A peer-reviewed version of this preprint was published in PeerJ on 8 May 2018.

View the peer-reviewed version (peerj.com/articles/4629), which is the preferred citable publication unless you specifically need to cite this preprint.

Duplouy A, Hornett EA. 2018. Uncovering the hidden players in Lepidoptera biology: the heritable microbial endosymbionts. PeerJ 6:e4629
https://doi.org/10.7717/peerj.4629
Uncovering the hidden players in Lepidoptera biology: the heritable microbial endosymbionts

Anne Duplouy ¹, Emily A Hornett Corresp. ²

¹ University of Helsinki, Helsinki, Finland
² Department of Zoology, University of Cambridge, Cambridge, United Kingdom

Corresponding Author: Emily A Hornett
Email address: eh481@cam.ac.uk

The Lepidoptera is one of the most widespread and recognisable insect orders. Due to their remarkable diversity, economic and ecological importance, moths and butterflies have been studied extensively over the last 200 years. More recently, the relationship between Lepidoptera and their heritable microbial endosymbionts has received increasing attention. Heritable endosymbionts reside within the host’s body and are often, but not exclusively, inherited through the female line. Advancements in molecular genetics have revealed that host-associated microbes are both extremely prevalent among arthropods and highly diverse. Furthermore, heritable endosymbionts have been repeatedly demonstrated to play an integral role in many aspects of host biology, particularly host reproduction. Here, we review the major findings of research of heritable microbial endosymbionts of butterflies and moths. We promote the Lepidoptera as important models in the study of reproductive manipulations employed by heritable endosymbionts, with the mechanisms underlying male-killing and feminisation currently being elucidated in both moths and butterflies. We also reveal that the vast majority of research undertaken of Lepidopteran endosymbionts concerns Wolbachia. While this highly prevalent bacteria is undoubtedly important, studies should move towards investigating the presence of other, and interacting endosymbionts, and we discuss the merits of examining the microbiome of Lepidoptera to this end. We finally consider the importance of understanding the influence of endosymbionts under global environmental change and when planning conservation management of endangered Lepidoptera species.
Uncovering the hidden players in Lepidoptera biology: the heritable microbial endosymbionts

Anne Duplouy\textsuperscript{1} & Emily A. Hornett\textsuperscript{2}

\textsuperscript{1} The University of Helsinki, PO Box 65 (Viiikinkaari 1), FI-00014 University of Helsinki, Finland.

\textsuperscript{2} The University of Cambridge, Department of Zoology, Cambridge, CB2 3EJ, United Kingdom

Corresponding author: Emily Hornett\textsuperscript{2}

Email address: eh481@cam.ac.uk
Abstract

The Lepidoptera is one of the most widespread and recognisable insect orders. Due to their remarkable diversity, economic and ecological importance, moths and butterflies have been studied extensively over the last 200 years. More recently, the relationship between Lepidoptera and their heritable microbial endosymbionts has received increasing attention. Heritable endosymbionts reside within the host’s body and are often, but not exclusively, inherited through the female line. Advancements in molecular genetics have revealed that host-associated microbes are both extremely prevalent among arthropods and highly diverse. Furthermore, heritable endosymbionts have been repeatedly demonstrated to play an integral role in many aspects of host biology, particularly host reproduction. Here, we review the major findings of research of heritable microbial endosymbionts of butterflies and moths. We promote the Lepidoptera as important models in the study of reproductive manipulations employed by heritable endosymbionts, with the mechanisms underlying male-killing and feminisation currently being elucidated in both moths and butterflies. We also reveal that the vast majority of research undertaken of Lepidopteran endosymbionts concerns *Wolbachia*. While this highly prevalent bacteria is undoubtedly important, studies should move towards investigating the presence of other, and interacting endosymbionts, and we discuss the merits of examining the microbiome of Lepidoptera to this end. We finally consider the importance of understanding the influence of endosymbionts under global environmental change and when planning conservation management of endangered Lepidoptera species.
Introduction

Symbiosis was originally described as the living together of dissimilar organisms in an intimate association (de Bary, 1879). This broad term is commonly used to encompass relationships between two or more organisms that range from parasitic, through commensal (one party gains a benefit, whilst the other is not significantly affected) to mutualistic (both parties benefit). We now know that the nature of an association is often much more complex, and varies greatly depending on factors such as the local environment, the host genetic background or condition, and the longevity of the relationship. Thus it is perhaps now more pertinent to understand symbiosis as an interaction in which two or more organisms of different species are in a persistent relationship, with no pre-conceived idea of the nature of the interaction.

One of the most intimate associations between species is that between a host organism and a microbial endosymbiont (a symbiont living within the body of its host). This lifestyle substantially affects the relationship between the two parties as survival and reproduction of host and microbe are intrinsically linked. Where the endosymbiont is intracellular - residing within the cytoplasm of host cells - it is predominantly inherited through the female line (although intrasperm paternal transmission has also been described (Watanabe, Yukuhiro & Matsuura, 2014)). Such maternal inheritance produces selection upon the symbiont to favour the cytoplasmic lineage of the host (in essence the females) - a phenomenon that has resulted in the evolution of remarkable manipulations of host reproductive biology, including sex ratio distortion (O’Neill, Hoffmann & Werren, 1998; Bandi et al., 2001; Engelstädt & Hurst, 2009). With increasing pace, evidence is gathering that diverse endosymbionts interact with many aspects of arthropod host biology including host reproduction (Werren, Zhang & Guo, 2004), development (Fraune & Bosch,
immunity (Gross et al., 2009; Nyholm & Graf, 2012), behaviour (Dion et al., 2011), body colour (Tsuchida et al., 2010), nutritional stress resistance (Brownlie et al., 2009), pathogen load (Graham & Wilson, 2012), dispersal (Goodacre et al., 2009), host plant specialisation (Leonardo & Muiru, 2003), thermal tolerance (Dunbar et al., 2007), nutrition (Douglas, 1998) and metabolism (McCutcheon, McDonald & Moran, 2009). Furthermore, symbiosis has been purported to be a key factor underlying natural variation, as well as an instigator of novelty and a promoter of speciation (Margulis & Fester, 1991; Brucker & Bordenstein, 2012).

Since the advent of the diagnostic PCR assay in the mid-1980s, organisms can be routinely screened for known endosymbionts. As a consequence of this development and recent advancements in genomics and bioinformatics (including high-throughput amplicon sequencing of microbial genes and metagenomics), we now recognise that all organisms are infected by a diverse range of microbes, including viruses, fungi and bacteria, and that many arthropods carry heritable endosymbionts. A recent study estimated that 52% of terrestrial arthropod species are infected with the intracellular bacteria *Wolbachia*, with a further 24% and 13% species infected with *Cardinium* and *Rickettsia* bacteria, respectively (Weinert et al., 2015). How species initially acquire heritable endosymbionts is not yet fully understood. While phylogenetic evidence suggests that horizontal transfer of endosymbionts on an evolutionary scale must be common, many barriers - ecological, geographical and physiological - exist that perturb the spread of endosymbionts between species and prevent the formation of novel symbioses. Successful transfer of an endosymbiont between species depends on the ability of the microbe to first enter and then survive in a novel host environment, followed by successful migration to the host germline to ensure propagation. The symbiont must then be able to invade the host population, or at least be maintained at low frequency. Thus the ‘fit’ between a host and symbiont can be quite
specific, with host biology playing an important role in the ability of the symbiont to thrive in the novel species. Failure in the formation of persistent associations may also be due to the endosymbiont causing harm to their new hosts (Hutchence et al., 2011). Where movement of heritable endosymbionts has been observed, it is often via ecological connectors such as shared host food sources (Huigens et al., 2000; Duron, Wilkes & Hurst, 2010; Caspi-Fluger et al., 2012; Chrostek et al., 2017) or common symbiont-vector parasites or parasitoids (Heath et al., 1999; Vavre et al., 1999; Huigens et al., 2004; Jaenike et al., 2007; Gehrer & Vorburger, 2012).

Horizontal transfer is perhaps more successful between related hosts (Russell et al., 2009); it has been suggested that within Acraea butterflies, Wolbachia has moved between species either via a common parasitoid, or through hybridisation and subsequent introgression. It is also possible that the different species inherited the bacteria from a recent common ancestor (Jiggins et al., 2000b).

The Lepidoptera are remarkably diverse and widely recognisable, encompassing butterflies and moths that are economically and ecologically important. While many aspects of Lepidopteran biology have been well studied, it is only recently that the pervasiveness of host-associated microbes in this group has been appreciated. Heritable endosymbionts have been the subject of several reviews (Bandi et al., 2001; Moran, McCutcheon & Nakabachi, 2008; Duron & Hurst, 2013), and here we focus upon studies of these influential elements in the Lepidoptera. Butterflies and moths are particularly important in the study of heritable endosymbionts due to the Lepidoptera sex determination system. In contrast to most other arthropod groups, the female is the heterogametic sex (females have one Z and one W sex chromosome, males have two Z chromosomes). The mechanisms and repercussions of reproductive manipulations caused by inherited microbial endosymbionts, which are commonly observed in butterflies and moths, are therefore likely to be very different from that observed in arthropods with alternative sex
determination systems. Furthermore, in the Lepidoptera heritable endosymbiont prevalence is
commonly very high, and vertical transmission of the infection is often near perfect. Together
with the maternal inheritance of intracellular endosymbionts such as *Wolbachia*, this creates
linkage of the infection not only with similarly maternally inherited host mitochondria, but also
with the female W chromosome. Formation of this wider co-inherited network may have
implications for host genetic diversity and even the sex determination system itself.

In this review we summarise the main body of research that has been conducted to date in
order to form a springboard for future work and to emphasise to researchers from traditionally
disparate fields as ecology, genomics and conservation, that in order to fully understand the
biology of an organism, one must take into account its endosymbionts. For clarity this review is
divided into areas of current research: 1) Manipulation of host reproduction; 2) Impact upon host
fitness; 3) Symbiont-mediated protection; 4) Host genetics and 5) Behavioural modification. We
then highlight outstanding questions and future directions, including consideration of the
influence of endosymbionts under global environmental change, and in species of conservation
concern.

Survey methodology
The authors have drawn upon knowledge gained from over a decade in butterfly-endosymbiont
research. Extensive literature searches were performed using repositories such as NCBI PubMed
and Google Scholar, and using keywords including “endosymbiont”, “microbe” and “heritable
symbiont”, along with “butterfly”, “moth” and “Lepidoptera”. Social media platforms such as
Twitter provided a useful tool to obtain up to date information of relevant publications. Research
on heritable endosymbionts of arthropods in general was also gathered with the aim to provide
information about areas in heritable endosymbiont-arthropod research that is lacking for Lepidoptera. Particular effort was made to compile a comprehensive list of butterfly and moth species that are published as infected with heritable endosymbionts.

The influence of heritable microbial endosymbionts on Lepidopteran biology

Concordant with general insect surveys, the Lepidoptera are commonly infected with heritable microbial endosymbionts. In an early screen of Panamanian arthropods, Wolbachia was detected in 16.3% of the 43 Lepidoptera species tested (Werren, Windsor & Guo, 1995). Further surveys identified Wolbachia in 29% of 24 species of Acraea butterflies from Uganda (Jiggins et al., 2001), 45% of 49 species of butterflies studied in Japan (Tagami & Miura, 2004), 50% of 56 Indian butterfly species (Salunke et al., 2012), 58.3% of 120 Lepidoptera species in West Siberia (Ilinsky & Kosterin, 2017), and 79% of 24 species of African Bicyclus butterflies (Duplouy & Brattström, 2017). Additionally, in a broad survey of ants, moths and butterflies (specifically Lycaenidae and Nymphalidae) for five heritable symbionts, Wolbachia (39 of 158 species) and Spiroplasma (5 of 200 species) were found to infect Lepidopteran species (Russell et al., 2012).

In general, these estimates are likely to be highly conservative, due to the presence of undetected low frequency infections, geographical and temporal variation in infection, tissue-specificity and PCR false negatives. Geographic structure in infection incidence and prevalence is a particularly important consideration and especially evident in endosymbiont-Lepidoptera systems e.g. Wolbachia-Hypolimnas bolina butterflies (Charlat et al., 2005). In a recent survey of published records of Wolbachia infections in the Lepidoptera, generalised geographic structure in infection frequency was observed, with lower frequencies towards higher latitudes (Ahmed et al., 2015).
In Table S1 and Table S2 we compile a comprehensive list of butterfly and moth species, respectively, reported as carrying heritable endosymbionts from published sources. We find that research of heritable endosymbionts in Lepidoptera is heavily dominated by studies of *Wolbachia* as opposed to that of other infections (*Wolbachia* in 248/253 butterfly species and in 109/115 moth species). While arthropod-infecting endosymbiont diversity is notable, including such divergent taxa as *Rickettsia, Spiroplasma, Arsenophonus, Flavobacteria, Cardinium* and the microsporidia, much of the early arthropod endosymbiont literature focused upon the Alphaproteobacteria genus *Wolbachia* (Hertig & Wolbach, 1924). Due to its presence in many agricultural pests and disease vectors, and also owing to the range of reproductive manipulations it employs in the host, *Wolbachia* is still widely, but justly, studied.

Tables S1 and S2 reveal that *Wolbachia* is common across Lepidopteran families, being found in all five families of ‘true’ butterflies (the Papilionoidea), and also in the skippers (Hesperiidae). *Wolbachia* strains have been divided into separate genetic lineages termed supergroups. It is clear from the compiled data that the *Wolbachia* strains carried by Lepidoptera are almost exclusively from supergroups A and B, with B group *Wolbachia* predominating over A group *Wolbachia*. Of species where the *Wolbachia* supergroup has been determined (80/109 moths and 208/248 butterflies), 85% of moth species carry B group and 25% A group; while 79% of butterfly species carry B group *Wolbachia* and 26% carry A group. Note that these data include multiple infections (*i.e.* some species harbour both A and B strains of *Wolbachia*). These findings concur with research analysing 90 *Wolbachia* strains associated with Lepidoptera: 84% of the strains belonged to supergroup B (76/90), with the remainder (14/90) belonging to supergroup A (Ahmed et al. 2016). A further study identified 22 *Wolbachia*-infected Lepidoptera species in Japan, 19 of which had infections from supergroup B (86%), with the remaining three
from supergroup A (Tagami & Miura, 2004). It is unclear why B group *Wolbachia* are particularly prevalent in the Lepidoptera; is there a greater ‘fit’ between Lepidoptera and B group *Wolbachia* i.e. are B group *Wolbachia* more likely to become established, or are B group *Wolbachia* those ancestrally associated with the Lepidoptera thus seeding this group stochastically? It is also interesting to note that there appears to be one particularly common strain of *Wolbachia* in Lepidoptera. In a study of 53 Lepidoptera species, 11 species across three families are infected with *Wolbachia* ST41, the next most common strain types (ST40 and ST125) were found in three species each (Ahmed et al. 2016). Whether *Wolbachia* ST41 is especially adept at moving between species, and/or whether it is particularly successful at establishing and maintaining itself with the host remains to be fully investigated.

The second most common heritable endosymbiont recorded in butterflies and moths is *Spiroplasma* - a bacterial genus belonging to the class Mollicutes (Table S1: 5/253 butterfly species and Table S2: 5/115 moth species). Until such endosymbionts receive the same level of attention as *Wolbachia*, or there is a move towards a generalised metagenomic approach to identify symbiotic microbes, little can be said of the extent of their presence or action in Lepidoptera. However, while there is a propensity for Lepidoptera to be specifically screened for *Wolbachia* infections (thereby creating a bias towards detection of *Wolbachia*), discovery of sex-ratio distorter identity is commonly a phenotype forward investigation i.e. a sex ratio bias in progeny or in a population is observed, and then the causative factor is identified. Given this, there should be no bias in the responsible infection found in these studies. Despite this, it appears that Lepidoptera are different from many other groups e.g. ladybirds, in that *Wolbachia* is almost always responsible for the observed sex ratio bias. In comparison with other arthropod groups such as the Diptera, Hymenoptera and Hemiptera, heritable endosymbiont diversity does appear
to be particularly low in the Lepidoptera. A systematic review (Russell et al. 2012) compiling
data of infection screens of arthropods for the heritable endosymbionts Arsenophonus,
Cardinium, Hamiltonella, Spiroplasma, and Wolbachia, found that only the latter two genera of
bacteria were present in Lepidoptera species (Spiroplasma: 5/205, Wolbachia: 140/481 species
infected). Thus we can say that for Arsenophonus, Cardinium and Hamiltonella, where 263, 183
and 251 Lepidopteran species were assayed respectively, such infections, should they exist at all
in Lepidoptera, are remarkably rare. A later compilation of data of arthropods screened for
heritable endosymbionts found that Rickettsia bacteria were also not commonly found in
Lepidoptera, with only one species (an unidentified Noctuidae moth) infected out of 14-32
species (variation in number reported here due to several individuals tested having no taxonomic
assignment in the study).

**Manipulation of host reproduction**

The Lepidoptera are becoming model systems for the study of endosymbiont manipulation of
host reproduction. Many species are infected with maternally inherited bacteria that have
evolved the ability to alter host reproduction to either increase the proportion of infected females
in the population, or increase the reproductive fitness of infected females relative to their
uninfected counterparts. In Lepidoptera endosymbionts are currently known to manipulate host
reproduction in three ways: through male-killing, feminisation and cytoplasmic incompatibility
(CI) (Fig. 1). While these methods facilitate the maintenance of the symbiont in the host
population, there are often severe repercussions for host biology and evolution. We provide a list
of butterflies and moths that have been recorded as being infected with endosymbionts that
manipulate the reproductive biology of the host (Table 1).
Male-killing (MK) is particularly well known in the Lepidoptera. Here, male offspring are killed early in development (most usually as an egg, but also as first instar larvae) producing a female biased sex ratio within an infected female’s offspring (Fig. 1). Should the male-killer infect many females, the host population as a whole may become female-biased. Several hypotheses have been proposed to explain why maternally inherited endosymbionts kill male hosts. If infected females gain a fitness benefit from the death of their male siblings over uninfected females (whose male siblings survive), the infection will invade and spread through the host population. Such benefits may include a reduction in the likelihood of detrimental inbreeding (as there are no brothers with which to mate) or a reduction in competition for resources (as there are half as many siblings with which to compete) (Hurst & Majerus, 1992; Hurst, Hurst & Majerus, 1997).

In ladybirds, Wolbachia-infected female neonates gain an important first meal by consuming their dead brothers, while uninfected females lack this ready source of nutrients (Elnagdy, Majerus & Handley, 2011). However, in Lepidopteran systems, the relative fitness benefit for infected females remains elusive as many of the species studied lay their eggs singly, thus making the likelihood of inbreeding, sibling egg cannibalism or competition unlikely (e.g. Danaus chrysippus: Jiggins et al., 2000a).

Despite the lack of evidence of any fitness benefit being provided to infected females, MK has been recorded numerous times in the Lepidoptera, possibly due to the readily observable phenotype of all-female broods and the long history of Lepidoptera being collected and reared in captivity. Early work recorded the presence of female-biases in wild-caught collections and captive bred broods in both Acraea encedon (Poulton, 1914; Owen, 1965; 1970), and
Hypolimnas bolina (Poulton, 1923; 1926) butterflies. Later, MK Wolbachia was identified as the causative agent in both A. encedon (Jiggins, Hurst & Majerus, 1998; Hurst et al., 1999) and H. bolina (Dyson, Kamath & Hurst, 2002). We now know that populations of Acraea butterflies carry highly prevalent MK Wolbachia infections, with more than 80% and 95% of Ugandan A. encedon and A. encedana females being infected, respectively (Jiggins et al., 2000a; Jiggins, Hurst & Majerus, 2000). The H. bolina system has become remarkable due to the extensive spatial and temporal variation in the dynamics of the interaction across the South-east Asian to Eastern Pacific range of the butterfly (Charlat et al., 2005; Hornett et al., 2009). The island of Samoa is particularly notable due to its well-documented history of a highly biased sex ratio of 100 females to every male, caused by 99% of female butterflies being infected with a MK Wolbachia (Dyson & Hurst, 2004). It appears that male-killers are often found at a particularly high frequency within butterfly populations, contrasting patterns seen in other taxa studied such as the ladybirds, where generally less than 49% of females carry an infection (Hurst & Jiggins, 2000). In the lycaenid Zizina emelina, at least one of the two Wolbachia strains described in Japanese populations is a male-killer that rapidly increased in prevalence from 65% to 86% within a 3 year period (Sakamoto et al., 2011).

The consequences of a highly distorted sex ratio are likely to be large (for discussions of evolutionary consequences see (Charlat, Hurst & Mercot, 2003; Engelstädter & Hurst, 2007)). As perhaps can be expected, one direct effect is that a large number of females remain unmated. In Makerere, Uganda, 94% of Wolbachia-infected A. encedon females were virgins (Jiggins, Hurst & Majerus, 2000). Of Samoan H. bolina 50% of infected females were unmated, with the females that did mate showing significant fertility deficiencies, implying sperm limitation (Dyson & Hurst, 2004). However, despite the detrimental impacts of male-killers upon the
reproductive biology of their hosts, natural host populations infected with high prevalence
infections can persist: the 100:1 female to male sex ratio of the Samoan H. bolina population
persisted for over 100 years (Dyson & Hurst, 2004). Only recently did the dynamics of this
interaction change, with the host evolving resistance of the MK activity (Hornett et al., 2006;
Charlat et al., 2007b).

Wolbachia are not the only endosymbionts that selectively kill male Lepidoptera. In the
nymphalid butterfly, Danaus chrysippus, a Spiroplasma bacteria, related to a MK strain
previously found in ladybirds, underlies the observed MK (Jiggins et al., 2000a). Similarly, while
Ostrinia corn borer moths are especially well-known to harbour MK Wolbachia strains (i.e. the
adzuki bean borer O. scapulalis (Kageyama & Traut, 2004), and the Asian corn borer O.
furnacalis (Sakamoto et al., 2007)), a MK Spiroplasma related to that found in D. chrysippus
infects the butterbur borer O. zaguliae (Tabata et al., 2011). Mirroring the pattern seen in H.
bolina, spatial variation of the MK Spiroplasma infection was observed in D. chrysippus (Smith
et al., 1998; Herren et al., 2007), with 40% of females infected in Uganda vs. 4% in East Kenya
(Jiggins et al., 2000a). Intriguingly, in this system infection appears to be correlated with a
colour pattern allele. Although the forces generating this correlation are unknown, it may be the
case that particular host genotypes are more susceptible to, or more efficient at transmitting, the
infection than others (Herren et al., 2007).

In most study systems the precise mechanisms of MK are unclear, and variation across
taxa is expected given that MK occurs in arthropods with widely disparate sex determination
systems. Dependent on host context several mechanisms have been proposed including defective
male chromatin remodelling (Wolbachia-infected Drosophila: (Riparbelli et al., 2012)); targeting
the dosage compensation complex (Spiroplasma-infected Drosophila: (Veneti et al., 2005));
damaging the host’s X chromosome to induce embryonic apoptosis (Spiroplasma-infected
Drosophila: (Harumoto et al., 2016)), and affecting maternally inherited centrosomes
(Arsenophonus-infected Nasonia wasps: (Ferree et al., 2008)). In a Wolbachia-infected moth,
Ostrinia scapulalis, male-killing is unusual in that males (genotype ZZ) selectively die early in
development, whereas females (ZW) die if cured of the Wolbachia infection following antibiotic
treatment (Fig. 2). Studies of this system suggest that MK Wolbachia interferes with the sex-
specific splicing pattern of the Ostrinia homologue of the sex determination gene doublesex,
Osdsx (Sugimoto et al., 2010), producing a mismatch between the genotypic sex and expression
of the phenotypic sex and leading to sex-specific death (Sugimoto & Ishikawa, 2012). Later
examination of the levels of dosage compensation (Z-linked gene expression) in male and female
embryos destined to die, revealed that misdirection of dosage compensation underlies the
observed mortality. Males destined to die (from Wolbachia-infected females) have higher levels
of expression of Z-linked genes than normal; while females destined to die (from females cured
of the Wolbachia infection) have lower expression levels of Z-linked genes than normal
(Sugimoto et al., 2015). In a related moth, O. furnacalis, RNA-Seq data of Wolbachia-infected
embryos demonstrated that MK Wolbachia down-regulated a masculinizing gene, Masc,
essential in controlling both sex determination and dosage compensation in Lepidoptera,
compared to uninfected embryos. The decrease in Masc mRNA levels is reported to cause the
MK phenotype via a failure of dosage compensation, and injection of in vitro transcribed Masc
cRNA into Wolbachia-infected embryos rescued male progeny (Fukui et al., 2015).
The mechanism of MK in Ostrinia moths may be different to that underlying MK in
other Lepidoptera. In H. bolina butterflies no female specific death is observed following
antibiotic treatment to reduce or remove MK Wolbachia (Charlat et al. 2007a). It is interesting to
note however, that the *doublesex* homologue in *H. bolina* may be involved in male-killing in this butterfly as it resides within the chromosomal region defined as containing a suppressor of male-killing action (Hornett et al., 2014). It would therefore be interesting to compare the *Wolbachia* strains and MK mechanisms of *Ostrinia* and *H. bolina*. Likewise, a comparison between the modes of action of the MK strain of *Wolbachia* in *Ostrinia* moths, which kills males as a consequence of feminising them through alteration of expression of *Osdsx*, and of ‘true’ feminising *Wolbachia* such as that infecting *Eurema* butterflies (see Feminisation) may shed light on how one genus of bacteria can induce multiple reproductive manipulations in their hosts and whether there is a functional link.

Finally, the Oriental tea tortrix moth *Homona magnanima* also carries a male-killer (Morimoto *et al.*, 2001), however male death in this case occurs much later in development (termed ‘late MK’), and appears to be associated with two novel RNA sequences (Nakanishi *et al.*, 2008). Late MK was originally only recorded in mosquitoes, with the causative agent being a microsporidian (Andreadis & Hall, 1979), however subsequent studies have now observed similar phenomena in other taxa including *Drosophila* flies (Jaenike, 2007). The extent of this type of manipulation, and the mechanisms underlying it, is still to be determined in insects, including Lepidoptera.

**Feminisation**

The feminisation of genetic males into functional phenotypic females (Stouthamer, Breeuwer & Hurst, 1999) is another strategy employed by maternally inherited endosymbionts to distort the host sex ratio towards the transmitting sex (females) (Fig. 1). While best known from the work on the association between *Wolbachia* and the terrestrial isopod *Armadillidium vulgare*
(Juchault, Rigaud & Mocquard, 1992; Rigaud, Juchault & Mocquard, 1997; Bouchon & Rigaud, 1998; Cordaux et al., 2004), feminisation also occurs in other female-heterogametic arthropods such as leafhoppers (XX/X0) (Negri et al., 2006), and Lepidoptera (ZZ/ZW). Other than *Wolbachia*, the Bacteroidetes bacterium *Cardinium* can also feminise males (Chigira & Miura, 2005; Groot & Breeuwer, 2006), however *Cardinium* has not yet been reported in butterflies or moths.

Observation of female-biased lines of pierid *Eurema* butterflies in Japan (Kato, 2000) led to the identification of a feminising *Wolbachia* in *E. mandarina* (formerly *E. hecabe* Y type) (Hiroki et al., 2002). *Eurema hecabe* (formerly *E. hecabe* B type) was later also discovered to carry a feminising *Wolbachia* indistinguishable from that of *E. mandarina*, thus suggesting that the infection transferred between the allopatric butterfly hosts via a shared predator or parasite, or via hybrid introgression between the species (Narita et al., 2011). When *E. mandarina* infected larvae were fed antibiotics to cure them of the infection, many of the adults emerged displaying sexually intermediate traits in their wings, reproductive organs and genitalia. Moreover, age at which antibiotics were administered was found to be important, with the highest level of intermediate sexual traits being exhibited when first instar larvae were treated. This work demonstrated that endosymbionts might continually influence and interact with their host (Narita et al., 2007) rather than have phenotypes that are effective only at a discrete time point in the lifecycle of the host.

The process of feminising in *E. mandarina* is more complex than originally thought. Against expectation, female butterflies infected with the feminising strain of *Wolbachia*, wFem, had only one, paternally derived, Z chromosome. This was proposed to be due to meiotic drive against the maternal Z, preventing the formation of the expected ZZ feminised males. It was also
suggested that wFem lines have lost the W chromosome, and rely on wFem for female
development as curing the infection with antibiotics results in all-male offspring (Kern et al.,
2015). Later work demonstrated that Wolbachia itself was responsible for the disruption of
maternal Z chromosome inheritance in wFem infected females, as well as the feminisation of
female ZO individuals that have lost the female-determining W chromosome (Kageyama et al.,
2017).

Cytoplasmic Incompatibility (CI)

Perhaps the most commonly observed reproductive manipulation employed by endosymbionts in
insects is cytoplasmic incompatibility (CI). Unlike for MK or feminisation, the sex ratio of host
populations infected by CI-inducing endosymbionts is generally not altered. Instead the symbiont
induces an incompatibility upon mating between infected males and females of a different
infection status (i.e. uninfected or infected with a different symbiont strain), leading to the death
of all or a proportion of the offspring (Yen & Barr, 1971; 1973) (Fig. 1). This incompatibility is
proposed to occur due to a modification of the infected male’s sperm that can be rescued when
the female is similarly infected (mod-res mechanism). This specific rescue function is lacking in
uninfected females or females carrying a different infection (Hoffman & Turelli, 1997; Charlat,
Calmet & Mercot, 2001; Poinso, Charlat & Merçot, 2003). Infected females therefore have a
reproductive benefit of successfully producing a full complement of progeny (relative to
uninfected females), when mated to uninfected or similarly infected males in the population.
While Wolbachia is often described as the causative agent, Cardinium also has this ability, e.g.
(Hunter, Perlman & Kelly, 2003; Perlman, Kelly & Hunter, 2008).
CI has been observed in a number of Lepidoptera including the Mediterranean flour moth *Ephestia kuehniella* and the almond moth *Cadra cautella* (Sasaki & Ishikawa, 1999).

Interestingly, CI *Wolbachia* was discovered to lower the amount of fertile sperm transferred in *Cadra cautella* during second matings. However, no effect was shown on the amount of apyrene (non-fertile) sperm suggesting that *Wolbachia* may only target fertile sperm production (Lewis *et al.*, 2011). Further work is required to expand our knowledge of CI mechanisms in Lepidoptera.

Similarly to MK, CI *Wolbachia* are often observed at high frequency in Lepidoptera populations. In a study of seven Japanese populations of the pierid butterfly *Colias erate poliographus*, CI *Wolbachia* occurred at 85-100% prevalence. The high infection frequency was ascribed to strong CI (*i.e.* a high proportion of the progeny from an incompatible cross die) and perfect vertical transmission of the bacteria (Tagami & Miura, 2004; Narita, Shimajiri & Nomura, 2008). Where CI reaches very high frequency within a host population, the incompatibility between infected males and uninfected females is rarely observed as few females remain uninfected. However, selection for beneficial effects of infection and an eventual shift towards a mutualistic relationship between host and symbiont would remain.

Symbioses can be extraordinarily complex; individuals can carry multiple endosymbionts with differing phenotypes. For instance, *E. hecabe* butterflies carry a feminising *Wolbachia* strain, but also a second strain that causes CI (Hiroki *et al.*, 2004). This was the first indication that different strains of *Wolbachia* could infect a single individual and cause different phenotypes. Host context is important in the expression of endosymbiont-induced phenotypes - one symbiont strain can have the ability to cause more than one phenotype, including reproductive manipulations that were originally assumed to be distinct from each other. This has been exemplified in the butterfly *H. bolina*: in populations where *H. bolina* has evolved
suppression of the action of MK, surviving infected males are incompatible with uninfected females in the population i.e. expression of the CI phenotype (Hornett et al., 2008). This finding indicates a potential functional or mechanistic link between the two phenotypes. However phenotypic switching between CI and MK through mutations cannot yet be ruled out. An intriguing possibility is whether feminisation is also mechanistically linked to CI and MK. Some evidence that may suggest this latter link is provided in studies of the moth O. scapularis. As mentioned above, male moths that die as a result of infection with MK Wolbachia, were found to carry the female isoform of a homologue of the sex-determining gene, doublesex, and hence were feminised prior to death (Sugimoto & Ishikawa, 2012).

Artificial transinfection of Wolbachia strains have provided further evidence of the relative importance of endosymbiont or host in determining the nature of the phenotype expressed. While in some cases transfer of Wolbachia from the natural host into a novel host did not alter the phenotype expressed (e.g. Wolbachia causes MK in the natural host O. scapulalis and in the transinfected host E. kuehniella (Fujii et al., 2001)), host context is important in others. Transfer of CI Wolbachia wCau-A from C. cautella to E. kuehniella resulted in the expression of MK in the novel host (Sasaki, Kubo & Ishikawa, 2002). The strength of the phenotype may also alter in the novel host: the level of CI induced by Wolbachia in the transinfected host O. scapulalis, was higher than that in its natural host E. kuehniella, indicating that host factors as well as endosymbiont strain are important in determining the phenotype expressed (Sakamoto et al., 2005).

Impact upon host fitness
It is becoming increasingly evident that many heritable endosymbionts do not manipulate host reproduction, and yet are still maintained within the host population. Host-associated microbes are now thought to be commonly beneficial to their host. For an inherited endosymbiont, the trade-off between virulence and transmission can lead to a reduction in its pathogenicity towards the host, and evolution towards mutualism (e.g. Weeks et al., 2007). At the extreme end of the spectrum are the obligatory endosymbionts, which are necessary for host survival or reproduction. The growing number of cases include: Wolbachia required for oogenesis in the wasp Asobara tabida (Dedeine et al., 2001; Dedeine, Bouletreau & Vavre, 2005); Wigglesworthia bacteria acting as an obligate nutritional mutualist in tsetse flies (Aksoy, 1995); and Buchnera bacteria providing essential nutrients to aphids (Buchner, 1965).

Many more endosymbionts are facultatively (non-essentially) beneficial, with fitness benefits including increasing host survival (e.g. (Fry & Rand, 2002)) or fecundity (e.g. (Vavre, Girin & Boulétreau, 1999; Weeks & Stouthamer, 2004). Studies of the beneficial effects of endosymbiont infection in the Lepidoptera provide an unusual example in Parnassius apollo. In one isolated population this near threatened butterfly regularly exhibits deformed or reduced wings, however while 86% of normal winged butterflies are found infected with Wolbachia, this percentage drops to 30% in individuals displaying deformed wings, and 0% in individuals with reduced wings. Although this is suggestive of a protective role of Wolbachia in the ontogenetic development of the butterfly, further study needs to be carried out to prove causality (Łukasiewicz, Sanak & Węgrzyn, 2016).

Microbial endosymbionts can contribute to insect adaptation by providing complementary or novel metabolic capacities, allowing the insect host to exploit host plant nutritional resources. One such instance has been observed in the phytophagous leaf-mining
moth *Phyllonorycter blancardella*. In this system, a bacterial endosymbiont, most likely *Wolbachia*, indirectly affects larval nutrition by manipulating the physiology of the host plant to create photosynthetically active green patches in otherwise senescent yellow leaves. The phenotype, termed ‘green-island’, produces areas of leaf viable for host feeding in a nutritionally constrained stage of the lifecycle. Curing the larvae of endosymbionts resulted in the non-production of ‘green-islands’, and consequent increased compensatory larval feeding and higher mortality (Kaiser *et al.*, 2010). The mechanism behind green island formation involves increased levels of cytokinins (CKs), plant hormones important in plant senescence and nutrient translocation. *Wolbachia* have been shown to be involved in the release of CKs by the larvae, creating these nutritionally enhanced areas of leaf. Whether the CKs are bacterial-derived or produced by the insect in response to *Wolbachia* infection (or a combination of both) remains to be fully understood (Body *et al.*, 2013; Giron & Glevarec 2014). Several strains of *Wolbachia* from both A- and B-supergroups have been identified in 13 Gracillariidae leaf-mining moth species, while none were found in ancestral Gracillariidae. Acquisition of the green-island phenotype appears to have occurred several times independently across the Gracillariidae in association with different *Wolbachia* infections (Gutzwiller *et al.*, 2015).

Generally, vertically inherited endosymbionts are unlikely to be maintained in host populations if they are highly costly. However, direct fitness or physiological costs of infection have been observed where the symbiont also manipulates host reproduction. CI *Wolbachia* are maintained in the host population despite reducing male fertility (Snook *et al.*, 2000) or female fecundity (Hoffman, Turelli & Harshman, 1990) in *Drosophila* flies, or detrimentally affecting fecundity, adult survival and locomotor performance in the parasitoid wasp *Leptopilina heterotoma* (Fleury *et al.*, 2000). Among Lepidoptera examples, presence of MK *Spiroplasma* in
D. chrysippus in Kenya was negatively correlated with forewing length, suggesting that the
bacteria may adversely affect development time or the growth rate of larvae (Herren et al.,
2007). Presumably these physiological costs are counter-balanced by the reproductive
manipulations employed by heritable endosymbionts, thus enabling the symbiont to persist.
Although not covered in detail here, we note that in contrast, a symbiont that is also (or only)
horizontally transmitted, can be highly detrimental to the host yet still be maintained in the host
population. Indeed, host death may be its source of transmission to a novel host.

The gregarine protozoan infection, Ophryocystis elektroscirrha, of the Monarch butterfly
(Danaus plexippus) is one of the most studied cases of direct fitness costs of symbionts in
Lepidoptera. While it is not heritable in the sense of being intracellular, we include it here as it is
passed vertically from mother to offspring via the surface of the egg. An infected female
inadvertently coats her eggs with protozoan spores that cover the outside of her abdomen during
oviposition. Newly hatched larvae ingest these spores while consuming the eggshell
(McLaughlin & Myers, 1970). The parasite, which requires the adult host stage for transmission,
rarely kills larvae or pupae under natural conditions, however the degree of virulence and
transmission trade-off varies depending on the level of infection at the adult stage. Where
individual D. plexippus butterflies carry high densities of the protozoa, they have both reduced
survival and flight capacity compared to individuals with lower density infections (Altizer &
Oberhauser, 1999; de Roode, Yates & Altizer, 2008; de Roode & Altizer, 2010).

Symbiont-mediated protection

Although understudied in Lepidopteran systems, an exciting avenue of research in arthropods
revolves around a symbiont’s ability to afford the host some level of resistance to its natural
enemies, often through interference with pathogen or parasite replication or transmission (reviewed in insects in (Brownlie & Johnson, 2009)). This may be particularly the case for heritable endosymbionts, where symbiont and host fitness is inextricably linked - competing infections may elicit a response by the endosymbiont to protect the host, and thus simultaneously itself (Haine, 2008). Such symbiont-mediated protection has been documented in numerous taxa, particularly the Diptera, including recent studies demonstrating the ability of *Wolbachia* to supply their *Drosophila* host with anti-viral protection (Hedges et al., 2008; Teixeira, Ferreira & Ashburner, 2008; Martinez et al., 2014). Similarly, maternally transmitted *Spiroplasma* were found to protect *D. neotestacea* against the sterilising effects of a parasitic nematode (Jaenike et al., 2010), and enhance the survival of *D. hydei* parasitized by wasps (Xie, Vilchez & Mateos, 2010).

Symbiont-mediated protection appears to be extremely diverse. Aphids are host to a range of inherited symbionts, several of which provide protection against parasitoid wasp attacks (Oliver et al., 2003; Ferrari et al., 2004) or fungal infections (Ferrari et al., 2004; Scarborough, Ferrari & Godfray, 2005). In the European beewolf wasp, *Philanthus triangulum*, *Streptomyces* bacteria are stored in special antennae glands and deposited together with the egg in the oviposition chamber. The bacteria secrete antibiotics protecting the developing wasp larvae against fungal pathogens (Kaltenpoth et al., 2005). There is even some evidence that symbionts can protect their host from predators by producing toxic compounds. For example, a bacterial endosymbiont (that is both vertically and horizontally transmitted) closely related to *Pseudomonas aeruginosa* produces the polyketide toxin pederin, which protects *Paederua* beetle larvae from predatory wolf spiders (Kellner & Dettner, 1996; Kellner, 1999; Piel, Höfer & Hui, 2004). Furthermore, endosymbionts may have the ability to inhibit a range of pathogens by
priming the host immune system (Braquart-Varnier et al., 2008; Moreira et al., 2009; Hughes et al., 2011), suggesting that symbionts can interact with, and alter integral components of, host biology.

Conversely, it is important to acknowledge that endosymbiont infection can also increase pathogen load. While Wolbachia confers protection against a variety of pathogens and parasites in a wide range of hosts, pathogen or parasite levels can also be enhanced by the presence of an endosymbiont (Hughes, Rivero & Rasgon, 2014): in the moth Spodoptera exempta, Wolbachia triggers a higher rate of virus infection and therefore lowers host fitness (Graham & Wilson, 2012).

Host genetics

Host population genetics

Sex ratio distorting symbionts are likely to have a severe impact upon host population biology (Engelstädter & Hurst, 2007). If the prevalence of a sex ratio distorer is high, the sex ratio of the population can become severely biased. In consequence, the hosts’ effective population size ($N_e$) will be reduced. Where there is little gene flow into the population (i.e. low immigration), a reduction of the effective population size may affect the amount of standing genetic variation and the potential for the host population to respond and adapt to environmental change. In contrast, if gene flow does occur, spatial variation in sex ratio (as seen in the butterfly H. bolina (Charlat et al., 2005; Hornett et al., 2009)) may result in asymmetric gene flow between populations. Although both sexes typically contribute equally to the gene pool of the next generation, immigration of an individual into a population in which the sex ratio is skewed against it (e.g. a male into a highly female-biased region) can have a much larger genetic impact (i.e. contribute
more) than if that individual immigrated into an unbiased sex ratio population (Telschow et al., 2006). MK symbionts are also thought to hinder the spread of beneficial alleles and facilitate the spread of deleterious alleles, due to constrained gene flow from infected to uninfected individuals within the population (Engelstädter & Hurst, 2007). In a further complication, strains expressing different reproductive manipulations may be incompatible. Although most famous for its MK *Wolbachia* infections, some populations of *H. bolina* also carry a CI-inducing strain of *Wolbachia*. This CI strain is phylogenetically distant from the MK strain, and crosses between MK-infected females and CI-infected males are fully incompatible with no progeny surviving. The incompatibility produced has led to strong competition between the two strains, with the CI-strain being able to not only spread successfully through uninfected populations, but to also resist invasion by the MK-strain carried by butterflies from neighbouring island populations (Charlat et al., 2006). Extending from this model, a study (Zug & Hammerstein, 2017) recently showed that when direct fitness benefits are taken into account in parallel to reproductive costs, the CI-strain is likely to also be able to spread across MK-infected *H. bolina* populations. Taken together, this suggests that successful establishment of particular butterfly genotypes is affected by the endosymbionts they harbour.

**Linkage with host mitochondrial DNA**

Maternally inherited symbionts residing within the cytoplasm of cells can alter the diversity and population genetics of the host’s mitochondrial genome (mtDNA). Co-inherited symbionts and mitochondria are in linkage disequilibrium, therefore when a cytoplasmic symbiont invades a population, the initially associated mitochondrial haplotype (mitotype) may ‘hitch-hike’ and correspondingly increase in frequency. Should such a selective sweep have occurred recently, the
effective population size and genetic diversity of mtDNA would be reduced to that of the infected individuals (Johnstone & Hurst, 1996), and the geographic structure of mitochondrial variation lost. The latter has been observed in Acraea butterflies (Jiggins, 2003) and the comma butterfly Polygonia c-album (Kodandaramaiah et al., 2011). The tight association between endosymbiont and mtDNA can therefore seriously confound the results of any study using mtDNA genes as neutral genetic markers (Hurst & Jiggins, 2005). Reconstruction of phylogenetic trees using mitochondrial markers are hence likely to be misleading, particularly within shallower branches, when the study species is infected. In the Diamondback moth, Plutella xylostella, the main correlate of mtDNA variation is presence or absence of the plutWB1 Wolbachia infection (Delgado & Cook, 2009), and the lycaenid butterfly Lampides boeticus may have experienced accelerated population differentiation due to Wolbachia infection (Lohman et al., 2008). Recognition of these processes should lead to an increasing number of Lepidopteran studies interested in using mtDNA markers to systematically screen for maternally inherited symbionts.

Where there is perfect transmission of the maternally inherited symbiont from the host to its offspring, infected individuals all carry the same mitotype, while uninfected individuals remain polymorphic. This pattern has been repeatedly observed in natural populations of insects, including in the Lepidoptera. In the butterfly H. bolina, a strong association between one specific Wolbachia strain and one particular mitotype supported the hypothesis that the MK infection occurred with very high vertical transmission efficiency and rare horizontal transmission. In H.bolina, this strain of Wolbachia is thought to have undergone a recent selective sweep and was introduced into this butterfly through introgression, potentially from another Hypolimnas species, H. alimena. Conversely the infection and associated mitotype may have been introgressed from...
H. bolina to H. alimena (Charlat et al., 2009; Duplouy et al., 2010; Sahoo et al., 2018). Similarly, in the Acraea butterflies, a study of mitochondrial variants demonstrated that a MK Wolbachia, together with the associated mitotype, had introgressed from A. encedana into A. encedon within the last 16,000 years. As female butterflies are heterogametic (ZW), this event could potentially also lead to the introgression of genes on the female W chromosome (Jiggins, 2003). This scenario appears to have occurred in D. chrysippus infected with a MK Spiroplasma, as all infected females carry the same W chromosome variant (Smith, Gordon & Allen, 2010). This aside, the nuclear DNA is generally less likely to be in linkage with inherited symbionts.

Gompert and colleagues studying North American Lycaeides butterflies reported that the spread of an endosymbiont (and associated mitotype) through the host population produced substantial mito-nuclear discordance. Therefore, the evolutionary history of an individual’s nuclear and mitochondrial genomes may be very different from each other (Gompert et al., 2008). Such discordance may have far-reaching effects on host metabolism and physiology, as coevolution between nuclear and mitochondrial components of essential pathways is broken down.

Speciation by symbiosis

The concept that symbionts can be important promoters of speciation and diversity has been around for a long time (Wallin, 1927; Laven, 1959; Thompson, 1987; Breeuwer & Werren, 1990; Hurst & Schilthuizen, 1998; Bordenstein, 2003), but has recently been rejuvenated with the development of microbiome analyses (Brucker & Bordenstein, 2012). Contemporary evidence of microbe-assisted speciation involves pre-mating reproductive isolation through behavioural barriers such as mate preference, associated with the microbiome of the potential partners (Koukou et al., 2006; Miller, Ehrman & Schneider, 2010; Sharon et al., 2010; Chafee et
The underlying mechanisms may involve alteration of the sex pheromones, interference with sensory organs, or effects upon immune-competence and hence mate attractiveness. Ecological isolation may also be heavily influenced by microbial symbionts. Although the genetic basis of niche or habitat specificity is widely accepted, there is also increasing evidence that symbionts may play a role in determining host resource availability (e.g. (Akman et al., 2002; Hosokawa et al., 2010)), and thus may facilitate niche separation.

Additionally, endosymbionts might enable host speciation through post-mating isolation. In particular, strong bi-directional CI may result in reproductive isolation between hosts carrying different CI symbiont strains (Hurst & Schilthuizen, 1998; Werren, 1998; Bordenstein, 2003). In order for speciation to follow CI, a stable infection polymorphism must be maintained across host populations. This has been demonstrated in many systems including the butterfly *H. bolina* (Charlat et al., 2006). Theory predicts that two bi-directional CI-inducing symbionts can be stable for even high migration rates (Telschow, Hammerstein & Werren, 2005). What is more contentious is that for speciation to occur, the CI produced must be very strong (*i.e.* no offspring surviving from such crosses), and the symbiont must be maintained at a high transmission rate over time, to allow significant nuclear divergence (Engelstädter & Hurst, 2009).

Male-killing has also been linked to speciation in the butterfly *D. chrysippus*. In Kenya two forms exist: *D. c. chrysippus* and *D. c. dorippus*, separated by a hybrid zone. Each subspecies has an individual colour pattern controlled by locus *C*, which is intermediate in the hybrid (*Cc*). The *C* locus lies on an autosome that has fused with the W chromosome within the hybrid zone, physically linking colour pattern with female determination. A locus on this same autosome has also been associated with susceptibility to male-killing by *Spiroplasma*. The hybrid zone is characterised by female-biased sex ratios, caused by MK *Spiroplasma* that infects
D. c. chrysippus or hybrid females, but rarely D. c. dorippus females. As immigrant males into the hybrid zone are predominantly D. c. dorippus, gene flow between the two subspecies is restricted: D. c. chrysippus/hybrid female x D. c. dorippus male crosses produce female-biased broods (Smith et al., 2016).

Sex determination

The maternal inheritance of intracellular endosymbionts has led to a great degree of interaction of the symbiont with the sex determination pathways of the host (reviewed in (Cordaux, Bouchon & Grève, 2011; Kageyama, Narita & Watanabe, 2012; Ma, Vavre & Beukeboom, 2014) and so not discussed in detail here). Maternally inherited endosymbionts distort the host sex ratio in order to enhance the fitness of the transmitting female sex. The mechanisms underlying these phenotypes often require considerable manipulation of host sex determination. We have seen above that in several cases MK and feminising Wolbachia can interfere with central components of the sex determination pathways in Lepidoptera. When a feminising element is highly prevalent in a host population, sex determination may be inextricably linked to the presence or absence of feminising activity (Hiroki et al., 2002), but may also enter into conflict with other genetic elements not under similar maternal inheritance. Furthermore, evolution of host suppressors of feminisation may move the system away from the original ZZ/ZW sex determination system. In E. mandarina, Wolbachia disrupts the inheritance of maternal Z chromosomes in Wolbachia-infected females, and feminises the resulting Z0 individuals that have lost the female-determining W chromosome (Kageyama et al., 2017). The host may then be prompted to evolve a strategy to counteract the feminising effects of the symbiont. It has been speculated that in the pillbug A. vulgare, a masculinising factor in the form
of a dominant autosomal $M$ gene has evolved in the host to counter the effect of the feminising endosymbiont (Rigaud & Juchault, 1993; Caubet et al., 2000).

Evolution of host resistance

Co-evolution between a host and a detrimental symbiont can result in the evolution of host genetic modifiers of symbiont presence or action. Despite this, and considering the wide array of costly effects that endosymbionts can impose on their hosts, it is perhaps surprising that there are relatively few well documented examples of the host having evolved genetic resistance to an endosymbiont. Indeed no suppression of the detrimental phenotype is observed in several studies where it may have been expected (Hurst, Jiggins & Robinson, 2001; Veneti, Toda & Hurst, 2004; Dyer & Jaenike, 2005). However, artificial transinfection experiments have provided an indirect method of discovering whether a host has evolved resistance to an endosymbiont, and have suggested that suppression of reproductive manipulation phenotypes may actually be common. In the moth *C. cautella*, which is naturally infected with two *Wolbachia* strains ($wCauA$ and $wCauB$), artificial transinfection of CI-inducing $wCauA$ to a sister host species, *E. kuehniella*, resulted in the transferred bacteria inducing MK instead of CI in the novel host (Sasaki, Kubo & Ishikawa, 2002; Sasaki, Massaki & Kubo, 2005). By interpreting these data in the light of the hidden MK theory (where MK is masked by the presence of a fixed suppressor), this switch in phenotype between species could be interpreted as the ‘unmasking’ of MK when released into a background devoid of host suppression genes. More generally, resistance may also underlie the loss of infections from populations or host species, however this is obviously hard to document in nature.
The selective pressure for host resistance is particularly strong when the sex ratio is severely biased (Düsing, 1884; Fisher, 1930; Hamilton, 1967), and therefore one would expect the evolution of resistance particularly in cases of highly prevalent sex ratio distorsers. As mentioned above, the Samoan population of the butterfly *H. bolina* had an extraordinarily female-biased sex ratio of 100 females per male, caused by 99% of the females being infected with MK *Wolbachia* (Dyson & Hurst, 2004). However between 2001 and 2006 the dynamics of the interaction changed dramatically when *H. bolina* evolved suppression of the MK trait, allowing infected males to survive and rapidly re-establishing a 1:1 sex ratio within approximately 10 generations of the host butterfly (Charlat et al., 2007b). The presence of a zygotically acting dominant suppressor locus had previously been documented in SE Asian *H. bolina* populations (Hornett et al., 2006).

Sex ratio distorting endosymbionsnts can also have much wider implications upon host genetics. Recent work on the same Samoan population of *H. bolina* investigating the genomic impact of the rapid spread of suppression revealed that a substantial selective sweep had taken place, covering at least 25cM of the chromosome carrying the suppressor locus. In addition to large changes in the frequency of genetic variants across this broad region, the sweep was associated with the appearance of several novel alleles. This suggests that the suppressor spread following migration of butterflies carrying the locus, potentially from SE Asia, rather than from a *de novo* mutation occurring within the population. It is also interesting to note that the suppressor of MK has been located to the chromosome containing *doublesex* (Hornett et al., 2014)– a sex determination gene demonstrated to be involved in *Wolbachia*-induced MK in *Ostrinia* moths.

*Horizontal transfer of genetic material*
While the extent of horizontal (lateral) gene transfer (HGT) between eukaryotes and prokaryotes remains uncertain, technological advances in genomics followed by an accumulation of microbial and host genomic data, have revealed that endosymbionts, particularly those that are vertically inherited, may readily exchange genetic material with their host. HGT from a prokaryote symbiont to its eukaryote host has been reported in many insects including beetles, flies, parasitoid wasps, mosquitoes and butterflies (e.g. (Hotopp et al., 2007; Nikoh et al., 2008; Klasson et al., 2009; Werren et al., 2010) and has recently been reviewed in detail (Husnik & McCutcheon, 2017). Such movement of genes can afford the receiving organism important benefits. For instance, horizontally transferred bacterial DNA that is involved in the detoxification of cyanide has been identified in several moths and butterflies, allowing these insects to utilise otherwise noxious plants (Wybouw et al., 2014). However, the discovery of bacterial DNA within the host’s genome does not necessarily imply functionality, and definitive proof of function is difficult to obtain, indeed many transferred Wolbachia genes are not expressed at a significant level in the host (Hotopp et al., 2007; Nikoh et al., 2008). To date, the identification of a 350bp long Wolbachia gene insert in the genome of the butterfly Melitaea cinxia, is the only reported example of an HGT from an endosymbiont to a Lepidoptera species (Ahmed et al. 2016), its origin and functionality have yet to be demonstrated.

Horizontal gene transfer is also known to occur in the opposite direction, from eukaryote host to symbiont. Wolbachia genome projects have indicated that genome fragments have been transferred from host to the bacteria, including in the H. bolina system. The MK Wolbachia strain sequenced appears to be extremely receptive to exogenous genetic material (Duplouy et al., 2013). In addition to cross-level transfer of genes, bacteria within a host may also exchange genetic material. Bacteria are known to be promiscuous with regard to DNA, with movement of
bacteriophages between co-infecting symbiont species providing a convenient method of transfer of genes. Some endosymbiont traits are associated with phage presence (Oliver et al., 2009) and thus this movement offers the potential for transfer of traits between co-infecting symbiont strains (Duron & Hurst, 2013). Indeed extensive HGT involving the bacteriophage \textit{WO} has been reported between several \textit{Wolbachia} strains infecting diverse hosts including within the Lepidoptera, Diptera and Hymenoptera (Masui et al., 2000; Bordenstein & Wernegreen, 2004).

### Behavioural modification

The transmission of many parasites is facilitated by their ability to manipulate the behaviour of their hosts (Lefevre et al., 2009). Reported cases are often restricted to viral and fungal pathogens, for instance, some baculoviruses and fungi cause summit disease – a syndrome that induce caterpillars to climb to high vegetation prior to being killed so that any spores released are carried further by the wind (Maitland, 1994; Yamazaki & Sugiura, 2004). Behavioural modification of arthropod hosts by heritable endosymbionts is less evident, and where observed are perhaps more attributable to indirect effects of infection. \textit{Rickettsia} bacteria have been associated with limiting long distance dispersal in a spider (Goodacre et al., 2009), and \textit{Wolbachia} has been demonstrated to reduce wasp locomotor performance (Fleury et al., 2000). Models of MK endosymbionts in metapopulations have suggested that male-killers can increase host dispersal rates (Bonte, Hovestadt & Poethke, 2008). These patterns may be attributed to the evolution of adaptive modifications by the symbiont to promote its own transmission. However another explanation is that these behavioural changes are merely side effects of physiological alterations without any adaptive causality.
In the butterfly, *D. plexippus*, the protozoan *O. elektroscirrha* has attracted much attention because of its potential involvement in the famous migratory behaviour of its host. This parasite is known to reduce the flight capacity of the host (Altizer & Oberhauser, 1999; Bradley & Altizer, 2005) – a trait that creates an important trade-off as the butterflies’ dispersive behaviour allows the spread of the protozoa across the species range, and thus increases the chance of it infecting naive populations. For the butterfly, migration offers an opportunity of escaping highly infected habitats where they may risk reduced fitness (Altizer, Bartel & Han, 2011). Altizer and colleagues demonstrated that variation in protozoa prevalence correlates with host movement - non-migratory populations have high infection prevalence whereas populations that migrate long distances show less than 10% prevalence of infection (Altizer, Oberhauser & Brower, 2000). More recently it was found that where migratory behaviour has been lost, the risk of infection is increased (Satterfield, Maerz & Altizer, 2015). Thus in part the presence of the protozoa may have led to Monarch butterflies forming both resident and migratory populations.

Further indirect behavioural consequences of microbial infection are also possible. In order to escape the fecundity and physiological costs of mating with an incompatible mate, individuals may evolve new adaptive mating strategies, including increased polyandry or mate discrimination (reviewed in (Miller & Schneider, 2012). *Wolbachia* influences mate-choice in the two-spotted spider mite, where uninfected females preferentially mate with uninfected males (Vala *et al.*, 2004), while in *Drosophila paulistorum*, *Wolbachia* titer and mate discrimination are positively correlated (Miller, Ehrman & Schneider, 2010). In *Acraea* butterfly populations harbouring high frequency MK bacteria (thereby having highly female-biased sex ratios), infected females more often remained unmated than uninfected females (Jiggins, Hurst & Majerus, 2000). While this is suggestive of preferential mating by the male, further work needs
to be carried out to test this. However, *Acraea* butterflies afford another example: in butterflies, males are often the competing sex and court the females. When the butterfly population is strongly female biased due to the presence of a highly prevalent sex ratio distorting endosymbiont, the roles of the sexes may reverse. Such sex-role reversal was observed in *Acraea* butterflies infected with MK *Wolbachia*. Although male ‘hill-topping’ (swarming at the tops of hills) is common throughout the genus (Jiggins, 2002), in *A. encedon* the lack of males induced females to swarm instead, and to exhibit behaviours soliciting the males’ attention (Jiggins, Hurst & Majerus, 2000).

MK endosymbionts may also result in female reproduction becoming sperm limited. In a comparison of *H. bolina* populations varying in MK *Wolbachia* prevalence, the prediction that female mating rates would decline with increasing MK infection prevalence as males became increasingly rare was not borne out. Unexpectedly the opposite occurs – as the population sex-ratio becomes more biased, the female mating rate increased until a point at which the lack of males makes it impossible for females to find a mate. It was suggested that female promiscuity increased in response to increasing male ‘fatigue’. Males from more highly female-biased populations produced smaller spermatophores thus necessitating females to become more solicitous (Charlat et al., 2007c).

**Outstanding questions and future directions**

We here are promoting the Lepidoptera as important models in the study of endosymbiont induced reproductive manipulations, with MK, feminisation and CI all being evident in butterflies and moths. Current research is uncovering the genetic and functional basis underlying these phenotypes but many outstanding questions remain: Are all three reproductive
manipulations found in Lepidoptera functionally linked? How commonly can a single endosymbiont strain confer more than one phenotype? How do different endosymbiont genera confer similar phenotypes in their host (e.g. both Wolbachia and Spiroplasma cause MK in Lepidoptera), and are the mechanisms related? How does MK, feminisation and CI in Lepidoptera differ from that expressed in taxa with divergent sex determination systems? Also, how do sex-ratio distorting endosymbionts affect the long-term evolution of the host. Given recent advances in genomics this now can include investigations of the genomic impact of a sustained population sex ratio bias. Sex-linked traits in particular may be expected to be affected.

More questions are provoked when research into heritable endosymbionts associated with other arthropod taxa is considered. Of particular interest is the evidence accruing that symbionts often afford the host some level of protection against pathogens and parasitoids. But how frequent is this phenomenon in butterflies and moths? Also, can we see these effects in combination with reproductive manipulations, producing a trade-off between detrimental and mutualistic effects of infection? Conversely, where we see highly prevalent and persistent endosymbiont infections in host populations that do not induce reproductive manipulations, do these symbionts offer the host protection? While there are clearly many outstanding questions to examine in the Lepidoptera, in this next section we focus upon four further areas of research that will move Lepidoptera-heritable endosymbiont research forward.

Comparative endosymbiont genomics

The genomes of many arthropod heritable endosymbionts have now been assembled, however very few of those sequenced are associated with Lepidoptera hosts. A comparative genomics approach can be used to elucidate endosymbiont evolution and function in its host including
identifying candidate genes involved in reproductive manipulations such as CI (as in *Drosophila*
(Sutton et al., 2014; LePage et al., 2017)), and parthenogenesis induction (in parasitoid wasps
(Newton et al., 2016; Lindsey et al., 2016)). A recent comparison of 16 *Wolbachia* genomes
identified a core *Wolbachia* genome of 496 sets of orthologous genes, 14 of which were unique
to *Wolbachia* among the Rickettsiales bacteria, of which it is a member (Lindsey et al., 2016).
This study included the MK *Wolbachia* strain wBol1b from *H. bolina* butterflies, which was
revealed to be closely related to a CI *Wolbachia* infecting *Culex pipiens* mosquitoes, wPip. A
comparison of the two strains identified a number of genes specific to wBol1b that could be
potential candidates involved in the induction of MK (Duplouy et al., 2013). An interesting
future research direction that may inform on the diversity and genetic basis of MK, would be to
expand this line of enquiry by comparing the genome of wBol1b with other MK and non-MK
*Wolbachia* genomes. Candidate loci could also be investigated in other MK-inducing symbiont
genomes such as *Spiroplasma*. While the genomes of many *Spiroplasma* bacteria have been
characterized from various arthropods (see (Bolaños, Servín-Garcidueñas & Martínez-Romero,
2015) for a minireview), including the MK *Spiroplasma* endosymbiont MSRO found in *D.
melanogaster* (Paredes et al., 2015), to our knowledge none have as yet been published that
specifically associate with Lepidoptera.

As high-throughput sequencing costs reduce, the genomes of increasing numbers of
Lepidoptera are being sequenced (for a review of the current status see (Triant, Cinel &
Kawahara, 2018)). A happy indirect consequence of this is that endosymbiont genome sequences
can be retrieved as a by-product of host genome sequencing. This is a particularly useful tool
when studying intracellular endosymbionts that are not readily culturable, and hence difficult to
directly isolate and sequence (such as *Wolbachia*). This approach has been used to reconstruct
the genome of *Wolbachia* infecting the moth *Operophtera brumata* (Derks et al., 2015), and that of *Wolbachia*, *wAus*, associated with the moth *Plutella australiana* (Ward & Baxter, 2017). Interestingly, and similarly to *wBoll1b* from *H. bolina*, both strains were most closely related to the CI *Wolbachia wPip* from the mosquito *C. pipiens* (Derks et al., 2015; Ward & Baxter, 2017), however in the case of *wAus*, two genes previously determined to be involved in CI caused by *Wolbachia* from *Drosophila melanogaster* were not found in the genome of *wAus* (Ward & Baxter, 2017). Further work needs to be conducted to characterize the nature of the interaction between *Wolbachia* and host before more insight can be gained through genomic comparisons.

**What else is in there? Moving towards a metagenomics approach**

This review has revealed a marked bias in Lepidopteran heritable endosymbiont research – *Wolbachia* is by far the most studied endosymbiont in butterflies and moths. While the incidence of *Wolbachia* is undoubtedly high in Lepidoptera and its effects upon its hosts important, the development of routine PCR assays and resources specific to this one genus of bacteria may have inflated its significance relative to other endosymbionts. Thus a practical limitation of the current methodology in the study of heritable endosymbionts in Lepidoptera is the lack of an unbiased approach to determine what microbes butterflies and moths carry. This is changing with the development of culture-independent methods of ascertaining what microbes, particularly bacteria, are present within an organism. High-throughput sequencing of the hypervariable bacterial 16S rRNA gene, and metagenomics allow the characterisation of whole bacterial communities of hosts. Particular to heritable endosymbiont research, attention should be given to the tissue from which DNA is sourced, as heritable bacteria are not necessarily found in the commonly sequenced gut tissue or lumen. Amplifying bacterial DNA from whole insects or the
reproductive tracts may yield a clearer idea of the vertically inherited symbionts present. We also have to consider what constitutes a heritable endosymbiont; many Lepidopteran gut bacteria are transitory and/or environmentally acquired for example via the food plant as larvae (Mason & Raffa, 2014; Hammer et al., 2017), or nectar as adults and as such may not evolve symbiotically with the host. However, gut bacteria may be transmitted by the female to the progeny via for example the egg coating, which neonates often consume upon hatching. One challenge will be to distinguish which of the microbes present in a community are symbiotic, and further, which are vertically transferred. Therefore close behind microbiome characterisations of Lepidoptera will be experimental manipulations of the microbiome and the sequencing of progeny to ascertain heritability.

Revealing the microbiome of Lepidoptera will open up a new set of questions such as do gut microbes and heritable endosymbionts interact? Can endosymbionts affect the composition of the microbiome? Do their effects interact? One promising avenue of research is the antimicrobial activity of gut symbionts. The moth *Spodoptera littoralis* harbours a gut bacterium *Enterococcus mundtii* that secretes an antimicrobial peptide (mundticin KS) against invading bacteria, but not against other resident gut bacteria. This antimicrobial activity directly inhibits competitors, but also potential pathogens, from the gut of its host. In *S. littoralis*, this extracellular symbiont persists across host developmental stages and is a major constituent of the microbiome across generations, suggesting that it can be vertically inherited, and that it may form a long-term symbiotic association with its host (Shao et al., 2017).

A further avenue for future research is the presence and impact of non-bacterial heritable endosymbionts. In particular there is increasing recognition that viruses may be vertically inherited and can have dynamic interactions with their host (reviewed in insects in (Longdon &
The moth *Helicoverpa armigera*, a crop pest, is infected with a vertically (and horizontally) inherited densovirus (HaDNV-1) that appears to be mutualistic. In wild larvae a negative interaction exists between the symbiotic densovirus and the presence of a nucleopolyhedrovirus (HaNPV) that is widely used as a pesticide against *H. armigera*. Laboratory work confirmed that larvae carrying HaDNV-1 had significantly higher resistance to the HaNPV pesticide, and also to low doses of *Bacillus thuringiensis* (Bt) toxin. Additionally, HaDNV-1 infected individuals have a higher developmental rate and higher fecundity than that of their uninfected counterparts (Xu et al., 2014). In contrast, in the moth *Homona magnanima* a novel RNA virus appears to be responsible for ‘late’ male-killing while being benign to female moths, thus acting as a reproductive manipulator (Nakanishi et al., 2008). Metagenomic sequencing has identified viruses across diverse arthropods (e.g. (Li et al., 2015)), and while often pathogenic a recent study identified a vertically inherited sigma virus in the nymphalid butterfly *Pararge aegeria*, that may have a more symbiotic role. In this species transmission of the virus was predominantly maternal (through eggs), with paternal (through sperm) transmission rates being much lower. Wild populations of *P. aegeria* experience high levels of infection, with a mean viral prevalence of 74%, and marked population structure in the genetic diversity of the virus (PAegRV). The nature of the relationship between *P. aegeria* and PAegRV remains to be determined (Longdon et al., 2017).

Global environmental change: can endosymbionts facilitate or constrain adaptation?

Predicting if or how organisms adapt to environmental change is a critical and timely question. Every organism interacts with a multitude of abiotic and biotic factors, including heritable endosymbionts, and knowledge of how these influence each other is imperative in understanding
an organism’s adaptive potential. Global environmental change is likely to alter the level and
direction of natural selection in host/symbiont co-evolution (Wolinska & King, 2009). In one
direction, endosymbionts may increase the host's potential repertoire for responding to
environmental changes such as temperature, while we also recognise that the destabilisation of
often finely tuned host-symbiont interactions may be severely detrimental for natural
populations.

As poikilotherms - organisms that do not maintain internal thermal homeostasis -
butterflies and moths are very susceptible to extreme temperatures (Denlinger & Yocum, 1998).
While they utilise a range of mechanisms, including behavioural and physiological responses, to
regulate temperature, every species is defined by thermal limits. Recent work has indicated that
microbial symbionts of insects can often facilitate or constrain adaptation to environmental
changes, including temperature. For instance, aphids carry symbionts that proffer heat stress
protection (Montllor, Maxmen & Purcell, 2002; Russell & Moran, 2006), including a point
mutation (a change in a single nucleotide), which governs host thermal tolerance (Dunbar et al.,
2007). The temperature insects are exposed to during development is also important in the
maintenance of symbionts (Anbutsu, Goto & Fukatsu, 2008), or to the phenotype expressed by
the symbiont in the host (Hurst et al., 2000). With global environmental change, de-stabilisation
of the host-symbiont interaction may become more frequent and have severe consequences for
many species. The sudden loss of an obligatory mutualistic symbiont, for example, would almost
certainly lead to a host population decline (for further discussion of host-symbiont interactions
and temperature see (Wernegreen, 2012; Corbin et al., 2016; Moran, 2016).

Furthermore, the nature of the relationship between host and symbiont may be indirectly
affected by the changing climate. A few degrees rise in temperature can alter the geographic
range of Lepidoptera (Parmesan et al., 1999). For many species, such range shifts and
colonisation events should only be possible if the plants they utilise were following a similar
expansion, such as in the host-limited butterfly Gonepteryx rhamni (Gutiérrez & Thomas, 2000).
Additionally, range shifts may lead to a switch in host plant species or increased generalisation
(Braschler & Hill, 2007), bringing subsequent repercussions for Lepidopteran-endosymbiont
interactions. For example, in the moth P. blancardella, where endosymbionts nutritionally
benefit the host by creating photosynthetically active green patches in otherwise senescent leaves
of the host plant (Kaiser et al., 2010), a shift in host plant use could make this “green-island”
strategy ineffective in a novel plant with a different chemical makeup. In contrast, novel host
plant utilisation may also be facilitated by endosymbionts, including through enhanced
provisioning of nutrients, or detoxification (reviewed in (Hansen & Moran, 2014)).
Finally, habitat degradation and fragmentation is likely to have several implications for
natural host-symbiont dynamics. Habitat destruction has the effect of crowding insect
populations into smaller patches, and through fragmentation and subsequent isolation, the
amount of gene flow between populations becomes reduced. These factors may increase disease
transmission within a population, and alter geographical variance in endosymbiont presence and
prevalence.

Screening butterflies and moths of conservation concern for endosymbionts

The Lepidoptera are model organisms in the fields of conservation and climate change research.
However, despite the high occurrence of endosymbionts in Lepidoptera, current conservation
planning rarely includes data on endosymbiont infections of the species under consideration, a
deficit that may profoundly influence the outcome of any management undertaken. For effective
conservation, or to understand how species will respond and adapt to environmental and
anthropogenic changes, it is important that we try to understand the intricate relationships that
microbes have with the hosts in which they reside. Fortunately there is increasing recognition of
this importance with several recent studies reporting endosymbiont infections in populations of
dangered or near threatened Lepidoptera (Nice et al., 2009; Sakamoto et al., 2011; Patricelli et
al., 2013; McHugh et al., 2013; Łukasiewicz, Sanak & Węgrzyn, 2016; Fenner et al., 2017). One
study surveying 22 species of conservation concern (comprising members of the Lycaenidae,
Nymphalidae, Hesperidae and Noctuidae) for Wolbachia found 19 to be infected (Hamm et al.,
2014). Nice and colleagues examined the nature of a Wolbachia infection in the North American
dangered Karner blue butterfly, Lycaeides melissa samuelis. Screening for endosymbionts
revealed that across the western edge of this butterfly’s range there was a widespread Wolbachia
infection. They went on to simulate demographic effects of the spread of Wolbachia into
uninfected populations and suggested that the spread of such an infection might further reduce
already small population sizes. The authors show concern that the Wolbachia infection was
prevalent in many of the largest and least impacted populations of this butterfly. This is
significant as these populations are likely candidates from whom captive propagation efforts
would draw individuals, and so the chance of inadvertently infecting a naturally uninfected
population is high (Nice et al., 2009).

Release of wild individuals or of those reared in captivity, either as part of conservation
management schemes or for commercial purposes (birthdays or weddings), might have
unexpected and undesirable impacts if not monitored correctly. Rearing Lepidoptera, which
often occurs at high densities, can allow the accumulation of pathogens. Releasing these
individuals back into the field may therefore alter the parasite load and consequent fitness of the
receiving population. Movement of individuals between populations may also affect the natural spatial pattern of endosymbiont diversity and prevalence: novel microbes may be introduced, or symbionts that have locally adapted in the donor population may affect the host in dramatically different ways in the novel population or environment. Further consequences with regard to endosymbiont infection are likely to be numerous, for example competition between native and novel infections may result in a shift in the natural equilibrium between the host and its native microbes or the introduction of cytoplasmic endosymbionts may also introduce linked variants such as host mtDNA haplotypes or female-linked nuclear DNA. Furthermore, as we have seen in the butterfly H. bolina, movement of individuals could also introduce host resistance loci that irrevocably alter the dynamics of host-symbiont interaction, and may have a wider impact upon the host genome. In general, if a novel association does form and/or spread, there follows rapid evolution of both host and symbiont, with phenotypic alterations that alter or optimise the new symbiosis (for an example see (Weeks et al., 2007).

Conclusion

The Lepidoptera have emerged as important models in the study of the genetic and functional basis of the reproductive manipulations heritable endosymbionts employ, particularly with regard to Wolbachia bacteria. The results of this cumulative work is suggestive of the role of endosymbionts in the evolution of host sex determination itself. We have no doubt Lepidopteran endosymbiont research will continue to highlight the omnipresence and importance of Wolbachia but we suggest that more attention should now be given to the presence and interaction of other heritable endosymbionts Lepidoptera carry. Metagenomic approaches enable an unbiased view of the microbial community residing within moths and butterflies, while
comparative endosymbiont genomics may illuminate the genetic mechanisms underlying the phenotypes endosymbionts induce in their host. Finally, given the importance of Lepidoptera as key indicators of climate change and the growing numbers of species listed as endangered, the study of heritable microbial endosymbiont in the Lepidoptera should transition from being a pure science filled with interesting curiosities, to a necessity that will contribute to the preservation of natural biodiversity and inform conservation management.

Acknowledgements
We wish to thank S. Charlat, J. Marden, A. Teacher, M. Riegler, H. Kokko, G. Hurst and an anonymous reviewer for constructive comments on the manuscript.

Figure and Table legends

Figure 1. Endosymbiont-induced manipulation of Lepidoptera reproduction. In the Lepidoptera, endosymbionts are currently known to manipulate host reproduction in three ways in order to increase their transmission to the next generation. Male-killing: female hosts infected with male-killing endosymbionts only give rise to infected female offspring, with male offspring dying early in development. Feminisation: female hosts infected with feminising endosymbionts only give rise to infected female offspring, with male offspring having been feminised so that they are genetically male (ZZ) but phenotypically female. Uninfected males may arise through inefficient transmission of the infection. Cytoplasmic incompatibility (CI): crosses between uninfected females and infected males result in few or no viable offspring, as the result of an incompatibility induced by the endosymbiont in the male. Infected females are able to rescue this incompatibility and hence are able to produce viable (infected) offspring when mated with
infected males. For male-killing and feminisation the endosymbiont acts as a sex-ratio distorfer, creating a female-bias in the offspring, and potentially in the population if the infection is highly prevalent.

**Figure 2. Wolbachia-induced male-killing and interference of sex determination in *Ostrinia scapulalis* moths.** A) Uninfected females gives rise to a normal 1:1 sex ratio in progeny: female offspring have ZW sex chromosomes and express the female isoform of the *Ostrinia* homologue of a gene in the sex determination cascade, *doublesex* (*dsx*), called *OsdsxF*; male offspring have two Z sex chromosomes and express the male *dsx* isoform *OsdsxM*. B) *Wolbachia* infected females only give rise to infected female progeny. Male offspring die early in development due to a mismatch between the genotypic sex (ZZ) and phenotypic sex (*OsdsxF*). C) *Wolbachia*-infected females cured of the infection as larvae by antibiotic treatment only give rise to uninfected males. Female offspring die early in development due to a mismatch between their genotypic sex (ZW) and phenotypic sex (*OsdsxM*). D) *Wolbachia*-infected females cured of the infection as adults prior to oviposition by antibiotic treatment give rise to sexual mosaics which have the male ZZ genotype but both *OsdsxF* and *OsdsxM*. Note: there are two female isoforms of *dsx in Ostrinia scapulalis*: *OsdsxF* and *OsdsxFs*; these are simplified to *OsdsxF* in this schematic.

White circles: uninfected individual; Red circles: *Wolbachia*-infected individual; Dark grey circle: *Wolbachia*-infected female cured as larva; Light grey circle: *Wolbachia*-infected female cured as adult.

**Table 1.** Butterfly and moth species recorded as carrying heritable endosymbionts that manipulate the reproduction of the host. Endosymbiont induced phenotypes are given as MK:
Male-killing, Late MK: Male-killing occurring late in development; CI: Cytoplasmic Incompatibility; Feminisation; or Sex-ratio distortion (where further investigation is needed to determine the nature of the sex-ratio bias).

References

Ahmed MZ, Araujo-Jnr EV, Welch JJ, Kawahara AY 2015. Wolbachia in butterflies and moths: geographic structure in infection frequency. Frontiers in Zoology 1:1–9. DOI: 10.1186/s12983-015-0107-z.

Akman L, Yamashita A, Watanabe H, Oshima K, Shiba T, Hattori M, Aksoy S 2002. Genome sequence of the endocellular obligate symbiont of tsetse flies, Wigglesworthia glossinidia. Nature Genetics 32:402–407. DOI: 10.1038/ng986.

Aksoy S 1995. Wigglesworthia gen. nov. and Wigglesworthia glossinidia sp. nov., taxa consisting of the mycetocyte-associated, primary endosymbionts of tsetse flies. International journal of systematic bacteriology 45:848–851.

Altizer SM, Oberhauser KS 1999. Effects of the Protozoan Parasite Ophryocystis elektroscirrha on the Fitness of Monarch Butterflies (Danaus plexippus). Journal of Invertebrate Pathology 74:76–88. DOI: 10.1006/jipa.1999.4853.

Altizer SM, Oberhauser KS, Brower LP 2000. Associations between host migration and the prevalence of a protozoan parasite in natural populations of adult monarch butterflies. Ecological Entomology 25:125–139. DOI: 10.1139/z78-240.

Altizer S, Bartel R, Han BA 2011. Animal migration and infectious disease risk. Science (New York, N.Y.) 331:296–302. DOI: 10.1126/science.1194694.

Anbutsu H, Goto S, Fukatsu T 2008. High and Low Temperatures Differently Affect Infection Density and Vertical Transmission of Male-Killing Spiroplasma Symbionts in Drosophila Hosts. Applied and Environmental Microbiology 74:6053–6059. DOI: 10.1128/AEM.01503-08.

Andreadis TG, Hall DW 1979. Significance of transovarial infections of Amblyospora sp. (Microspora: Thelohaniidae) in relation to parasite maintenance in the mosquito Culex salinarius. Journal of Invertebrate Pathology 34:152–157. DOI: 10.1016/0022-2011(79)90095-8.

Ankola K, Brueckner D, Puttaraju HP 2011. Wolbachia endosymbiont infection in two Indian butterflies and female-biased sex ratio in the Red Pierrot, Tlacida nyseus. Journal of Biosciences 36:845–850. DOI: 10.1098/rspb.2000.1139.

Bandi C, Dunn AM, Hurst GD, Rigaud T 2001. Inherited microorganisms, sex-specific virulence and reproductive parasitism. Trends in Parasitology 17:88–94.

Body, M, Kaiser, W, Dubreuil, G, Casas, J & Giron, D 2013. Leaf-Miners Co-opt Microorganisms to Enhance their Nutritional Environment. Journal of Chemical Ecology 39:969–977.

Bolaños LM, Servín-Garcidueñas LE, Martínez-Romero E 2015. Arthropod-Spiroplasma relationship in the genomic era. FEMS Microbiology Ecology 91:1–8. DOI: 10.1093/femsec/fiu008.
Bonte D, Hovestadt T, Poethke H-J 2008. Male-killing endosymbionts: influence of environmental conditions on persistence of host metapopulation. *BMC evolutionary biology* 8:243. DOI: 10.1186/1471-2148-8-243.

Bordenstein SR 2003. Symbiosis and the origin of species. In: Bourtizis K, Miller TA eds. *Insect Symbiosis*. CRC Press, 283–304.

Bordenstein SR, Wernegreen JJ 2004. Bacteriophage flux in endosymbionts (*Wolbachia*): infection frequency, lateral transfer, and recombination rates. *Molecular Biology and Evolution* 21:1981–1991. DOI: 10.1093/molbev/msh211.

Bouchon D, Rigaud T 1998. Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. *Proceedings of the Royal Society B: Biological Sciences* 265: 1081-1090 DOI: 10.1098/rspb.1998.0402

Bradley CA, Altizer S 2005. Parasites hinder monarch butterfly flight: implications for disease spread in migratory hosts. *Ecology Letters* 8: 290-300 DOI: 10.1111/j.1550-7408.1970.tb02375.x

Bouchon D, Rigaud T 1998. Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. *Proceedings of the Royal Society B: Biological Sciences* 265: 1081-1090 DOI: 10.1098/rspb.1998.0402

Brownlie JC, Johnson KN 2009. Symbiont-mediated protection in insect hosts. *Trends in Microbiology* 17:348–354. DOI: 10.1016/j.tim.2009.05.005.

Brownlie JC, Cass BN, Riegler M, Witsenburg JJ, Iturbe-Ormaetxe I, McGraw EA, O’Neill SL 2009. Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress. *PLoS Pathogens* 5:e1000368. DOI: 10.1371/journal.ppat.1000368.

Buchner P 1965. Endosymbiosis of animals with plant microorganisms. Interscience Publishers: John Wiley

Caspi-Fluger A, Inbar M, Mozes-Daube N, Katzir N, Portnoy V, Belausov E, Hunter MS, ZCHORI-FEIN E 2012. Horizontal transmission of the insect symbiont *Rickettsia* is plant-mediated. *Proceedings of the Royal Society B: Biological Sciences* 279:1791–1796. DOI: 10.1098/rspb.2011.2095.

Chafee ME, Zecher CN, Gourley ML, Schmidt VT, Chen JH, Bordenstein SR, Clark ME, Bordenstein SR 2011. Decoupling of Host-Symbiont-Phage Coadaptations Following Transfer Between Insect Species. *Gene* 187:203–215. DOI: 10.1534/genetics.110.120675.
Charlat S, Calmet C, Mercot H 2001. On the mod resc model and the evolution of Wolbachia compatibility types. *Gene* 159:1415–1422.

Charlat S, Davies N, Roderick GK, Hurst GDD. 2007a. Disrupting the timing of Wolbachia-induced male-killing. *Biology Letters* 3:154-156 DOI: 10.1098/rsbl.2006.0584

Charlat S, Duplouy A, Hornett EA, Dyson EA, Davies N, Roderick GK, Wedell N, Hurst GDD 2009. The joint evolutionary histories of Wolbachia and mitochondria in *Hypolimnas bolina*. *BMC evolutionary biology* 9:64. DOI: 10.1186/1471-2148-9-64.

Charlat S, Engelstädter J, Dyson EA, Hornett EA, Duplouy A, Tortosa P, Davies N, Roderick GK, Wedell N, Hurst GDD 2006. Competing selfish genetic elements in the butterfly *Hypolimnas bolina*. *Current biology : CB* 16:2453–2458. DOI: 10.1016/j.cub.2006.10.062.

Chigira A, Miura K 2005. Detection of “candidatus Cardinium” bacteria from the haploid host *Brevipalpus californicus* (Acari: Tenuipalpidae) and effect on the host. *Experimental and Applied Acarology* 37:107–116. DOI: 10.1007/s10493-005-0592-4.

Chrostek E, Pelz-Stelinski K, Hurst GDD, Hughes GL 2017. Horizontal transmission of intracellular insect symbionts via plants. *Frontiers in microbiology* 8:628. DOI: 10.1371/journal.pone.0048148.

Corbin C, Heyworth ER, Ferrari J, Hurst GDD 2016. Heritable symbionts in a world of varying temperature. *Heredity* 118:10–20. DOI: 10.1111/j.1420-9101.2012.02601.x.

Cordeaux R, Bouchon D, Grève P 2011. Evidence for a new feminizing *Wolbachia* strain in the isopod *Armadillidium vulgare*: evolutionary implications. *Heredity* 93:78–84. DOI: 10.1038/sj.hdy.6800482.

de Bary A 1879. *Die Erscheinungen der Symbiose*. Strassburg: Verlag von Karl J. Trübner.

de Roode JC, Altizer S 2010. Host-parasite genetic interactions and virulence-transmission relationships in natural populations of monarch butterflies. *Evolution; international journal of organic evolution* 64:502–514. DOI: 10.1111/j.1558-5646.2009.00845.x.

de Roode JC, Yates AJ, Altizer S 2008. Virulence-transmission trade-offs and population divergence in virulence in a naturally occurring butterfly parasite. *PNAS* 105:7489–7494. DOI: 10.1073/pnas.0710909105.

Dedeine F, Bouletreau M, Vavré F 2005. *Wolbachia* requirement for oogenesis: occurrence within the genus *Asobara* (Hymenoptera, Braconidae) and evidence for intraspecific variation in *A. tabida*. *Heredity* 95:394–400. DOI: 10.1038/sj.hdy.6800739.

Dedeine F, Vavré F, Fleury F, Loppin B, Hochberg ME, Bouletreau M 2001. Removing
symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *PNAS* 98:6247–6252. DOI: 10.1073/pnas.101304298.

Delgado AM, Cook JM 2009. Effects of a sex-ratio distorting endosymbiont on mtDNA variation in a global insect pest. *BMC evolutionary biology* 9:49. DOI: 10.1186/1471-2147-9-49.

Denlinger D, Yocum G 1998. Physiology of heat sensitivity. In: Hallman G, Denlinger D eds. *Temperature sensitivity in insects and application in integrated pest management*. Boulder: Westview Press, 7–57.

Derks MFL, Smit S, Salis L, Schijlen E, Bossers A, Mateman C, Pijl AS, de Ridder D, Groenen MAM, Visser ME, Megens H-J 2015. The genome of winter moth (*Operophtera brumata*) provides a genomic perspective on sexual dimorphism and phenology. *Genome Biology and Evolution* 7:2321–2332. DOI: 10.1093/gbe/evv145.

Dion E, Polin SE, Simon J-C, Outreman Y 2011. Symbiont infection affects aphid defensive behaviours. *Biology Letters* 7:743–746.

Douglas AE 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology* 43:17–37. DOI: 10.1146/annurev.ento.43.1.17.

Dunbar HE, Wilson ACC, Ferguson NR, Moran NA 2007. Aphid thermal tolerance is governed by a point mutation in bacterial symbionts. *PLoS biology* 5:e96 DOI: 10.1371/journal.pbio.0050096.

Duplouy A, Brattström O 2017. *Wolbachia* in the genus Bicyclus: a forgotten player. *Microbial Ecology* 75: 255-263 DOI: 10.1007/s00248-017-1024-9

Duplouy A, Hurst GDD, O'Neill SL, Charlat S 2010. Rapid spread of male-killing *Wolbachia* in the butterfly *Hypolimnas bolina*. *Journal of Evolutionary Biology* 23:231–235. DOI: 10.1111/j.1420-9101.2009.01891.x.

Duplouy A, Iturbe-Ormaetxe I, Beatson SA, Szubert JM, Brownlie JC, McMeniman CJ, McGraw EA, Hurst GDD, Charlat S, O’Neill SL, Woolfit M 2013. Draft genome sequence of the male-killing *Wolbachia* strain wBol1 reveals recent horizontal gene transfers from diverse sources. *BMC genomics* 14:20–13. DOI: 10.1186/1471-2164-14-20.

Duron O, Hurst GD 2013. Arthropods and inherited bacteria: from counting the symbionts to understanding how symbionts count. *BMC Biology* 11:45–4. DOI: 10.1186/1741-7007-11-45.

Duron O, Wilkes TE, Hurst GDD 2010. Interspecific transmission of a male-killing bacterium on an ecological timescale. *Ecology Letters* 13:1139–1148. DOI: 10.1111/j.1461-0248.2010.01502.x.

Düsing C 1884. Die Regulierung des Geschlechtsverhältnisses bei der Vermehrung der Menschen, Tiere und Pflanzen. *Jenaische Zeitschrift für Naturwissenschaft* 17:593–940.

Dyer KA, Jaenike J 2005. Evolutionary dynamics of a spatially structured host-parasite association: *Drosophila innubila* and male-killing *Wolbachia*. *Evolution; international journal of organic evolution* 59:1518–1528.

Dyson EA, Hurst G 2004. Persistence of an extreme sex-ratio bias in a natural population. *PNAS* 101:6520–6523.

Dyson EA, Kamath MK, Hurst GDD 2002. *Wolbachia* infection associated with all-female broods in *Hypolimnas bolina* (Lepidoptera: Nymphalidae): evidence for horizontal transmission of a butterfly male killer. *Heredity* 88:166 EP —6. DOI: 10.1186/1741-7007-6-27.
Elnagdy S, Majerus MEN, Handley L-JL 2011. The value of an egg: resource reallocation in ladybirds (Coleoptera: Coccinellidae) infected with male-killing bacteria. *Journal of Evolutionary Biology* 24:2164–2172. DOI: 10.1111/j.1420-9101.2011.02346.x.

Engelstädter J, Hurst GDD 2007. The impact of male-killing bacteria on host evolutionary processes. *Gene* 175:245–254. DOI: 10.1016/j.ymgene.2006.060921.

Engelstädter J, Hurst GDD 2009. The ecology and evolution of microbes that manipulate host reproduction. *Annual Review of Ecology, Evolution, and Systematics* 40:127–149. DOI: 10.1146/annurev.ecolsys.110308.120206.

Fenner J, Seltzer J, Peyton S, Sullivan H, Tolson P, Walsh R, Hill J, Counterman B 2017. Demographic variation of *Wolbachia* infection in the endangered Mitchell’s Satyr butterfly. *Insects* 8:50. DOI: 10.3390/insects8020050.

Ferrari J, Darby AC, Daniell TJ, Godfray HCJ, Douglas AE 2004. Linking the bacterial community in pea aphids with host-plant use and natural enemy resistance. *Ecological Entomology* 29:60–65.

Ferree PM, Avery A, Azpurua J, Wilkes T, Werren JH 2008. A bacterium targets maternally inherited centrosomes to kill males in *Nasonia*. *Current biology : CB* 18:1409–1414. DOI: 10.1016/j.cub.2008.07.093.

Fisher RA 1930. *The genetical theory of natural selection*. Clarendon Press.

Fleury F, Vavre F, Ris N, Fouillet P, Bouletreau M 2000. Physiological cost induced by the maternally-transmitted endosymbiont *Wolbachia* in the *Drosophila* parasitoid *Leptopilina heterotoma*. *Parasitology* 121 Pt 5:493–500.

Fraune S, Bosch TCG 2010. Why bacteria matter in animal development and evolution. *BioEssays* 32:571–580. DOI: 10.1002/bies.200900192.

Fry AJ, Rand DM 2002. *Wolbachia* interactions that determine *Drosophila melanogaster* survival. *Evolution; international journal of organic evolution* 56:1976–1981.

Fujii Y, Kageyama D, Hoshizaki S, Ishikawa H, Sasaki T 2001. Transfection of *Wolbachia* in Lepidoptera: the feminizer of the adzuki bean borer *Ostrinia scapulalis* causes male killing in the Mediterranean flour moth *Ephestia kuehniella*. *Proceedings of the Royal Society B: Biological Sciences* 268:855–859. DOI: 10.1098/rspb.2001.1593.

Fukui T, Kawamoto M, Shoji K, Kiuchi T, Sugano S, Shimada T, Suzuki Y, Katsuma S 2015. The endosymbiotic bacterium *Wolbachia* selectively kills male hosts by targeting the masculinizing gene. *PLoS Pathogens* 11:e1005048. DOI: 10.1371/journal.ppat.1005048.s003.

Fraune S, Bosch TCG 2010. Why bacteria matter in animal development and evolution. *BioEssays* 32:571–580. DOI: 10.1002/bies.200900192.

Gehrer L, Vorburger C 2012. Parasitoids as vectors of facultative bacterial endosymbionts in aphids. *Biology Letters* 8:613–615. DOI: 10.1098/rsbl.2012.0144.

Geier PW, Briese DT, Lewis T 1978. The light-brown apple moth, *Epiphyas postvittana* (Walker) 2. Uneven sex ratios and a condition contributing to them in the field. *Australian Journal of Ecology* 3:467–488. DOI: 10.1111/j.1442-9906.1978.tb01192.x.

Giron, D & Glevarec, G 2014. Cytokinin-induced phenotypes in plant-insect interactions: learning from the bacterial world. *Journal of Chemical Ecology* 40:826–835.

Gompert Z, Forister ML, Fordyce JA, Nice CC 2008. Widespread mito-nuclear discordance with evidence for introgressive hybridization and selective sweeps in Lycaenides. *Molecular ecology* 17:5231–5244. DOI: 10.1111/j.1365-294X.2008.03988.x.

Goodacre SL, Martin OY, Bonte D, Hutchings L, Woolley C, Ibrahim K, Thomas CG, Hewitt GM 2009. Microbial modification of host long-distance dispersal capacity. *BMC Biology* 7:32–32. DOI: 10.1186/1741-7007-7-32.
Graham RI, Wilson K 2012. Male-killing Wolbachia and mitochondrial selective sweep in a migratory African insect. *BMC evolutionary biology* 12:204–11. DOI: 10.1186/1471-2148-12-204.

Graham RI, Hartley L, Wilson K 2011. Characterisation of a nucleopolyhedrovirus and *Spiroplasma* sp. bacterium associated with outbreaksing populations of the Antler moth *Cerapteryx graminis*. *Journal of Invertebrate Pathology* 107:90–93. DOI: 10.1016/j.jip.2011.01.006.

Groot TVM, Breeuwer JAJ 2006. Cardinium symbionts induce haploid thelytoky in most clones of three closely related Brevipalpus species. *Experimental and Applied Acarology* 39:257–271. DOI: 10.1007/s10493-006-9019-0.

Gross R, Vavre F, Heddi A, Hurst GDD, Zchori-Fein E, Bourtzis K. 2009. Immunity and symbiosis. *Molecular Microbiology* 73:751–759. doi:10.1111/j.1365-2958.2009.06820.x

Gutiérrez D, Thomas CD 2000. Marginal range expansion in a host-limited butterfly species *Gonepteryx rhamni*. *Ecological Entomology*.

Gutzwiller F, Dedeine F, Kaiser W, Giron D, López-Vaamonde C 2015. Correlation between the green-island phenotype and Wolbachia infections during the evolutionary diversification of Gracillariidae leaf-mining moths. *Ecology and Evolution* 5:4049–4062. DOI: 10.1111/nph.12886.

Haine ER 2008. Symbiont-mediated protection. *Proceedings of the Royal Society B: Biological Sciences* 275:353–361. DOI: 10.1098/rspb.2007.1211.

Hamilton WDW 1967. Extraordinary sex ratios. A sex-ratio theory for sex linkage and inbreeding has new implications in cytogenetics and entomology. *Science* 156:477–488. DOI: 10.1126/science.156.3774.477.

Hamm CA, Handley CA, Pike A, Forister ML, Fordyce ML, Fordyce JA, Nice CC 2014. Wolbachia infection and Lepidoptera of conservation concern. *Journal of Insect Science* 14:6.

Hammer TJ, Janzen DH, Hallwachs W, Jaffe SP, Fierer N 2017. Caterpillars lack a resident gut microbiome. *Proceedings of the National Academy of Sciences* 114:9641–9646. DOI: 10.1073/pnas.1707186114.

Hansen AK, Moran NA 2014. The impact of microbial symbionts on host plant utilization by herbivorous insects. *Molecular ecology* 23:1473–1496. DOI: 10.1111/mec.12421.

Harumoto T, Anbutsu H, Lemaitre B, Fukatsu T 2016. Male-killing symbiont damages host's dosage-compensated sex chromosome to induce embryonic apoptosis. *Nature communications* 7:12781–12. DOI: 10.1038/ncomms12781.

Hassan SSM, Idris E 2013. Male-killing in African butterflies. *Trends in Evolutionary Biology* 5:2. DOI: 10.4081/eb.2013.e2.

Heath BD, Butcher RD, Whitfield WG, Hubbard SF 1999. Horizontal transfer of Wolbachia between phylogenetically distant insect species by a naturally occurring mechanism. *Current biology* 9:313–316.

Hedges LM, Brownlie JC, O’Neill SL, Johnson KN 2008. Wolbachia and virus protection in insects. *Science (New York, N.Y.)* 322:702–702. DOI: 10.1126/science.1162418.

Herren JK, Gordon I, Holland PWH, Smith D 2007. The butterfly Danaus chrysippus (Lepidoptera: Nymphalidae) in Kenya is variably infected with respect to genotype and body size by a maternally transmitted male-killing endosymbiont (Spiroplasma). *International Journal of Tropical Insect Science* 27:62. DOI: 10.1017/S1742758407818327.

Hertig M, Wolbach SB 1924. Studies on Rickettsia-Like Micro-Organisms in Insects. *The
Higashiura Y, Ishihara M, Schaefer PW 1999. Sex ratio distortion and severe inbreeding depression in the gypsy moth Lymantria dispar L. in Hokkaido, Japan. Heredity 83:290–297.

Hiroki M, KATO Y, Kamito T, Miura K 2002. Feminization of genetic males by a symbiotic bacterium in a butterfly, Eurema hecabe (Lepidoptera: Pieridae). Die Naturwissenschaften 89:167–170.

Hiroki M, Tagami Y, Miura K, Kato Y 2004. Multiple infection with Wolbachia inducing different reproductive manipulations in the butterfly Eurema hecabe. Proceedings of the Royal Society B: Biological Sciences 271:1751–1755. DOI: 10.1098/rspb.2004.2769.

Hoffman AA, Turelli M 1997. Cytoplasmic incompatibility in insects. In: O’Neill SL, Hoffman AA, Werren JH eds. Influential Passengers: Inherited Microorganisms and Arthropod Reproduction. Oxford: Oxford University Press, 42–80.

Hoffman AA, Turelli M, Harshman LG 1990. Factors affecting the distribution of cytoplasmic incompatibility in Drosophila simulans. Gene 126:933–948.

Hornett EA, Charlat S, Duplouy AMR, Davies N, Roderick GK, Wedell N, Hurst GDD 2006. Evolution of male-killer suppression in a natural population. PLoS biology 4:e283. DOI: 10.1371/journal.pbio.0040283.

Hornett EA, Charlat S, Wedell N, Jiggins CD, Hurst GDD 2009. Rapidly shifting sex ratio across a species range. Current biology 19:1628–1631. DOI: 10.1016/j.cub.2009.07.071.

Hornett EA, Duplouy AMR, Davies N, Roderick GK, Wedell N, Hurst GDD, Charlat S 2008. You can't keep a good parasite down: evolution of a male-killer suppressor uncovers cytoplasmic incompatibility. Evolution 62:1258–1263. DOI: 10.1111/j.1558-5646.2008.00353.x.

Hornett EA, Moran B, Reynolds LA, Charlat S, Tazzyman S, Wedell N, Jiggins CD, Hurst GDD 2014. The evolution of sex ratio distorter suppression affects a 25 cM genomic region in the butterfly Hypolimnas bolina. PLoS genetics 10:e1004822. DOI: 10.1371/journal.pgen.1004822.s011.

Hughes GL, Koga R, Rasgon JL 2011. Wolbachia infections are virulent and inhibit the human malaria parasite Plasmodium falciparum in Anopheles gambiae. PLoS Pathogens 7:e1002043. DOI: 10.1371/journal.ppat.1002043.

Hughes GL, Rivero A, Rasgon JL 2014. Wolbachia can enhance Plasmodium infection in mosquitoes: implications for malaria control? PLoS Pathogens 10:e1004182. DOI: 10.1371/journal.ppat.1004182.

Huigens ME, de Almeida RP, Boons PAH, Luck RF, Stouthamer R 2004. Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing Wolbachia in Trichogramma wasps. Proceedings of the Royal Society B: Biological Sciences 271:509–515. DOI: 10.1098/rspb.2003.2640.

Huigens ME, Luck RF, Klaffenbach RHG, Maas MFPM, Timmermans MJTN, Stouthamer R 1999. Infectious parthenogenesis. Nature 405:178–179. DOI: 10.1093/aesa/91.4.410.
Hunter MS, Perlman SJ, Kelly SE 2003. A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp Encarsia pergandiella. Proceedings of the Royal Society B: Biological Sciences 270:2185–2190. DOI: 10.1098/rspb.2003.2475.

Hurst GDD, Majerus MEN 1992. Why do maternally inherited microorganisms kill males? Heredity 71, 81–95.

Hurst G, Schilthuizen M 1998. Selfish genetic elements and speciation. Heredity 71, 81–95.

Hurst GDD, Jiggins FM 2000. Male-killing bacteria in insects: mechanisms, incidence, and implications. Emerging Infectious Diseases 6:329–336.

Hurst GDD, Jiggins FM 2005. Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. Proceedings of the Royal Society B: Biological Sciences 272:1525–1534. DOI: 10.1098/rspb.2005.3056.

Hurst GDD, Hurst LD, Majerus M 1997. Cytoplasmic sex-ratio distorters. In: O’Neill SL, Hoffmann AA, Werren JH eds. Influential Passengers: Inherited Microorganisms and Arthropod Reproduction. Oxford: Oxford University Press, 125–154.

Hurst GDD, Jiggins FM, Hinrich Graf von der Schulenburg J, Bertrand D, West SA, Goriacheva II, Zakharov IA, Werren JH, Stouthamer R, Majerus MEN 1999. Male-killing Wolbachia in two species of insect. Proceedings of the Royal Society B: Biological Sciences 266:735–740. DOI: 10.1098/rspb.1999.0698.

Hurst GD, Jiggins FM, Robinson SJ 2001. What causes inefficient transmission of male-killing Wolbachia in Drosophila? Heredity 87:220–226.

Hurst GD, Johnson AP, Schulenburg JH, Fuyama Y 2000. Male-killing Wolbachia in Drosophila: a temperature-sensitive trait with a threshold bacterial density. Gene 156:699–709.

Husnik F, McCutcheon JP 2017. Functional horizontal gene transfer from bacteria to eukaryotes. Nature Publishing Group:1–13. DOI: 10.1038/nrmicro.2017.137.

Hutchence KJ, Fischer B, Paterson S, Hurst GDD 2011. How do insects react to novel inherited symbionts? A microarray analysis of Drosophila melanogaster response to the presence of natural and introduced Spiroplasma. Molecular ecology 20:950–958. DOI: 10.1111/j.1365-294X.2010.04974.x.

Ilinsky Y, Kosterin OE 2017. Molecular diversity of Wolbachia in Lepidoptera: Prevalent allelic content and high recombination of MLST genes. Molecular Phylogenetics and Evolution 109:164–179. DOI: 10.1016/j.ympev.2016.12.034.

Jaenike J 2007. Spontaneous emergence of a new Wolbachia phenotype. Evolution 61:2244–2252. DOI: 10.1111/j.1558-5646.2007.00180.x.

Jaenike J, Polak M, Fiskin A, Helou M, Minhas M 2007. Interspecific transmission of endosymbiotic Spiroplasma by mites. Biology Letters 3:23–25.

Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ 2010. Adaptation via symbiosis: recent spread of a Drosophila defensive symbiont. Science 329:212–215. DOI: 10.1126/science.1188235.

Jiggins FM 2002. Widespread “hilltopping” in Acraea butterflies and the origin of sex-role-reversed swarming in Acraea encedon and A. encedana. African Journal of Ecology. 40: 228–231. DOI: 10.1146/annurev.ento.42.1.587

Jiggins FM 2003. Male-killing Wolbachia and mitochondrial DNA: selective sweeps, hybrid introgression and parasite population dynamics. Gene 164:5–12.

Jiggins FM, Bentley JK, Majerus MEN, Hurst GDD 2001. How many species are infected with Wolbachia? Cryptic sex ratio distorters revealed to be common by intensive sampling.
Proceedings of the Royal Society B: Biological Sciences 268:1123–1126. DOI: 10.1098/rspb.2001.1632.

Jiggins FM, Hurst GDD, Majerus MEN 1998. Sex ratio distortion in Acraea encedon (Lepidoptera: Nymphalidae) is caused by a male-killing bacterium. Heredity 81:87-91. DOI: 10.1046/j.1365-2540.1998.00357.x.

Jiggins FM, Hurst GD, Jiggins CD, d Schulenburg von JH, Majerus ME 2000a. The butterfly Danaus chrysippus is infected by a male-killing Spiroplasma bacterium. Parasitology 120:439–446.

Jiggins FM, Hurst G, Majerus M 2000. Sex-ratio-distorting Wolbachia causes sex-role reversal in its butterfly host. Proceedings of the Royal Society B: Biological Sciences 267:69–73.

Jiggins, Hurst, Dolman, Majerus 2000b. High-prevalence male-killing Wolbachia in the butterfly Acraea encedana. Journal of Evolutionary Biology 13:495–501. DOI: 10.1098/rspb.1998.0324.

Johnstone RA, Hurst GDD 1996. Maternally inherited male-killing microorganisms may confound interpretation of mitochondrial DNA variability. Biological Journal of the Linnean Society 58:453–470. DOI: 10.1006/bijl.1996.0047.

Juchault P, Rigaud T, Mocquard JP 1992. Evolution of sex-determining mechanisms in a wild population of Armadillidium vulgare Latr.(Crustacea, Isopoda): competition between two feminizing parasitic sex factors Heredity. 69:382-390

Kageyama D, Nishimura G, Hoshizaki S, Ishikawa Y 2002. Feminizing Wolbachia in an insect, Ostrinia furnacalis (Lepidoptera: Crambidae). Heredity 88:444–449.

Kageyama D, Nishimura G, Ohno S, Takanashi T, Hoshizaki S, Ishikawa Y 2004. Wolbachia infection and an all-female trait in Ostrinia orientalis and Ostrinia zagliaevi. Entomologia Experimentalis et Applicata 111:79–83. DOI: 10.1098/rspb.1998.0324.

Kageyama D, Traut W 2004. Opposite sex-specific effects of Wolbachia and interference with the sex determination of its host Ostrinia scapulalis. Proceedings of the Royal Society B: Biological Sciences 271:251–258. DOI: 10.1098/rspb.2003.2604.

Kageyama D, Narita S, Watanabe M 2012. Insect sex determination manipulated by their endosymbionts: incidences, mechanisms and implications. Insects 3:161–199. DOI: 10.3390/insects3010161.

Kageyama D, Ohno M, Sasaki T, Yoshido A, Konagaya T, Jouraku A, Kuwazaki S, Kanamori H, Katayose Y, Narita S, Miyata M, Riegler M, Sahara K 2017. Feminizing Wolbachia endosymbiont disrupts maternal sex chromosome inheritance in a butterfly species. Evolution Letters 1:232–244. DOI: 10.1038/hdy.2015.110.

Kaiser W, Huguet E, Casas J, Commin C, Giron D 2010. Plant green-island phenotype induced by leaf-miners is mediated by bacterial symbionts. Proceedings of the Royal Society B: Biological Sciences 277:2311–2319. DOI: 10.1098/rspb.2010.0214.

Kaltenpoth M, Göttler W, Herzner G, Strohm E 2005. Symbiotic bacteria protect wasp larvae from fungal infestation. Current biology : CB 15:475–479. DOI: 10.1016/j.cub.2004.12.084.

Kato Y 2000. Overlapping distribution of two groups of the butterfly Eurema hecabe differing in the expression of seasonal morphs on Okinawa-jima Island. Zoological Science. 17:539-547. DOI: 10.2108/0289-0003(2000)17[539:ODOTGO]2.0.CO;2

Kellner R 1999. What is the basis of pederin polymorphism in Paederus riparius rove beetles? The endosymbiotic hypothesis. Entomologia Experimentalis et Applicata. 93:41-49. DOI: 10.1046/j.1570-7458.1999.00560.x

Kellner R, Dettner K 1996. Differential efficacy of toxic pederin in deterring potential arthropod
predators of Paederus (Coleoptera: Staphylinidae) offspring. *Oecologia*. 107:293-300. DOI: 10.1007/s00442-006-0489-z.

Kern P, Cook JM, Kageyama D, Riegler M 2015. Double trouble: combined action of meiotic drive and *Wolbachia* feminization in *Euereia* butterflies. *Biological Letters* 11:20150095–20150095. DOI: 10.1098/rsbl.2015.0095.

Klassen L, Kambris Z, Cook PE, Walker T, Sinkins SP 2009. Horizontal gene transfer between *Wolbachia* and the mosquito *Aedes aegypti*. *BMC genomics* 10:33. DOI: 10.1186/1471-2164-10-33.

Kodandaramaiah U, Weingartner E, Janz N, Dalén L, Nylin S 2011. Population structure in relation to host-plant ecology and *Wolbachia* infestation in the comma butterfly. *Journal of Evolutionary Biology* 24:2173–2185. DOI: 10.1111/j.1420-9101.2011.02352.x.

Koukou K, Pavlikaki H, Kilias G, Werren JH, Bourtzis K, Alahiotis SN 2006. Influence of antibiotic treatment and *Wolbachia* curing on sexual isolation among *Drosophila melanogaster* cage populations. *Evolution* 60:87–96. DOI: 10.1111/j.0014-3820.2006.tb01084.x.

Laven H 1959. Speciation by cytoplasmic isolation in the *Culex pipiens*-complex. *Cold Spring Harbor symposia on quantitative biology* 24:166–173.

Lefèvre T, Adamo S, Biron DG, Hughes D, Misse D 2009. Invasion of the body snatchers: the diversity and evolution of manipulative strategies in host-parasite interactions. In: Webster J ed. *Advances in Parasitology*. Academic Press, 45–83.

Leonardo TE, Muiru GT 2003. Facultative symbionts are associated with host plant specialization in pea aphid populations. *Proceedings of the Royal Society B: Biological Sciences* 270 Suppl 2:S209–S212. DOI: 10.1098/rsbl.2003.0064.

LePage DP, Metcalf JA, Bordenstein SR, On J, Perlmutter JI, Shropshire JD, Layton EM, Funkhouser-Jones LJ, Beckmann JF, Bordenstein SR. 2017. Prophage WO Genes Recapitulate and Enhance *Wolbachia*-induced Cytoplasmic Incompatibility. *Nature* 543:243-247. DOI: 10.1038/nature21391.

Lewis Z, Champion de Crespigny FE, Sait SM, Tregenza T, Wedell N 2011. *Wolbachia* infection lowers fertile sperm transfer in a moth. *Biology Letters* 7:187–189. DOI: 10.1098/rsbl.2010.0605.

Li C-X, Shi M, Tian J-H, Lin X-D, Kang Y-J, Chen L-J, Qin X-C, Xu J, Holmes EC, Zhang Y-Z 2015. Unprecedented genomic diversity of RNA viruses in arthropods reveals the ancestry of negative-sense RNA viruses. *eLIFE* 4:e05378–26. DOI: 10.7554/eLife.05378.

Lindsey ARI, Werren JH, Richards S, Stouthamer R 2016. Comparative genomics of a parthenogenesis-inducing *Wolbachia* symbiont. *G3* 6:2113–2123. DOI: 10.1534/g3.116.028449.

Lohman DJ, Peggie D, Pierce NE, Meier R 2008. Phylogeography and genetic diversity of a widespread Old World butterfly, *Lampides boeticus* (Lepidoptera: Lycaenidae). *BMC evolutionary biology* 8:301. DOI: 10.1186/1471-2148-8-301.

Longdon B, Jiggins FM 2012. Vertically transmitted viral endosymbionts of insects: do sigma viruses walk alone? *Proceedings of the Royal Society B: Biological Sciences* 279:3889–3898. DOI: 10.1371/journal.pone.0026564.

Longdon B, Day JP, Schulz N, Leftwich PT, de Jong MA, Breuker CJ, Gibbs M, Obbard DJ, Wilfert L, Smith SCL, McGonigle JE, Houslay TM, Wright LI, Livraghi L, Evans LC, Friend LA, Chapman T, Vontas J, Kambouraki N, Jiggins FM 2017. Vertically transmitted rhabdoviruses are found across three insect families and have dynamic interactions with their
Łukasiewicz K, Sanak M, Węgrzyn G 2016. A lack of Wolbachia-specific DNA in samples from apollo butterfly (Parnassius apollo, Lepidoptera: Papilionidae) individuals with deformed or reduced wings. *Journal of Applied Genetics* 57:271–274. DOI: 10.1007/978-94-009-0343-2_2.

Ma W-J, Vavre F, Beukeboom LW 2014. Manipulation of arthropod sex determination by endosymbionts: diversity and molecular mechanisms. *Sexual Development* 8:59–73. DOI: 10.1159/000357024.

Maitland DP 1994. A parasitic fungus infecting yellow dungflies manipulates host perching behaviour. *Proceedings of the Royal Society B: Biological Sciences* 258:187-193 DOI: 10.1098/rspb.1994.0161

Margulis L, Fester R 1991. Symbiosis as a source of evolutionary innovation: speciation and morphogenesis. The MIT Press, Cambridge (Massachusetts)

Martinez J, Ben Longdon, Bauer S, Chan Y-S, Miller WJ, Bourtzis K, Teixeira L, Jiggins FM 2014. Symbionts commonly provide broad spectrum resistance to viruses in insects: a comparative analysis of Wolbachia strains. *PLoS Pathogens* 10:e1004369–e1004369. DOI: 10.1371/journal.ppat.1004369.

Mason CJ, Raffa KF 2014. Acquisition and structuring of midgut bacterial communities in Gypsy Moth (Lepidoptera: Erebidae) larvae. *Environmental entomology* 43:595–604. DOI: 10.1603/EN14031.s1.

Masui S, Kamoda S, Sasaki T, Ishikawa H 2000. Distribution and evolution of bacteriophage WO in Wolbachia, the endosymbiont causing sexual alterations in arthropods. *Journal of Molecular Evolution* 51:491–497. DOI: 10.1007/s002390010112.

McCutcheon JP, McDonald BR, Moran NA 2009. Convergent evolution of metabolic roles in bacterial co-symbionts of insects. *PNAS* 106:15394–15399. DOI: 10.1073/pnas.0906424106.

McHugh A, Bierzychudek P, Greever C, Marzulla T, Van Buskirk R, Binford G 2013. A molecular phylogenetic analysis of Speyeria and its implications for the management of the threatened Speyeria zerene hippolyta. *Journal of Insect Conservation* 17:1237–1253. DOI: 10.1038/hdy.2009.26.

McLaughlin RE, Myers J 1970. *Ophryocystis elektroscirrhra* sp. n., a neogregarine pathogen of the Monarch butterfly Danaus plexippus (L.) and the Florida Queen butterfly D. gilippus berenice Cramer. *Journal of Protozoology* 17:300–305.

Miller WJ, Schneider D 2012. Endosymbiotic microbes as adaptive manipulators of arthropod behavior and natural driving sources of host speciation. In: Hughes D, Brodeur J, Thomas F eds. *Host manipulation by parasites*. Oxford: Oxford University Press, 119–137.

Miller WJ, Ehrman L, Schneider D 2010. Infectious speciation revisited: impact of symbiont-depletion on female fitness and mating behavior of Drosophila paulistorum. *PLoS Pathogens* 6:e1001214–e1001214. DOI: 10.1371/journal.ppat.1001214.

Montllor CB, Maxmen A, Purcell AH 2002. Facultative bacterial endosymbionts benefit pea aphids Acyrthosiphon pisum under heat stress. *Ecological Entomology*. 27:189-195. DOI: 10.1006/jipa.2000.4927

Moran NA 2016. When obligate partners melt down. *mBio* 7:e01904–16. DOI: 10.1128/mBio.01904-16.

Moran NA, McCutcheon JP, Nakabachi A 2008. Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics* 42:165–190. DOI:
Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van den Hurk AF, Ryan PA, O’Neill SL 2009. A Wolbachia symbiont in Aedes aegypti limits infection with dengue, Chikungunya, and Plasmodium. Cell 139:1268–1278. DOI: 10.1016/j.cell.2009.11.042.

Morimoto S, Nakai M, Ono A, Kunimi Y 2001. Late male-killing phenomenon found in a Japanese population of the oriental tea tortrix, Homona magnanima (Lepidoptera: Tortricidae). Heredity 87:435–440.

Nakanishi K, Hoshino M, Nakai M, Kunimi Y 2008. Novel RNA sequences associated with late male killing in Homona magnanima. Proceedings of the Royal Society B: Biological Sciences 275:1249–1254. DOI: 10.1098/rspb.2008.0013.

Narita S, Kageyama D, HIROKI M, SANPEI T, HASHIMOTO S, KAMITOH T, KATO Y 2011. Wolbachia-induced feminisation newly found in Eurema hecabe, a sibling species of Eurema mandarina (Lepidoptera: Pieridae). Ecological Entomology 36:309–317. DOI: 10.1109/rspb.1998.0324.

Narita S, Kageyama D, Nomura M, Fukatsu T 2007. Unexpected mechanism of symbiont-induced reversal of insect sex: feminizing Wolbachia continuously acts on the butterfly Eurema hecabe during larval development. Applied and Environmental Microbiology 73:4332–4341. DOI: 10.1128/AEM.00145-07.

Narita S, Shimajiri Y, Nomura M 2008. Strong cytoplasmic incompatibility and high vertical transmission rate can explain the high frequencies of Wolbachia infection in Japanese populations of Colias erate polygraphus (Lepidoptera: Pieridae). Bulletin of Entomological Research 99:385. DOI: 10.1017/S0007485308006469.

Negri I, Pellecchia M, Mazzoglio PJ, Patetta A, Alma A 2006. Feminizing Wolbachia in Zyginidia pullula (Insecta, Hemiptera), a leafhopper with an XX/X0 sex-determination system. Proceedings of the Royal Society B: Biological Sciences 273:2409–2416. DOI: 10.1098/rspb.2006.3592.

Newton ILG, Clark ME, Kent BN, Bordenstein SR, Qu J, Richards S, Kelkar YD, Werren JH 2016. Comparative genomics of two closely related Wolbachia with different reproductive effects on hosts. Genome Biology and Evolution 8:1526–1542. DOI: 10.1093/gbe/evw096.

Nice CC, Gompert Z, Forister ML, Fordyce JA 2009. An unseen foe in arthropod conservation efforts: The case of Wolbachia infections in the Karner blue butterfly. Biological Conservation 142:3137–3146. DOI: 10.1016/j.biocon.2009.08.020.

Nikoh N, Tanaka K, Shibata F, Kondo N, Hizume M, Shimada M, Fukatsu T 2008. Wolbachia genome integrated in an insect chromosome: evolution and fate of laterally transferred endosymbiont genes. Genome Research 18:272–280. DOI: 10.1101/gr.7144908.

Nyholm SV, Graf J 2012. Knowing your friends: invertebrate innate immunity fosters beneficial bacterial symbioses. Nature Reviews Microbiology 10:815–827. DOI: 10.1038/nrmicro2894.

Oliver KM, Degnan PH, Hunter MS, Moran NA 2009. Bacteriophages encode factors required for protection in a symbiotic mutualism. Science 325:992–994. DOI: 10.1126/science.1174463.

Oliver KM, RUSSELL JA, Moran NA, Hunter MS 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. PNAS 100:1803–1807. DOI: 10.1073/pnas.0335320100.

Owen DF 1965. Change in sex ratio in an African butterfly. Nature 206:744.
Owen DF 1970. Inheritance of Sex ratio in the butterfly *Acraea encedon*. Nature 225:662–663. DOI: 10.1038/225662a0.

O’Neill SL, Hoffmann AA, Werren JH 1998. *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction*. Oxford University Press.

Paredes JC, Herren JK, Schüpfer F, Marin R, Claverol S, Kuo C-H, Lemaitre B, Béven L 2015. Genome sequence of the *Drosophila melanogaster* male-killing Spiroplasma strain MSRO endosymbiont. *mBio* 6:e02437–14. DOI: 10.1128/mBio.02437-14.

Parmesan C, Ryholm N, Stefanescu C, Hill JK 1999. Poleward shifts in geographical ranges of butterfly species associated with regional warming. *Nature*. 399:579-583

Patricelli D, Sielezniew M, Ponikwicka-Tyszko D, Ratkiewicz M, Bonelli S, Barbero F, Witek M, Buś MM, Rutkowski R, Balletto E 2013. Contrasting genetic structure of rear edge and continuous range populations of a parasitic butterfly infected by *Wolbachia*. *BMC evolutionary biology* 13:14. DOI: 10.1111/j.1420-9101.2011.02352.x.

Perlman SJ, Kelly SE, Hunter MS 2008. Population biology of cytoplasmic incompatibility: maintenance and spread of *Cardinium* symbionts in a parasitic wasp. *Gene* 178:1003–1011. DOI: 10.1534/genetics.107.083071.

Piel J, Höfer I, Hui D 2004. Evidence for a symbiosis island involved in horizontal acquisition of pederin biosynthetic capabilities by the bacterial symbiont of *Paederus fuscipes* beetles. *Journal of Bacteriology* 186:1280–1286.

Poinso D, Charlat S, Merçot H 2003. On the mechanism of *Wolbachia*-induced cytoplasmic incompatibility: Confronting the models with the facts. *BioEssays* 25:259–265. DOI: 10.1002/bies.10234.

Poulton EB 1914. W. A. Lamborn's Breeding Experiments upon *Acræa encedon* (Linn.), in the Lagos District of West Africa, 1910–1912. *Zoological Journal of the Linnean Society* 32:391–416. DOI: 10.1111/j.1096-3642.1914.tb01463.x.

Poulton EB 1923. All female families of *Hypolimnas bolina* L., bred in Fiji by H. W. Simmonds. *Proceedings of the Royal Entomological Society London*:9–12.

Poulton EB 1926. Sex-ratio of *Hypolimnas bolina* in Viti Levu, Fiji. *Proceedings of the Royal Entomological Society London* 1:29–32.

Rigaud T, Juchault P 1993. Conflict between feminizing sex ratio distorters and an autosomal masculinizing gene in the terrestrial isopod *Armadillidium vulgare* Latr. *Gene* 133:247–252.

Rigaud T, Juchault P, Mocquard JP 1997. The evolution of sex determination in isopod crustaceans. *BioEssays* 19:409416.

Riparbelli MG, Giordano R, Ueyama M, Callaini G 2012. *Wolbachia*-mediated male killing is associated with defective chromatin remodeling. *PLoS ONE* 7:e30045. DOI: 10.1371/journal.pone.0030045.s003.

Russell JA, Moran NA 2006. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proceedings of the Royal Society B: Biological Sciences* 273:603–610. DOI: 10.1098/rspb.2005.3348.

Russell JA, Funaro CF, Giraldo YM, Goldman-Huertas B, Suh D, Kronauer DJC, Moreau CS, Pierce NE 2012. A veritable menagerie of heritable bacteria from ants, butterflies, and beyond: broad molecular surveys and a systematic review. *PLoS ONE* 7:e51027. DOI: 10.1371/journal.pone.0051027.s014.

Russell JA, Goldman-Huertas B, Moreau CS, Baldo L, Stahlhut JK, Werren JH, Pierce NE 2009. Specialization and geographic isolation among *Wolbachia* symbionts from ants and lycaenid butterflies. *Evolution* 63:624–640. DOI: 10.1111/j.1558-5646.2008.00579.x.
Sahoo RK, Lohman DJ, Wahlberg N, Müller CJ, Brattström O, Collins SC, Peggie D, Aduse-Poku K, Kodandaramaiah U. 2018. Evolution of *Hypolimnas* butterflies (Nymphalidae): Out-of-Africa origin and Wolbachia-mediated introgression. *Molecular Phylogenetics and Evolution* 123:50-58. DOI: 10.1016/j.ympev.2018.02.001.

Sakamoto H, Ishikawa Y, Sasaki T, Kikuyama S, Tatsuki S, Hoshizaki S 2005. Transinfection reveals the crucial importance of Wolbachia genotypes in determining the type of reproductive alteration in the host. *Genetical Research* 85:205. DOI: 10.1017/S0016672305007573.

Sakamoto H, Kageyama D, Hoshizaki S, Ishikawa Y 2007. Sex-specific death in the Asian corn borer moth (*Ostrinia furnacalis*) infected with Wolbachia occurs across larval development. *Genome* 50:645–652.

Salunke BK, Salunkhe RC, Dhotre DP, Walujkar SA, Khandagale AB, Chaudhari R, Chandode RK, Ghate HV, Patole MS, Werren JH, Shouche YS 2012. Determination of Wolbachia diversity in butterflies from Western Ghats, India, by a multigene approach. *Applied and Environmental Microbiology* 78:4458–4467. DOI: 10.1128/AEM.07298-11.

Sasaki T, Ishikawa H 1999. Wolbachia infections and cytoplasmic incompatibility in the Almond moth and the Mediterranean Flour moth. *Zoological Science* 16:739–744. DOI: 10.2108/zsj.16.739.

Sasaki T, Kubo T, Ishikawa H 2002. Interspecific transfer of Wolbachia between two Lepidopteran insects expressing cytoplasmic incompatibility: A Wolbachia variant naturally infecting *Cadra cautella* causes male killing in *Ephestia kuehniella*. *Gene* 162:1313–1319.

Shao Y, Chen B, Sun C, Ishida K, Hertweck C, Boland W 2017. Symbiont-derived antimicrobials contribute to the control of the Lepidopteran gut microbiota. *Cell Chemical Biology* 24:66–75. DOI: 10.1016/j.chembior.2016.11.015.

Smith DAS, Gordon IJ, Allen JA 2010. Reinforcement in hybrids among once isolated semispecies of *Danaus chrysippus*(L.) and evidence for sex chromosome evolution. *Ecological Entomology* 35:77–89. DOI: 10.1111/j.1365-2311.1943.tb00432.x.

Smith DAS, Gordon IJ, Traut W, Herren J, Collins S, Martins DJ, Saitoti K, Ireri P, ffrench-Constant R 2016. A neo-W chromosome in a tropical butterfly links colour pattern, male-killing, and speciation. *Proceedings of the Royal Society B: Biological Sciences* 283:20160821. DOI: 10.1007/s10577-011-9262-z.
(L.) in a broad hybrid zone, with special reference to sex ratio, polymorphism and intragenomic conflict. *Biological Journal of the Linnean Society* 65:1–40. DOI: 10.1006/bijl.1998.0240.

Snook RR, Cleland SY, Wolfrer MF, Karr TL 2000. Offsetting effects of *Wolbachia* infection and heat shock on sperm production in *Drosophila simulans*: analyses of fecundity, fertility and accessory gland proteins. *Gene* 155:167–178.

Stouthamer R, Breeuwer JAJ, Hurst GDD 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Microbiology* 53:71–102. DOI: 10.1146/annurev.micro.53.1.71.

Sugimoto TN, Ishikawa Y 2012. A male-killing *Wolbachia* carries a feminizing factor and is associated with degradation of the sex-determining system of its host. *Biology Letters* 8:412–415. DOI: 10.1007/s00114-002-0303-5.

Sugimoto TN, Fujii T, Kayukawa T, Sakamoto H, Ishikawa Y 2010. Expression of a doublesex homologue is altered in sexual mosaics of *Ostrinia scapulalis* moths infected with *Wolbachia*. *Insect Biochemistry and Molecular Biology* 40:847–854. DOI: 10.1016/j.ibmb.2010.08.004.

Sugimoto TN, Kayukawa T, Shinoda T, Ishikawa Y, Tsuchida T 2015. Misdirection of dosage compensation underlies bidirectional sex-specific death in *Wolbachia*-infected *Ostrinia scapulalis*. *Insect Biochemistry and Molecular Biology* 66:72–76. DOI: 10.1016/j.ibmb.2015.10.001.

Sutton ER, Harris SR, Parkhill J, Sinkins SP 2014. Comparative genome analysis of *Wolbachia* strain wAu. *BMC genomics* 15:928. DOI: 10.1016/0378-1119(95)00657-5.

Tabata J, Hattori Y, Sakamoto H, Yukuhiro F, Fujii T, Kugimiya S, Mochizuki A, Ishikawa Y, Kageyama D 2011. Male killing and incomplete inheritance of a novel *Spiroplasma* in the moth *Ostrinia zagulaevi*. *Microbial Ecology* 61:254–263. DOI: 10.1007/s00248-010-9799-y.

Tagami Y, Miura K 2004. Distribution and prevalence of *Wolbachia* in Japanese populations of Lepidoptera. *Insect Molecular Biology* 13:359–364. DOI: 10.1111/j.0962-1075.2004.00492.x.

Teixeira L, Ferreira A, Ashburner M 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS biology* 6:e2–e2. DOI: 10.1371/journal.pbio.1000002.

Telschow A, Engelstader J, Yamamura N, Hammerstein P, Hurst GDD 2006. Asymmetric gene flow and constraints on adaptation caused by sex ratio distorters. *Journal of Evolutionary Biology* 19:869–878. DOI: 10.1111/j.1420-9101.2005.01049.x.

Telschow A, Hammerstein P, Werren JH 2005. The effect of *Wolbachia* versus genetic incompatibilities on reinforcement and speciation. *Evolution* 59:1607–1619. DOI: 10.2307/3449068.

Thompson JN 1987. Symbiont-induced speciation. *Biological Journal of the Linnean Society*. 32:385-393.

Triant DA, Cinel SD, Kawahara AY 2018. Lepidoptera genomes: current knowledge, gaps and future directions. *Current Opinion in Insect Science* 25:99–105. DOI: 10.1016/j.cois.2017.12.004.

Tsuchida T, Koga R, Horikawa M, Tsunoda T, Maoka T, Matsumoto S, Simon J-C, Fukatsu T 2010. Symbiotic bacterium modifies aphid body color. *Science* 330:1102–1104. DOI: 10.1146/annurev-ento-112408-085305.

Vala F, Egas M, Breeuwer JAJ, Sabelis MW 2004. *Wolbachia* affects oviposition and mating
behaviour of its spider mite host. *Journal of Evolutionary Biology* 17:692–700. DOI: 10.1046/j.1420-9101.2003.00679.x.

Vavre FF, Girin CC, Boulétreau MM 1999. Phylogenetic status of a fecundity-enhancing *Wolbachia* that does not induce thelytoky in *Trichogramma*. *Insect Molecular Biology* 8:67–72. DOI: 10.1046/j.1365-2583.1999.810067.x.

Vavre F, Fleury F, Lepetit D, Fouillet P, Boulétreau M 1999. Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. *Molecular Biology and Evolution* 16:1711–1723.

Veneti Z, Bentley JK, Koana T, Braig HR, Hurst GDD 2005. A functional dosage compensation complex required for male killing in *Drosophila*. *Science* 307:1461–1463. DOI: 10.1126/science.1107182.

Veneti Z, Toda MJ, Hurst GD 2004. Host-resistance does not explain variation in incidence of male-killing bacteria in *Drosophila bifasciata*. *BMC evolutionary biology* 4:52. DOI: 10.1186/1471-2148-4-52.

Wallin IE 1927. *Symbionticism and the Origin of Species*. Baltimore: Williams & Wilkins.

Ward CM, Baxter SW 2017. Draft genome assembly of a *Wolbachia* endosymbiont of *Plutella australiana*. *Genome Announcements* 5:e01134–17. DOI: 10.1101/189266.

Watanabe K, Yukuhiro F, Matsuura Y 2014. Intrasperm vertical symbiont transmission. *PNAS*. 111:7433-7437 DOI: 10.1186/1741-7007-7-6

Weeks AR, Stouthamer R 2004. Increased fecundity associated with infection by a cytophaga-like intracellular bacterium in the predatory mite, *Metaseiulus occidentalis*. *Proceedings of the Royal Society B: Biological Sciences* 271:S193–5. DOI: 10.1098/rsbl.2003.0137.

Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA 2007. From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *PLoS biology* 5:e114. DOI: 10.1371/journal.pbio.0050114.

Weinert LA, Araujo-Jnr EV, Ahmed MZ, Welch JJ 2015. The incidence of bacterial endosymbionts in terrestrial arthropods. *Proceedings of the Royal Society B: Biological Sciences* 282:20150249–20150249. DOI: 10.1186/1471-2148-7-238.

Werren JH 1998. *Wolbachia* and speciation. In: Howard DJ, Berlocher SH eds. *Endless forms: species and speciation*. New York: Oxford University Press, 245–260.

Werren JH, Richards S, Desjardins CA, Niehuis O, Gadau J, Colbourne JK, Nasonia Genome Working Group, Werren JH, Richards S, Desjardins CA, Niehuis O, Gadau J, Colbourne JK, Beukeboom LW, Desplan C, Elsik CG, Grimmelikhuijzen CJP, Kitts P, Lynch JA, Murphy T, Oliveira DCSG, Smith CD, van de Zande L, Worley KC, Zdobnov EM, Aerts M, Albert S, Anaya VH, Anzola JM, Barchuk AR, Behura SK, Bera AN, Berenbaum MR, Bertossa RC, Bitondi MMG, Bordenstein SR, Bork P, Bornberg-Bauer E, Brunain M, Cazzamali G, Chabouab L, Chacko J, Chavez D, Childers CP, Choi J-H, Clark ME, Claudianos C, Clinton RA, Cree AG, Cristino AS, Dang PM, Darby AC, de Graaf DC, Devreese B, Dinh HH, Edwards R, Elango N, Elhaik E, Ermolaeva O, Evans JD, Foret S, Fowler GR, Gerlach D, Gibson JD, Gilbert DG, Graur D, Gründler S, Hagen DE, Han Y, Hauser F, Hultmark D, Hunter HC, Hurst GDD, Jhangian SN, Jiang H, Johnson RM, Jones AK, Junier T, Kadokawa T, Kamping A, Kapustin Y, Kecharavzi B, Kim J, Kim J, Kiryutin B, Koevoets T, Kovar CL, Kriventseva EV, Kucharski R, Lee H, Lee SL, Lees K, Lewis LR, Loehlin DW, Logsdon JM, Lopez JA, Lozano RJ, Maglott D, Maleszka R, Mayampurath A, Mazur DJ,
McClure MA, Moore AD, Morgan MB, Muñoz Torres MC, Muzny DM, Nazareth LV, Neupert S, Nguyen NB, Nunes FMF, Oakeshott JG, Okwuonu GO, Pannebakker BA, Pejaver VR, Peng Z, Pratt SC, Predel R, Pu L-L, Ranson H, Raychoudhury R, Rechtsteiner A, Reese JT, Reid JG, Riddle M, Robertson HM, Romero-Severson J, Rosenberg M, Sackton TB, Satelle DB, Schlühs H, Schmitt T, Schneider M, Schüler A, Schurko AM, Shuker DM, Simões ZLP, Sinha S, Smith Z, Solovyev V, Souvorov A, Springauf A, Stafflinger E, Stage DE, Stanke M, Tanaka Y, Telschow A, Trent C, Vattathil S, Verhulst EC, Viljakainen L, Wanner KW, Waterhouse RM, Whitfield JB, Wilkes TE, Williamson M, Willis JH, Wolschin F, Wyder S, Yamada T, Yi SV, Zecher CN, Zhang L, Gibbs RA 2010. Functional and evolutionary insights from the genomes of three parasitoid Nasonia species. Science 327:343–348. DOI: 10.1126/science.1178028.

Werren JH, Windsor D, Guo L 1995. Distribution of Wolbachia among neotropical arthropods. Proceedings of the Royal Society B: Biological Sciences 262:197–204. DOI: 10.1098/rspb.1995.0196.

Werren JH, Zhang W, Guo LR 2004. Evolution and phylogeny of Wolbachia: reproductive parasites of arthropods. 261:55–71.

Wolinska J, King KC 2009. Environment can alter selection in host–parasite interactions. Trends in Parasitology 25:236–244. DOI: 10.1016/j.pt.2009.02.004.

Wybouw N, Dermauw W, Tirry L, Stevens C, Grbić M, Feyereisen R, Van Leeuwen T 2014. A gene horizontally transferred from bacteria protects arthropods from host plant cyanide poisoning. eLIFE 3:e02365. DOI: 10.7554/eLife.02365.019.

Xie J, Vilchez I, Mateos M 2010. Spiroplasma bacteria enhance survival of Drosophila hydei attacked by the parasitic wasp Leptopilina heterotoma. PLoS ONE 5:e12149. DOI: 10.1371/journal.pone.0012149.t001.

Xu P, Liu Y, Graham RI, Wilson K, Wu K 2014. Densovirus is a mutualistic symbiont of a global crop pest (Helicoverpa armigera) and protects against a Baculovirus and Bt biopesticide. PLoS Pathogens 10:e1004490. DOI: 10.1371/journal.ppat.1004490.s008.

Yamazaki K, Sugiura S 2004. Epizootics and behavioral alteration in the arctiid caterpillar Chionarctia nivea (Lepidoptera: Arctiidae) caused by an entomopathogenic fungus, Entomophaga aulicae (Zygomycetes: Entomophthorales). Entomological Science. 7: 219-223. DOI: 10.1111/j.1479-8298.2004.00066.x

Yen JH, Barr AR 1971. New hypothesis of the cause of cytoplasmic incompatibility in Culex pipiens L. Nature 232:657–658. DOI: 10.1038/232657a0.

Yen JH, Barr AR 1973. The etiological agent of cytoplasmic incompatibility in Culex pipiens. Journal of Invertebrate Pathology 22:242–250.

Zug R, Hammerstein P 2017. Evolution of reproductive parasites with direct fitness benefits. Heredity:1–16. DOI: 10.1038/s41437-017-0022-5.
Table 1

Lepidoptera species carrying heritable endosymbionts that manipulate host reproduction

A list of butterfly and moth species that have been recorded as carrying heritable endosymbionts that manipulate the reproduction of the host. Endosymbiont induced phenotypes are given as MK: Male-killing, Late MK: Male-killing occurring late in development; CI: Cytoplasmic Incompatibility; Feminisation; or Sex-ratio distortion (where further investigation is needed to determine the nature of the sex-ratio bias).
| Host          | Endosymbiont | Phenotype               | Source                                      |
|--------------|--------------|-------------------------|---------------------------------------------|
| **Butterflies** |              |                         |                                             |
| **Lycaenidae** |              |                         |                                             |
| Talicada nyseus | Wolbachia    | Sex-ratio distortion    | Ankola et al. 2011                          |
| Zizina emelina | Wolbachia    | MK                      | Sakamoto et al. 2011                        |
| **Nymphalidae** |              |                         |                                             |
| Acraea acerata | Wolbachia    | CI                      | Jiggins et al. 2001                         |
| Acraea ecedana | Wolbachia    | MK                      | Jiggins et al. 2000a                        |
| Acraea encledon| Wolbachia    | MK                      | Jiggins et al. 1998; Jiggins et al. 2000a   |
| Acraea eponina | Wolbachia    | MK                      | Jiggins et al. 2001                         |
| Acraea stoikensis | Wolbachia | MK                      | Hassan & Idris 2013                         |
| Danaus chrysippus |             | Spiroplasma ixodetis    | Jiggins et al. 2000b; Dyson et al. 2002; Charlat et al. 2006; Hornett et al. 2008 |
| Hypolimnas bolina | Wolbachia    | MK &/or CI              |                                             |
| **Pieridae**   |              |                         |                                             |
| Colias erate poligraphus | Wolbachia | CI                      | Narita et al. 2009                          |
| Eurema hecabe  | Wolbachia    | Feminisation, CI        | Narita et al. 2011                          |
| Eurema mandarina | Wolbachia    | Feminisation, CI        | Hiroki et al. 2002; Hiroki et al. 2004      |
| **Moths**      |              |                         |                                             |
| **Crambidae**  |              |                         |                                             |
| Ostrinia furnacalis | Wolbachia | MK                      | Kageyama et al. 2002                        |
| Ostrinia orientalis | Wolbachia | Sex-ratio distortion    | Kageyama et al. 2004                        |
| Ostrinia scapulalis | Wolbachia | MK                      | Kageyama & Traut 2004                       |
| Ostrinia zagulaevi |             | Wolbachia, Spiroplasma ixodetis | Kageyama et al. 2004; Tabata et al. 2011 |
| Ostrinia zealis | Undefined agent | Sex-ratio distortion | Kageyama et al. 2004                       |
| **Erebidae**   |              |                         |                                             |
| Lymnantria dispar | Undefined agent | MK                      | Higashiru et al. 1999                       |
| **Noctuidae**  |              |                         |                                             |
| Cerapteryx graminis | Spiroplasma sp. | Sex-ratio distortion | Graham et al. 2011                          |
| Spodoptera exempta | Wolbachia    | MK                      | Graham & Wilson 2012                        |
| Spodoptera littoralis | Undefined agent |                                           | Brimacombe 1980                             |
| **Plutellidae** |              |                         |                                             |
| Plutella xylostella | Wolbachia    | Sex-ratio distortion    | Delgado & Cook 2009                         |
| **Pyralidae**  |              |                         |                                             |
| Cadra cautella  | Wolbachia    | CI                      | Sasaki & Ishikawa 1999                      |
| *Ephestia kuehniella* | Wolbachia    | CI                      | Sasaki & Ishikawa 1999                      |
| **Tortricidae** |              |                         |                                             |
| Epiphyas postvittana | Undefined agent | MK                      | Geier et al. 1978                           |
| Homona magnanima | RNA virus    | Late MK                 | Morimoto et al. 2001; Nakanishi et al. 2008 |
In the Lepidoptera, endosymbionts are currently known to manipulate host reproduction in three ways in order to increase their transmission to the next generation. Male-killing: female hosts infected with male-killing endosymbionts only give rise to infected female offspring, with male offspring dying early in development. Feminisation: female hosts infected with feminising endosymbionts only give rise to infected female offspring, with male offspring having been feminised so that they are genetically male (ZZ) but phenotypically female. Uninfected males may arise through inefficient transmission of the infection. Cytoplasmic incompatibility (CI): crosses between uninfected females and infected males result in few or no viable offspring, as the result of an incompatibility induced by the endosymbiont in the male. Infected females are able to rescue this incompatibility and hence are able to produce viable (infected) offspring when mated with infected males. For male-killing and feminisation the endosymbiont acts as a sex-ratio distorter, creating a female-bias in the offspring, and potentially in the population if the infection is highly prevalent.
|                  | Uninfected | Male-killing (MK) | Feminisation | Cytoplasmic incompatibility (CI) |
|------------------|------------|-------------------|--------------|-------------------------------|
| ![endosymbiont](image) | ![endosymbiont](image) | ![endosymbiont](image) | ![endosymbiont](image) | ![endosymbiont](image) |
| ![endosymbiont](image) | ![endosymbiont](image) | ![endosymbiont](image) | ![endosymbiont](image) | ![endosymbiont](image) |
| ![endosymbiont](image) | ![endosymbiont](image) | ![endosymbiont](image) | ![endosymbiont](image) | ![endosymbiont](image) |
| SRD              | SRD        |                   |              |                               |
| ![injured](image) | ![injured](image) | ![injured](image) | ![injured](image) | ![injured](image) |

- **Endosymbiont uninfected**
- **Endosymbiont infected**
- **Injured/**killed**
- **SRD:** Sex ratio distortion
Figure 2

*Wolbachia*-induced male-killing and interference of sex determination in *Ostrinia scapulalis* moths

A) Uninfected females gives rise to a normal 1:1 sex ratio in progeny: female offspring have ZW sex chromosomes and express the female isoform of the *Ostrinia* homologue of a gene in the sex determination cascade, *doublesex (dsx)*, called *OsdsxF*; male offspring have two Z sex chromosomes and express the male *dsx* isoform *OsdsxM*. B) *Wolbachia* infected females only give rise to infected female progeny. Male offspring die early in development due to a mismatch between the genotypic sex (ZZ) and phenotypic sex (*OsdsxF*). C) *Wolbachia*-infected females cured of the infection as larvae by antibiotic treatment only give rise to uninfected males. Female offspring die early in development due to a mismatch between their genotypic sex (ZW) and phenotypic sex (*OsdsxM*). D) *Wolbachia*-infected females cured of the infection as adults prior to oviposition by antibiotic treatment give rise to sexual mosaics which have the male ZZ genotype but both *OsdsxF* and *OsdsxM*. Note: there are two female isoforms of *dsx in Ostrinia scapulalis*: *OsdsxFL* and *OsdsxFS*; these are simplified to *OsdsxF* in this schematic. White circles: uninfected individual; Red circles: *Wolbachia*-infected individual; Dark grey circle: *Wolbachia*-infected female cured as larva; Light grey circle: *Wolbachia*-infected female cured as adult.
|   | A) Uninfected | B) Wolbachia infected (larval antibiotic treatment) | C) Wolbachia cured (adult antibiotic treatment) |
|---|----------------|--------------------------------------------------|-----------------------------------------------|
|   | ![Diagram](image1) | ![Diagram](image2) | ![Diagram](image3) |
| ZW | Osdx^f          | ZW Osdx^f & Osdx^m                                | ZZ Osdx^m & Osdx^m |
| ZZ | Osdx^m          | ZZ Osdx^m                                        | ZZ Osdx^m |

Notes: ( diagrams for interpretation)