (case 2a in Fig. 2, see Section VI.3; another evolutionary path to obligate mutualism involves compensatory evolution in the host; see Sections VI.2 and VI.4). However, some authors have refrained from classifying such relationships

as mutualisms and prefer the term 'obligatory parasitism' because *Wolbachia* does not provide any additional new function, but only ensures that pre-exisiting processes function properly, thus rendering the host incapable of independence (Dedeine *et al.*, 2003). The conflict can be resolved by the disambiguation put forward by De Mazancourt *et al.* (2005): symbioses in which the host requires *Wolbachia* are classified as proximate mutualisms (the host derives a benefit from *Wolbachia*'s presence) and as obligate mutualisms (arising from evolved dependence), but usually not as ultimate mutualisms (there is no additional benefit) (see Table 1).

Just as with facultative benefits, some obligate mutualisms are associated with reproductive manipulation, while others are not. In addition, there are certain kinds of obligate mutualism that could only arise because of the reproductive phenotype. We present in detail a case study for each of the three scenarios. Here, a main focus will be on the question how these dependencies evolved, both in evolutionary and developmental terms. This allows us to highlight both commonalities and differences between different forms of dependence, but also between mutualistic and parasitic *Wolbachia* phenotypes.

(2) Dependence without a reproductive phenotype: the case of *Asobara tabida*

The first report of an arthropod species being completely dependent on its *Wolbachia* symbiont comes from the parasitoid wasp *Asobara tabida* (Fig. 1D). Here, females that are cured of their *Wolbachia* (i.e. aposymbiotic females) fail to produce mature oocytes (Dedeine *et al.*, 2001). This case has risen to prominence even beyond the *Wolbachia* community and is frequently used to illustrate the role of symbiosis in animal development (Gilbert *et al.*, 2010; McFall-Ngai *et al.*, 2013). *A. tabida* is infected with three distinct *Wolbachia* strains, only one of which is required for oogenesis, whereas the two other strains induce CI (Dedeine *et al.*, 2004). No other species of the genus *Asobara* depends on *Wolbachia* for oogenesis, suggesting that the dependence in *A. tabida* has evolved recently (Dedeine, Boulétreau & Vavre, 2005). Although the oogenesis defects in *A. tabida* resemble those in *Sxl* female-sterile mutants in *Drosophila melanogaster* (Starr & Cline, 2002; see Section VI.1), recent findings suggest that dependence in *A. tabida* did not evolve by *Wolbachia* simply taking over control of some host function. In the following, we summarize what we know about the mechanisms underlying this dependence, and how it might have evolved.

The failure of oogenesis in aposymbiotic females has been shown to be due to extensive apoptosis of nurse cells in mid-stage egg chambers (Pannebakker *et al.*, 2007). Apoptosis is an essential part of insect oogenesis. At the end of *Drosophila* oogenesis, developmental apoptosis of nurse cells occurs after their cytoplasmic contents have been transferred to the oocyte (a process called dumping). In addition, cell death may be triggered at distinct checkpoints during early and mid-oogenesis in response to adverse stimuli (McCall, 2004). The results of Pannebakker *et al.* (2007) suggest that *Wolbachia* is necessary for egg chambers to pass the mid-oogenesis checkpoint by preventing apoptosis. The authors outline a co-evolutionary scenario in which *A. tabida* responds to infection with apoptosis, which is then suppressed by *Wolbachia*, and the host in turn compensates for suppression by further increasing apoptosis because it is essential to complete oogenesis (Pannebakker *et al.*, 2007). This scenario is based on the pleiotropic role of programmed cell death in development and immunity (Vavre *et al.*, 2008), and there is good empirical support for it. Although evidence for apoptosis as a host defence against *Wolbachia* is rather scant, apoptotic cell death is a common immune response to viral infections among insects (Clarke & Clem, 2003). Moreover, autophagic cell death was recently shown to regulate *Wolbachia* populations in mosquito and *D. melanogaster* cell lines (Voronin *et al.*, 2012). Further support for the involvement of cell death pathways in the insect immune response comes from the fact that bacterial suppression of such pathways is widespread (reviewed in Faherty & Maurelli, 2008; Böhme & Rudel, 2009). Strikingly, a native *Wolbachia* strain in *Drosophila mauritiana* is able to significantly decrease apoptosis in the female ovary (Fast *et al.*, 2011). So far, there are two candidate genes whose expression might be manipulated by *Wolbachia* in order to decrease apoptosis. Both *chico* and *lola* are involved in the apoptotic pathway of the *Drosophila* mid-oogenesis checkpoint (McCall, 2004; Bass, Cullen & McCall, 2007). In a recent gene-expression analysis of *D. melanogaster* larval testes, expression of both genes was found to be altered in *Wolbachia*-infected flies compared to uninfected flies (Zheng *et al.*, 2011). Moreover, in *chico* mutant lines infected with *Wolbachia*, symbiont removal results in complete lethality of homozygous mutants, although this *Wolbachia* effect appears to be not directly linked to *chico* (Clark *et al.*, 2005). These results suggest that *Wolbachia* might interfere with apoptosis in the *Drosophil*a ovary by targeting *chico* and/or *lola*. Lastly, the *Wolbachia* surface protein (WSP) of nematode-associated *Wolbachia* inhibits apoptosis in human neutrophils (Bazzocchi *et al.*, 2007). Taken together, these findings indicate that *Wolbachia* could directly manipulate apoptotic pathways in *A. tabida* ovaries.

There is also evidence that *Wolbachia* can act indirectly on host apoptotic processes. Kremer *et al.* (2009*b*) showed that *Wolbachia* interferes with iron metabolism in *A. tabida*, particularly with the expression of ferritin, a protein involved in iron storage and oxidative stress regulation. The authors suggest that *Wolbachia*, which is known to induce oxidative stress in another host system (Brennan *et al.*, 2008), can thus disrupt cellular physiology, including apoptosis. Regardless of whether the effect is direct or indirect, *Wolbachia*-induced suppression of apoptosis in the ovaries should select for increased apoptotic signalling in the host to enable developmental apoptosis of nurse cells after dumping. Thus, the host should evolve some form of tolerance (a strategy to reduce fitness costs of infection) to compensate for the harmful effects of the symbiont. In the absence of *Wolbachia*, this compensatory evolution would result in excessive apoptosis and therefore inhibition of oogenesis,

rendering *A. tabida* completely dependent on its symbiont (Aanen & Hoekstra, 2007). Is there any molecular evidence for such compensatory evolution in *A. tabida*?

Interestingly, there is a high level of intraspecific variation in *A. tabida* regarding the degree to which wasps depend on *Wolbachia* for oogenesis. Whereas most aposymbiotic females are unable to produce mature oocytes, there are *A. tabida* lines in which cured females can produce some eggs; however, larvae hatched from these eggs die early during development (Dedeine *et al.*, 2005). Very few eggs laid by aposymbiotic females even develop to adulthood; however, lines derived from these individuals are unable to be maintained (Kremer *et al.*, 2010). Therefore, despite considerable variation in the phenotype of aposymbiotic females (also termed the 'ovarian phenotype'), in no case are viable offspring produced, implying that the ovarian phenotype cannot be subject to direct selection. In an elegant work, Kremer *et al.* (2010) offer a possible explanation for this puzzle. They propose that the ovarian phenotype could be indirectly selected if it is correlated with traits that are under direct selection. The authors then argue that host mutations that compensate for the anti-apoptotic effects of *Wolbachia* are likely to be selected for. Indeed, they could show that the ovarian phenotype is correlated with the expression of genes that are involved in iron metabolism and oxidative stress control, e.g. ferritin (Kremer *et al.*, 2010, 2012). Exactly those genes are manipulated by *Wolbachia* to interfere with host apoptosis and therefore are likely to be used by *A. tabida* to counteract the symbiont's harmful effects. Moreover, these differences in gene expression are also present in *Wolbachia*-infected females (which do produce viable offspring), making direct selection possible (Kremer *et al.*, 2010). Thus, these findings strongly suggest that complete dependence of *A. tabida* on its symbiont is the result of compensatory evolution in the host.

The ability of *Wolbachia* to interfere with host apoptosis and iron metabolism pathways is not restricted to the *A. tabida* mutualism, but has also been observed in other relationships, both mutualistic and parasitic. This fact allows us to compare the mechanisms that are used by *Wolbachia* to interact with the host in different symbiotic relationships. Kremer *et al.* (2009*b*) showed that *Wolbachia* affect iron metabolism not only in the obligate mutualism with *A. tabida*, but also in the facultative parasitism with *Drosophila simulans* (where *Wolbachia* induces CI) and in *Aedes aegypti* cells. In a facultative mutualism with *D. melanogaster*, *Wolbachia* has a positive fecundity effect in low- or high-iron environments, again suggesting that the symbiont is involved in iron homeostasis (Brownlie *et al.*, 2009). Furthermore, interference with both host iron metabolism and apoptosis has been suggested to be involved in the *Wolbachia*–nematode mutualism. The biosynthetic pathway of heme (which plays a central role in iron metabolism) is absent in the nematode *Brugia malayi* (Ghedin *et al.*, 2007), whereas its *Wolbachia* symbiont has all but one gene for heme biosynthesis, suggesting that worms depend on acquiring heme from their symbionts (Foster *et al.*, 2005). In addition, it has recently been shown that depletion of *Wolbachia* from *B. malayi* leads to extensive apoptosis in the adult germline, which offers another potential basis for this mutualistic symbiosis and, moreover, mirrors the situation in *A. tabida* (Landmann *et al.*, 2011). These results indicate that parasitic and mutualistic *Wolbachia* use the same molecular mechanisms to interact with their hosts.

Taken together, the dependence of *A. tabida* on *Wolbachia* nicely illustrates the role of tolerance or compensatory evolution in the transition from parasitism to mutualism (Aanen & Hoekstra, 2007; Edwards, 2009) (case 2b in Fig. 2). Moreover, although probably no ultimate mutualism, it serves as a prime example of evolved dependence and obligate mutualism (De Mazancourt *et al.*, 2005; Edwards, 2009).

(3) Dependence associated with a reproductive phenotype: *Ostrinia scapulalis* and other lepidopterans

Here we present examples of obligate mutualisms where dependence has evolved concomitantly with reproductive manipulation. In the adzuki bean borer, *Ostrinia scapulalis*, *Wolbachia*-infected females produce all-female broods, indicative of a sex-ratio-distorting phenotype. Such all-female broods were shown to be due to the death of genetic males, suggesting a male-killing effect of *Wolbachia*. Unexpectedly, however, cured females give rise to all-male progeny, which is due to the death of genetic females (Kageyama & Traut, 2004). This finding indicates that *O. scapulalis* has evolved some form of dependence on its *Wolbachia* symbionts as the latter appear to be required for female development. Moreover, the sex-specificity of death suggests that *Wolbachia* somehow interferes with the sex-determination system of its host. Indeed, a recent study shows that *Wolbachia*'s manipulation of *O. scapulalis* sex determination plays a crucial role in both the sex-ratio-distorting and the dependence phenotype.

In many insect species, it is the chromosomal constitution that serves to start the sex-determination pathway. As in most lepidopterans, *O. scapulalis* has a female heterogametic sex chromosome system, i.e. females are heterogametic (ZW), and males are homogametic (ZZ) (Kageyama & Traut, 2004). The gene *doublesex* (*dsx*) is the conserved master switch at the bottom of the insect sex-determination cascade. Due to sex-specific splicing, *dsx* exists as a male or a female isoform, which starts male- or female-specific development, respectively (Sánchez, 2008). Recently, a *dsx* homologue was identified in *O. scapulalis*, which was termed *Osdsx* (Sugimoto *et al.*, 2010). Interestingly, in *Wolbachia*-infected individuals, the female-type *Osdsx* is expressed irrespective of the genetic sex. By contrast, in individuals that have been cured of infection, the male-specific splice form is expressed irrespective of the genetic sex (Sugimoto & Ishikawa, 2012). Death occurs if there is a mismatch between genetic sex (ZW or ZZ) and phenotypic sex (male- or female-specific *Osdsx*).

The expression of the female-specific *Osdsx* in genetic males demonstrates that *Wolbachia* has a feminizing effect in *O. scapulalis*, suggesting that male-killing occurs through lethal feminization. The feminizing factor is assumed to

interfere with some step in the sex-determination cascade upstream of, or directly at the splicing of, *Osdsx*. Moreover, the fact that genetic females develop a male phenotype in the absence of *Wolbachia* suggests that a component in the female-determining cascade of *O. scapulalis* is degraded which is functionally substituted by *Wolbachia* (Sugimoto & Ishikawa, 2012). This component might be a homologue of the *Bombyx mori* female-determining factor which is located on the W chromosome and serves as the primary signal in the sex-determination pathway (Fujii & Shimada, 2007). Taken together, the findings of Sugimoto & Ishikawa (2012) suggest that *Wolbachia*'s feminizing factor performs the function of the degraded component in the female-determining cascade of *O. scapulalis* (case 2a in Fig. 2). Their study thus provides novel insights into microbial manipulation of host sex determination (Beukeboom, 2012). Note that, in this case, bacterial disturbance of host sex determination is facilitated by female heterogamety: since the female-specific W chromosome is co-inherited with *Wolbachia*, W-specific functions can be lost and substituted by the symbiont. This is not possible in hosts with male heterogamety.

The pattern of opposite sex-specific effects of *Wolbachia* (male-killing when present, female-killing when removed) was also observed in the closely related species *Ostrinia furnacalis* (Sakamoto *et al.*, 2007). Therefore, this species is likely to have evolved essentially the same dependence on *Wolbachia* as *O. scapulalis*. Although the underlying mechanism has not yet been analysed in *O. furnacalis*, it seems likely that, given the close relatedness of both species, *Wolbachia* uses the same process to interfere with host sex determination.

In the butterfly *Eurema hecabe*, *Wolbachia* also has a feminizing effect which, in contrast to *Ostrinia*, results in viable feminized individuals (Hiroki *et al.*, 2002). One important evolutionary outcome of feminization is the elimination of the female sex determinant, i.e. the W chromosome in species with female heterogamety (because feminized XX individuals produce females without any W chromosome; Rigaud, 1997). Accordingly, adult females treated with antibiotics give rise to all-male broods (Hiroki *et al.*, 2002). Interestingly, however, antibiotic treatment at larval stages leads to intersexual phenotypes and high pupal mortality, particularly when treatment began at early larval stages (Narita *et al.*, 2007; Fig. 1E). One possible explanation for these findings is that feminizing *Wolbachia* are required for proper larval development. If this conclusion is correct, it demonstrates that evolved dependencies on *Wolbachia* can arise not only in the context of early developmental processes such as oogenesis or sex determination, but also during later stages of host development. However, another possible explanation is that sexually intermediate individuals die just because of intersexual defects (Narita *et al.*, 2007).

(4) Dependence through a reproductive phenotype: parthenogenesis-inducing *Wolbachia* and their hosts

Many insects, including wasps, bees, ants, and thrips, exhibit haplodiploid sex determination: unfertilized (haploid) eggs develop into males, while fertilized (diploid) eggs develop into females. In many haplodiploid species, *Wolbachia* are able to induce thelytokous parthenogenesis, i.e. the diploidization of unfertilized haploid eggs which thus develop into diploid females (Huigens & Stouthamer, 2003). Parthenogenesis-inducing (PI) *Wolbachia* benefit from this reproductive manipulation because it enhances the proportion of transmitting individuals (females). Furthermore, PI might even lead to the complete elimination of males in populations where infection has gone to fixation. These populations consist entirely of infected females which reproduce parthenogenetically; sexual reproduction is no longer present. The loss of sexual functionality makes *Wolbachia* an obligate mutualist for daughter production in all-female populations. Such obligate mutualism between arthropod hosts and their PI-*Wolbachia* has evolved in numerous haplodiploid species, mainly hymenopterans, but also in the mite species *Bryobia praetiosa* and in the thysanopteran *Franklinothrips vespiformis* (see Table 6; Fig. 1F). In what follows, we summarize evidence of how this dependence could evolve.

Table 6. Loss of sexual function due to infection with PI-*Wolbachia*

Host species	References
ACARI	
Bryobia praetiosa	Weeks & Breeuwer (2001)
INSECTA	
Hymenoptera	
Aphytis diaspidis	Zchori-Fein *et al.* (1995)
Aphytis lingnanensis	Zchori-Fein *et al.* (1995)
Apoanagyrus diversicornis	Pijls *et al.* (1996)
Asobara japonica	Kremer *et al.* (2009*a*)
Encarsia formosa	Zchori-Fein *et al.* (1992)
Eretmocerus mundus	De Barro & Hart (2001)
Gronotoma micromorpha	Arakaki *et al.* (2001*a*)
Leptopilina clavipes	Pannebakker *et al.* (2005)
Muscidifurax uniraptor	Gottlieb & Zchori-Fein (2001)
Telenomus nawai	Arakaki *et al.* (2000) and Jeong & Stouthamer (2005)
Trichogramma cordubensis	Silva & Stouthamer (1997)
Trichogramma pretiosum	Russell & Stouthamer (2011)
Thysanoptera	
Franklinothrips vespiformis	Arakaki *et al.* (2001*b*)

Interestingly, the lack of sex in fixed populations is due to a complete loss of sexual function in females, but not in males. This was shown in host species in which both fixed (asexual) and uninfected (sexual) populations exist. Males can be derived from fixed populations by antibiotic treatment. When such males are mated with females from sexual populations, the latter successfully fertilize the eggs. However, when females from fixed populations are exposed to males, they do not fertilize their eggs (Pijls, van Steenbergen & van Alphen, 1996; Kremer *et al.*, 2009*a*; Russell & Stouthamer, 2011). In some cases, the morphological or physiological aberrations underlying the failure of fertilization are known: in *Muscidifurax uniraptor*,

females lack a spermathecal muscle, and in *Trichogramma cordubensis*, females are not attractive to conspecific males, possibly due to lacking pheromone production (Gottlieb & Zchori-Fein, 2001; Silva & Stouthamer, 1997).

Several hypotheses have been proposed to explain the female-specific decay of sexual function in fixed populations and the concomitant evolution of dependence on PI-*Wolbachia*. The 'costly female trait' hypothesis says that in the absence of sex, costly female traits involved in sexual reproduction, e.g. pheromone production, will be selected against (Pijls *et al.*, 1996). The 'functional virginity' hypothesis proposes that the female-biased sex ratio in populations with a spreading PI-*Wolbachia* infection selects for mutations that increase the production of males in order to restore the optimal sex ratio. In haplodiploid species, this is achieved by lowering the fertilization rate. Thus, any mutation occurring in females that reduces the fertilization frequency will be selected, including even 'virginity' alleles which disable any trait required for successful sexual reproduction (Huigens & Stouthamer, 2003; Jeong & Stouthamer, 2005). Recent modelling favours the latter hypothesis: Stouthamer *et al.* (2010) showed that selection for lower fertilization rates ultimately results in the population becoming fixed for both the PI-*Wolbachia* infection and the virginity alleles. Once infection is fixed, mutations interfering with costly female traits will spread (Pijls *et al.*, 1996), and other genes involved in reproduction will accumulate mutations both in males and females (Carson, Chang & Lyttle, 1982). Thus, the nucleo-cytoplasmic conflict over sex ratio is eventually resolved by an irreversible loss of sexual reproduction (Stouthamer *et al.*, 2010). In line with theory, putative 'functional virginity' loci responsible for the loss of female sexual function have been identified in *Telenomus nawai* and *Trichogramma pretiosum* (Jeong & Stouthamer, 2005; Russell & Stouthamer, 2011). These findings show that selection can promote the evolutionary transition to obligate asexuality, associated with complete dependence on *Wolbachia* (King & Hurst, 2010).

The evolution of obligate mutualism involving PI-*Wolbachia* demonstrates another example of how dependence can result from compensatory evolution (tolerance) in the host (case 2b in Fig. 2), which, in this case, involves decreasing the fertilization rate to counteract the female-biased sex ratio. Exactly this host compensatory mechanism has also evolved in the haplodiploid mite *Tetranychus urticae*. Although not due to parthenogenesis induction, *Wolbachia*-infected *T. urticae* females produce more female-biased sex ratios than cured females. Interestingly, it is the sex ratio produced by infected females that best approaches the optimal sex ratio (which, due to local mate competition, is female-biased in *T. urticae*) (Vala *et al.*, 2003). Why is the sex ratio produced by cured females less than optimal? Vala *et al.* (2003) propose that, in response to the *Wolbachia*-induced shift in sex ratio (which initially was too female-biased), *T. urticae* decreased the fertilization rate in order to restore the optimal sex ratio. However, this compensatory mechanism is costly in the absence of *Wolbachia* because then the sex ratio is too male-biased. Again, obligate mutualism is a likely outcome of such compensatory evolution.

If the functional virginity hypothesis is correct, it has some interesting implications. Firstly, the theoretical finding by Stouthamer *et al.* (2010) that any allele that lowers the fertilization rate will become fixed nicely corroborates the prediction that any tolerance gene should be driven to fixation by natural selection (Roy & Kirchner, 2000). Secondly, it is only selection on the host that eventually leads to the evolution of dependence on PI-*Wolbachia*. This stands in contrast to other cases of evolved dependence in which selection on *Wolbachia* causes, or at least contributes to, the dependence phenotype (in addition to selection that gave rise to the reproductive phenotype itself). This issue can be exemplified by two closely related wasp species that both depend on *Wolbachia* for reproduction, but for completely different reasons. In *Asobara tabida*, dependence on *Wolbachia* for oogenesis could emerge ultimately because the symbiont evolved the ability to interfere with host apoptotic processes (see Section VI.2). In *A. japonica*, by contrast, infection with PI-*Wolbachia* has selected for lower fertilization rates and, eventually, led to the decay of sexual function in females and thus dependence (Kremer *et al.*, 2009*a*). Hence, although compensatory mechanisms underlie the dependence in both *Asobara* species, the evolutionary trajectories leading there are distinct.

(5) Resistance, tolerance and dependence

Resistance and tolerance are two distinct host strategies to cope with infection. Whereas resistance aims at limiting the infection, tolerance does not reduce the infection itself, but limits its fitness consequences (Roy & Kirchner, 2000). This review shows that tolerance to *Wolbachia* has evolved repeatedly in arthropod hosts. By contrast, resistance alleles have rarely been found in host species, although there is strong selection to counteract *Wolbachia*'s reproductive parasitism (Charlat *et al.*, 2007; Koehncke *et al.*, 2009). Why might host resistance to *Wolbachia* be rare? A possible reason is that, once resistance has led to the loss of infection, costly but redundant resistance alleles are likely to be lost as well. While this conjecture does not rule out that resistance itself evolves frequently (transition 8 in Fig. 2), there might be circumstances in which resistance is not the best strategy of responding to *Wolbachia* infection. Here we show two barriers to the evolution of host resistance, both of which are associated with *Wolbachia* mutualisms. Obviously, resistance should not evolve if *Wolbachia* confers a net fitness benefit to the host ('fitness benefit' barrier to resistance). A second barrier to resistance is closely linked to the evolution of tolerance in response to *Wolbachia* infection. This can be illustrated by any host compensatory mechanism, e.g. the decrease in fertilization rate to counteract the symbiont-induced female-biased sex ratio (see Section VI.4). Such compensatory mechanisms leading to host tolerance are costly in the absence of infection. If these costs are too high, there will be selection on females to foster vertical transmission of their symbiont (Law & Dieckmann, 1998).

Thus, selection for tolerance favours the evolution of dependence (obligate mutualism) (Roy & Kirchner, 2000; Aanen & Hoekstra, 2007; Edwards, 2009; see Sections VI.2 and VI.4). Once the host depends on *Wolbachia*, resistance is no longer an option ('dependence' barrier to resistance). On these grounds, both beneficial effects of, and tolerance to, *Wolbachia* can hinder the evolution of host resistance.

VII. ANTIBIOTIC TREATMENT AND *WOLBACHIA* EFFECTS: A CRITICAL NOTE

Most experimental approaches to identify mutualisms have investigated the performance of a given host in the presence and absence of its symbiont (Douglas & Smith, 1989). In the case of *Wolbachia*, infected hosts are cured of the infection by antibiotic treatment to compare the performance of cured individuals with that of their untreated counterparts. Usually, the broad-spectrum antibiotic tetracycline is used (Li *et al.*, 2014). It is implicitly assumed that tetracycline treatment has no other effect than removing *Wolbachia*. However, this is not always the case. Other symbionts (e.g. gut bacteria) are likely to be removed as well. Therefore, effects attributed to *Wolbachia* might in fact be caused by other bacteria. This can be exemplified by the role of symbionts in reproductive isolation in *Drosophila melanogaster*. Koukou *et al.* (2006) eliminated *Wolbachia* from *D. melanogaster* cage populations by tetracycline treatment and found that the preexisting sexual isolation between populations was reduced by about 50%. However, this effect could be due to any tetracycline-sensitive bacteria of the *D. melanogaster* microbiota. Indeed, recent results suggest that, rather than *Wolbachia*, *Lactobacillus* bacteria are responsible for the mating preference in *D. melanogaster* (Sharon *et al.* 2010).

Another largely disregarded effect of tetracycline concerns mitochondrial metabolism and mitochondrial DNA (mtDNA) density. Tetracycline works by blocking the 30S subunit of prokaryotic ribosomes, thus inhibiting translation and protein synthesis. Descending from bacterial ancestors, mitochondria have bacteria-type ribosomes, and thus tetracycline also inhibits mitochondrial protein synthesis (Zhang *et al.*, 2005). Indeed, in *Drosophila simulans*, tetracycline treatment reduces mitochondrial efficiency and probably leads to decreased ATP production. This could have a direct influence on fecundity or longevity, which may easily be confused with a *Wolbachia* effect (Ballard & Melvin, 2007). Moreover, tetracycline treatment causes an increase in mtDNA copy number in *Wolbachia*-uninfected fly lines, which is probably a consequence of tetracycline-induced inhibition of mtDNA translation. In infected flies, by contrast, tetracycline has no effect on mtDNA copy number because the presence of *Wolbachia* dilutes the concentration of the antibiotic in the mitochondria (Ballard & Melvin 2007). This differential effect of tetracycline on infected and uninfected flies might impair experimental controls in the laboratory. In the field, on the other hand, the antibiotic-diluting effect of *Wolbachia* will be beneficial only if the host is exposed to antibiotics in its environment, and if this antibiotic is somehow detrimental to host fitness. Finally, the effects of tetracycline on mitochondria in *D. simulans* were observed two generations after treatment (see also Zeh *et al.*, 2012). In light of these findings, it is essential that researchers carefully control for antibiotic effects other than *Wolbachia* removal. Otherwise, *Wolbachia* might be held responsible for effects that either are caused by other symbionts or actually do not exist in the field.

VIII. THE EVOLUTIONARY FATE OF *WOLBACHIA*–ARTHROPOD MUTUALISMS

What will happen to *Wolbachia* that have evolved a mutualistic association with their arthropod hosts? The question of whether mutualistic relationships are evolutionarily stable or whether transitions between mutualism and parasitism occur is an ongoing debate in evolutionary biology (Moran & Wernegreen, 2000; Sachs & Simms, 2006; Sachs *et al.*, 2011*b*). In the following, we briefly present possible evolutionary outcomes of mutualism in *Wolbachia*–arthropod associations (Fig. 2).

Facultative mutualisms that are based on environment-dependent fitness benefits might easily break down if the environment changes so that the cost–benefit ratio (i.e. the net effect on host fitness) becomes unfavourable. In the case of 'Jekyll and Hyde' infections, this would leave *Wolbachia* as pure reproductive parasites (transition 3 in Fig. 2). Moreover, such transitions from mutualism to reproductive parasitism probably are particularly relevant in the context of temporary benefits which help *Wolbachia* to invade a population. After such benefits have helped to overcome the invasion threshold, they might attenuate over time so that *Wolbachia* would have to rely on a reproductive manipulation to be maintained. Alternatively, facultative mutualisms might break down in 'stand-alone benefit' infections. In this case, *Wolbachia* would be prone to extinction in the absence of any mechanism to maintain them in the population (transition 4 in Fig. 2). In sum, facultative *Wolbachia* mutualisms seem to be relatively unstable and, owing to shifts in the cost–benefit ratio, might easily switch to parasitism. Given that there is also good evidence for the reverse switch (from parasitism to mutualism, which is the subject matter of this review), these findings together indicate that the two forms of symbiosis are often dynamic in *Wolbachia*–arthropod associations.

Facultative mutualisms might become obligate if dependence is evolving in the context of a developmental or reproductive pathway that is already manipulated by *Wolbachia* to provide the facultative benefit (transition 5 in Fig. 2). For example, *Wolbachia*'s ability to interfere with iron metabolism is presumably used both in the facultative mutualism with *Drosophila melanogaster* and in the obligate mutualism with *Asobara tabida* (Brownlie *et al.*, 2009; Kremer *et al.*, 2009*b*; see Section VI.2). Under such circumstances, one could imagine a scenario in which the facultative benefit comes about by the provisioning of an additional amount

of some factor. If the host ceased to produce this factor on its own, this would turn the facultative mutualism into an obligate symbiosis. It is unclear, however, how likely such shifts are in nature.

Lastly, facultative or obligate mutualisms might evolve into stable ultimate mutualisms (transition 6 in Fig. 2). In particular, evolved dependence is considered a possible precursor to ultimate mutualism because it couples the evolutionary fates of host and symbiont. Subsequent selection could then fine-tune the interaction and act on *Wolbachia* to confer some 'extra' benefit (Aanen & Hoekstra, 2007). Furthermore, mutual dependence should lead to co-speciation between host and symbiont. However, although host dependence on *Wolbachia* has evolved frequently (see Section VI), co-speciation between *Wolbachia* and arthropod hosts has never been found among mutualistic strains (and only rarely among parasitic strains; Raychoudhury *et al.*, 2009). This might suggest that obligate mutualisms between *Wolbachia* and arthropods are not stable on an evolutionary timescale or at least too short-lived to evolve into ultimate mutualisms. Obligate mutualisms might become unstable if the host is able to overcome the dependence (transition 7 in Fig. 2); in addition, these relationships could in general be more prone to extinction (Kremer *et al.*, 2009*a*). On the whole, the fact that mutualisms between *Wolbachia* and arthropod hosts appear to be quite dynamic, even on ecological timescales, makes it hard to predict the evolutionary fate of such associations.

IX. FUTURE DIRECTIONS

The study of *Wolbachia* mutualisms in arthropods is a young field of research, and several issues await further investigation. Here, we point to some promising avenues for future research.

(1) How are *Wolbachia*-induced mutualisms achieved mechanistically?

It will be of great importance to elucidate in more detail the mechanisms that underlie mutualistic effects. So far, some insights have been gained regarding obligate mutualisms (e.g. *Wolbachia*'s role in progressing *A. tabida* egg chambers past the mid-oogenesis checkpoint by preventing apoptosis of nurse cells). Still, the mechanisms underlying other cases of evolved dependence remain unclear. Furthermore, the molecular nature of most mutualisms is unknown, particularly that of facultative benefits. In light of common mechanisms involved in mutualistic and parasitic phenotypes, unravelling the mechanistic basis of *Wolbachia* mutualisms might also help to understand better how these symbionts manipulate host reproduction.

(2) Is host protection only a temporary phenomenon?

Wolbachia frequently triggers immune responses in newly infected hosts, but does so rarely in hosts adapted to infection. This suggests that protection associated with immune upregulation might only be a transient effect. On the other hand, *Wolbachia*-induced protection is not necessarily associated with immune activation. It is therefore crucial to elucidate the mechanism(s) underlying anti-pathogenic effects of natural *Wolbachia* infections, particularly the exact role of the host immune system (Cook & McGraw, 2010; Eleftherianos *et al.*, 2013). A better understanding of the physiological causes of anti-pathogenic effects will help to characterize such effects as largely ephemeral or as effective benefits of *Wolbachia* infection.

(3) How stable are mutualistic interactions between *Wolbachia* and arthropods?

Not only protective effects, but *Wolbachia*–arthropod mutualisms in general should be tested for their stability. This issue revolves around the question of how likely transitions between different forms of symbiosis are (see Fig. 2). In other words, how frequently do mutualisms arise, and how fast are they lost? Our understanding of this matter with respect to *Wolbachia*–arthropod relationships is still very limited. In contrast to the situation in *Wolbachia*-infected nematodes, co-speciation between mutualistic *Wolbachia* and arthropods has not yet been found, indicating that these mutualisms are relatively short-lived and might easily break down in evolutionary (or even in ecological) time. On the other hand, mutualisms might just be difficult to detect at all. These questions require further investigation.

(4) Can we identify ultimate benefits provided by *Wolbachia*?

The search for *Wolbachia*-induced ultimate mutualisms is still in its infancy. Ultimate mutualisms relate to interactions in which a partner could never have performed as well without the other (as opposed to evolved dependencies). In order to detect ultimate benefits, it is necessary to compare the performance of two different host genotypes, one being infected with and adapted to *Wolbachia* and the other one being uninfected and adapted to the symbiont's absence (De Mazancourt *et al.*, 2005). Future studies should try to apply this method to identify ultimate benefits (although they are difficult to measure).

(5) Are insects more prone to *Wolbachia* mutualisms than other arthropods?

It is striking that almost all cases of mutualistic *Wolbachia*–arthropod relationships have been found among insect species. This may be because non-insect arthropods have only rarely been tested for *Wolbachia* mutualisms, or because mutualisms have evolved less frequently in these host species. To discern between these possibilities, future work should intensify the search for mutualistic *Wolbachia* effects in non-insect host species such as spiders, isopods, and mites.

(6) Did *Wolbachia* mutualisms foster the evolution of haplodiploidy?

Although not discussed in this review, *Wolbachia* mutualisms could also be relevant to the question of whether male-killing endosymbionts possibly play a role in the evolution of haplodiploidy in their hosts. Recent theory suggests that slight benefits accruing from infection facilitate the evolution of haplodiploidy, whereas earlier models that do not consider possible mutualisms often fail to explain the evolution of haplodiploidy by endosymbionts (Kuijper & Pen, 2010). Researchers could look for empirical support for these theoretical findings.

X. CONCLUSIONS

(1) *Wolbachia* are the most widespread endosymbiotic bacteria among arthropods where they are notorious for their reproductive parasitism. Nevertheless, these symbionts also have the potential to engage in mutualistic relationships with their hosts. As mutualists, *Wolbachia* either provide facultative fitness benefits or are required for host survival or reproduction (obligate mutualism).

(2) Not only can *Wolbachia* be mutualistic, they also frequently act as a mutualist and as a reproductive parasite at the same time ('Jekyll and Hyde' type of infection). Moreover, they can induce both mutualism and reproductive parasitism by interfering with the same host process (e.g. iron metabolism). These findings argue against a clear-cut distinction between parasitic and mutualistic *Wolbachia* and imply that transitions between both forms of symbiosis might occur relatively easily.

(3) Facultative mutualisms arise through selection on maternally transmitted *Wolbachia* to enhance the fitness of their female hosts. Such fitness benefits have been found in different arthropod species and include increases in fecundity and longevity, nutritional provisioning, and protection against pathogens. Obligate mutualisms arise through the evolution of dependence, either *via* compensatory evolution in the host (tolerance) or *via* the takeover of some host function by *Wolbachia*. Tolerance has evolved frequently in arthropod hosts as a means to cope with the harmful effects of *Wolbachia* infection. Since tolerance strategies tend to render hosts dependent on *Wolbachia*, they are a potential barrier to the evolution of host resistance (as are direct fitness benefits, too).

(4) In contrast to the abundance of experimental studies that found *Wolbachia*-induced pathogen interference (anti-pathogenic effects), there is only limited support for a fitness-enhancing effect of such interference in natural interactions (i.e. for host protection). Many studies observed anti-pathogenic effects after *Wolbachia* had been artificially introduced into naturally uninfected insects. While these findings may offer great potential for disease control strategies, they say little about natural *Wolbachia*-host interactions. Moreover, there is evidence that protection that is based on a host immune response might only be a temporary phenomenon.

(5) Once *Wolbachia* are established in a host population, providing a fitness benefit is not necessary for them to be maintained, because reproductive parasitism is sufficient for this purpose. However, reproductive parasitism alone is often insufficient for *Wolbachia* to invade a population (for example because the bacteria fail to overcome the invasion threshold). By contrast, facultative mutualisms enable *Wolbachia* to establish and spread from low initial frequencies and therefore facilitate the invasion into novel hosts (this holds even if beneficial effects are only temporary).

(6) Both facultative and obligate *Wolbachia* mutualisms have further important consequences for the ecology, evolution, and development of their arthropod hosts. Effects can be as diverse as the requirement of bacterial signalling for oogenesis, manipulation of host plant physiology (e.g. induction of green islands on yellow leaves), or irreversible loss of sexual reproduction. The mechanisms that underlie *Wolbachia* mutualisms are likewise diverse (as far as they are known), including alterations in gene expression and interference with crucial host processes such as apoptosis and sex determination.

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