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.

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2 **endosymbionts**

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4 Anne Duplouy[1] & Emily A. Hornett[2]

5 [1] The University of Helsinki, PO Box 65 (Viikinkaari 1), FI-00014 University of Helsinki,

6 Finland.

7 [2] The University of Cambridge, Department of Zoology, Cambridge, CB2 3EJ, United Kingdom

8

9 Corresponding author:

10 Emily Hornett[2]

11 Email address: eh481@cam.ac.uk

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13   **Abstract**

14   The Lepidoptera is one of the most widespread and recognisable insect orders. Due to their

15   remarkable diversity, economic and ecological importance, moths and butterflies have been

16   studied extensively over the last 200 years. More recently, the relationship between Lepidoptera

17   and their heritable microbial endosymbionts has received increasing attention. Heritable

18   endosymbionts reside within the host's body and are often, but not exclusively, inherited through

19   the female line. Advancements in molecular genetics have revealed that host-associated microbes

20   are both extremely prevalent among arthropods and highly diverse. Furthermore, heritable

21   endosymbionts have been repeatedly demonstrated to play an integral role in many aspects of

22   host biology, particularly host reproduction. Here, we review the major findings of research of

23   heritable microbial endosymbionts of butterflies and moths. We promote the Lepidoptera as

24   important models in the study of reproductive manipulations employed by heritable

25   endosymbionts, with the mechanisms underlying male-killing and feminisation currently being

26   elucidated in both moths and butterflies. We also reveal that the vast majority of research

27   undertaken of Lepidopteran endosymbionts concerns *Wolbachia*. While this highly prevalent

28   bacteria is undoubtedly important, studies should move towards investigating the presence of

29   other, and interacting endosymbionts, and we discuss the merits of examining the microbiome of

30   Lepidoptera to this end. We finally consider the importance of understanding the influence of

31   endosymbionts under global environmental change and when planning conservation

32   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; Engelstädter & 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,

56   2010), immunity (Gross et al., 2009; Nyholm & Graf, 2012), behaviour (Dion *et al.*, 2011), body

57   colour (Tsuchida et al., 2010), nutritional stress resistance (Brownlie *et al.*, 2009), pathogen load

58   (Graham & Wilson, 2012), dispersal (Goodacre *et al.*, 2009), host plant specialisation (Leonardo

59   & Muiru, 2003), thermal tolerance (Dunbar *et al.*, 2007), nutrition (Douglas, 1998) and

60   metabolism (McCutcheon, McDonald & Moran, 2009). Furthermore, symbiosis has been

61   purported to be a key factor underlying natural variation, as well as an instigator of novelty and a

62   promoter of speciation (Margulis & Fester, 1991; Brucker & Bordenstein, 2012).

63   Since the advent of the diagnostic PCR assay in the mid-1980s, organisms can be

64   routinely screened for known endosymbionts. As a consequence of this development and recent

65   advancements in genomics and bioinformatics (including high-throughput amplicon sequencing

66   of microbial genes and metagenomics), we now recognise that all organisms are infected by a

67   diverse range of microbes, including viruses, fungi and bacteria, and that many arthropods carry

68   heritable endosymbionts. A recent study estimated that 52% of terrestrial arthropod species are

69   infected with the intracellular bacteria *Wolbachia*, with a further 24% and 13% species infected

70   with *Cardinium* and *Rickettsia* bacteria, respectively (Weinert et al., 2015). How species initially

71   acquire heritable endosymbionts is not yet fully understood. While phylogenetic evidence

72   suggests that horizontal transfer of endosymbionts on an evolutionary scale must be common,

73   many barriers - ecological, geographical and physiological - exist that perturb the spread of

74   endosymbionts between species and prevent the formation of novel symbioses. Successful

75   transfer of an endosymbiont between species depends on the ability of the microbe to first enter

76   and then survive in a novel host environment, followed by successful migration to the host

77   germline to ensure propagation. The symbiont must then be able to invade the host population, or

78   at least be maintained at low frequency. Thus the 'fit' between a host and symbiont can be quite

79   specific, with host biology playing an important role in the ability of the symbiont to thrive in the

80   novel species. Failure in the formation of persistent associations may also be due to the

81   endosymbiont causing harm to their new hosts (Hutchence *et al.*, 2011). Where movement of

82   heritable endosymbionts has been observed, it is often via ecological connectors such as shared

83   host food sources (Huigens et al., 2000; Duron, Wilkes & Hurst, 2010; Caspi-Fluger et al., 2012;

84   Chrostek et al., 2017) or common symbiont-vector parasites or parasitoids (Heath et al., 1999;

85   Vavre et al., 1999; Huigens et al., 2004; Jaenike et al., 2007; Gehrer & Vorburger, 2012).

86   Horizontal transfer is perhaps more successful between related hosts (Russell et al., 2009); it has

87   been suggested that within *Acraea* butterflies, *Wolbachia* has moved between species either via a

88   common parasitoid, or through hybridisation and subsequent introgression. It is also possible that

89   the different species inherited the bacteria from a recent common ancestor (Jiggins et al., 2000b).

90   The Lepidoptera are remarkably diverse and widely recognisable, encompassing

91   butterflies and moths that are economically and ecologically important. While many aspects of

92   Lepidopteran biology have been well studied, it is only recently that the pervasiveness of host-

93   associated microbes in this group has been appreciated. Heritable endosymbionts have been the

94   subject of several reviews (Bandi et al., 2001; Moran, McCutcheon & Nakabachi, 2008; Duron

95   & Hurst, 2013), and here we focus upon studies of these influential elements in the Lepidoptera.

96   Butterflies and moths are particularly important in the study of heritable endosymbionts due to

97   the Lepidoptera sex determination system. In contrast to most other arthropod groups, the female

98   is the heterogametic sex (females have one Z and one W sex chromosome, males have two Z

99   chromosomes). The mechanisms and repercussions of reproductive manipulations caused by

100   inherited microbial endosymbionts, which are commonly observed in butterflies and moths, are

101   therefore likely to be very different from that observed in arthropods with alternative sex

102 determination systems. Furthermore, in the Lepidoptera heritable endosymbiont prevalence is

103 commonly very high, and vertical transmission of the infection is often near perfect. Together

104 with the maternal inheritance of intracellular endosymbionts such as *Wolbachia*, this creates

105 linkage of the infection not only with similarly maternally inherited host mitochondria, but also

106 with the female W chromosome. Formation of this wider co-inherited network may have

107 implications for host genetic diversity and even the sex determination system itself.

108 In this review we summarise the main body of research that has been conducted to date in

109 order to form a springboard for future work and to emphasise to researchers from traditionally

110 disparate fields as ecology, genomics and conservation, that in order to fully understand the

111 biology of an organism, one must take into account its endosymbionts. For clarity this review is

112 divided into areas of current research: 1) Manipulation of host reproduction; 2) Impact upon host

113 fitness; 3) Symbiont-mediated protection; 4) Host genetics and 5) Behavioural modification. We

114 then highlight outstanding questions and future directions, including consideration of the

115 influence of endosymbionts under global environmental change, and in species of conservation

116 concern.

117

118 **Survey methodology**

119 The authors have drawn upon knowledge gained from over a decade in butterfly-endosymbiont

120 research. Extensive literature searches were performed using repositories such as NCBI PubMed

121 and Google Scholar, and using keywords including "endosymbiont", "microbe" and "heritable

122 symbiont", along with "butterfly", "moth" and "Lepidoptera". Social media platforms such as

123 Twitter provided a useful tool to obtain up to date information of relevant publications. Research

124 on heritable endosymbionts of arthropods in general was also gathered with the aim to provide

125 information about areas in heritable endosymbiont-arthropod research that is lacking for

126 Lepidoptera. Particular effort was made to compile a comprehensive list of butterfly and moth

127 species that are published as infected with heritable endosymbionts.

128

129 **The influence of heritable microbial endosymbionts on Lepidopteran biology**

130 Concordant with general insect surveys, the Lepidoptera are commonly infected with heritable

131 microbial endosymbionts. In an early screen of Panamanian arthropods, *Wolbachia* was detected

132 in 16.3% of the 43 Lepidoptera species tested (Werren, Windsor & Guo, 1995). Further surveys

133 identified *Wolbachia* in 29% of 24 species of *Acraea* butterflies from Uganda (Jiggins *et al.*,

134 2001), 45% of 49 species of butterflies studied in Japan (Tagami & Miura, 2004), 50% of 56

135 Indian butterfly species (Salunke *et al.*, 2012), 58.3% of 120 Lepidoptera species in West Siberia

136 (Ilinsky & Kosterin, 2017), and 79% of 24 species of African *Bicyclus* butterflies (Duplouy &

137 Brattström, 2017). Additionally, in a broad survey of ants, moths and butterflies (specifically

138 Lycaenidae and Nymphalidae) for five heritable symbionts, *Wolbachia* (39 of 158 species) and

139 *Spiroplasma* (5 of 200 species) were found to infect Lepidopteran species (Russell et al., 2012).

140 In general, these estimates are likely to be highly conservative, due to the presence of undetected

141 low frequency infections, geographical and temporal variation in infection, tissue-specificity and

142 PCR false negatives. Geographic structure in infection incidence and prevalence is a particularly

143 important consideration and especially evident in endosymbiont-Lepidoptera systems e.g.

144 *Wolbachia-Hypolimnas bolina* butterflies (Charlat et al., 2005). In a recent survey of published

145 records of *Wolbachia* infections in the Lepidoptera, generalised geographic structure in infection

146 frequency was observed, with lower frequencies towards higher latitudes (Ahmed et al., 2015).

147   In Table S1 and Table S2 we compile a comprehensive list of butterfly and moth species,

148   respectively, reported as carrying heritable endosymbionts from published sources. We find that

149   research of heritable endosymbionts in Lepidoptera is heavily dominated by studies of

150   *Wolbachia* as opposed to that of other infections (*Wolbachia* in 248/253 butterfly species and in

151   109/115 moth species). While arthropod-infecting endosymbiont diversity is notable, including

152   such divergent taxa as *Rickettsia, Spiroplasma, Arsenophonus, Flavobacteria, Cardinium* and the

153   microsporidia, much of the early arthropod endosymbiont literature focused upon the

154   Alphaproteobacteria genus *Wolbachia* (Hertig & Wolbach, 1924). Due to its presence in many

155   agricultural pests and disease vectors, and also owing to the range of reproductive manipulations

156   it employs in the host, *Wolbachia* is still widely, but justly, studied.

157   Tables S1 and S2 reveal that *Wolbachia* is common across Lepidopteran families, being

158   found in all five families of 'true' butterflies (the Papilionoidea), and also in the skippers

159   (Hesperiidae). *Wolbachia* strains have been divided into separate genetic lineages termed

160   supergroups. It is clear from the compiled data that the *Wolbachia* strains carried by Lepidoptera

161   are almost exclusively from supergroups A and B, with B group*Wolbachia* predominating over

162   A group *Wolbachia*. Of species where the *Wolbachia* supergroup has been determined (80/109

163   moths and 208/248 butterflies), 85% of moth species carry B group and 25% A group; while

164   79% of butterfly species carry B group *Wolbachia* and 26% carry A group. Note that these data

165   include multiple infections (*i.e.* some species harbour both A and B strains of *Wolbachia*). These

166   findings concur with research analysing 90 *Wolbachia* strains associated with Lepidoptera: 84%

167   of the strains belonged to supergroup B (76/90), with the remainder (14/90) belonging to

168   supergroup A (Ahmed et al. 2016). A further study identified 22 *Wolbachia*-infected Lepidoptera

169   species in Japan, 19 of which had infections from supergroup B (86%), with the remaining three

170   from supergroup A (Tagami & Miura, 2004). It is unclear why B group *Wolbachia* are

171   particularly prevalent in the Lepidoptera; is there a greater 'fit' between Lepidoptera and B group

172   *Wolbachia i.e.* are B group *Wolbachia* more likely to become established, or are B group

173   *Wolbachia* those ancestrally associated with the Lepidoptera thus seeding this group

174   stochastically? It is also interesting to note that there appears to be one particularly common

175   strain of *Wolbachia* in Lepidoptera. In a study of 53 Lepidoptera species, 11 species across three

176   families are infected with *Wolbachia* ST41, the next most common strain types (ST40 and

177   ST125) were found in three species each (Ahmed et al. 2016). Whether *Wolbachia* ST41 is

178   especially adept at moving between species, and/or whether it is particularly successful at

179   establishing and maintaining itself with the host remains to be fully investigated.

180   The second most common heritable endosymbiont recorded in butterflies and moths is

181   *Spiroplasma* - a bacterial genus belonging to the class Mollicutes (Table S1: 5/253 butterfly

182   species and Table S2: 5/115 moth species). Until such endosymbionts receive the same level of

183   attention as *Wolbachia*, or there is a move towards a generalised metagenomic approach to

184   identify symbiotic microbes, little can be said of the extent of their presence or action in

185   Lepidoptera. However, while there is a propensity for Lepidoptera to be specifically screened for

186   *Wolbachia* infections (thereby creating a bias towards detection of *Wolbachia*), discovery of sex-

187   ratio distorter identity is commonly a phenotype forward investigation *i.e.* a sex ratio bias in

188   progeny or in a population is observed, and then the causative factor is identified. Given this,

189   there should be no bias in the responsible infection found in these studies. Despite this, it appears

190   that Lepidoptera are different from many other groups e.g. ladybirds, in that *Wolbachia* is almost

191   always responsible for the observed sex ratio bias. In comparison with other arthropod groups

192   such as the Diptera, Hymenoptera and Hemiptera, heritable endosymbiont diversity does appear

193   to be particularly low in the Lepidoptera. A systematic review (Russell et al. 2012) compiling

194   data of infection screens of arthropods for the heritable endosymbionts *Arsenophonus,*

195   *Cardinium, Hamiltonella, Spiroplasma,* and *Wolbachia*, found that only the latter two genera of

196   bacteria were present in Lepidoptera species (*Spiroplasma*: 5/205, *Wolbachia*: 140/481 species

197   infected). Thus we can say that for *Arsenophonus*, *Cardinium* and *Hamiltonella*, where 263, 183

198   and 251 Lepidopteran species were assayed respectively, such infections, should they exist at all

199   in Lepidoptera, are remarkably rare. A later compilation of data of arthropods screened for

200   heritable endosymbionts found that *Rickettsia* bacteria were also not commonly found in

201   Lepidoptera, with only one species (an unidentified Noctuidae moth) infected out of 14-32

202   species (variation in number reported here due to several individuals tested having no taxonomic

203   assignment in the study).

204

205   **Manipulation of host reproduction**

206   The Lepidoptera are becoming model systems for the study of endosymbiont manipulation of

207   host reproduction. Many species are infected with maternally inherited bacteria that have

208   evolved the ability to alter host reproduction to either increase the proportion of infected females

209   in the population, or increase the reproductive fitness of infected females relative to their

210   uninfected counterparts. In Lepidoptera endosymbionts are currently known to manipulate host

211   reproduction in three ways: through male-killing, feminisation and cytoplasmic incompatibility

212   (CI) (Fig. 1). While these methods facilitate the maintenance of the symbiont in the host

213   population, there are often severe repercussions for host biology and evolution. We provide a list

214   of butterflies and moths that have been recorded as being infected with endosymbionts that

215   manipulate the reproductive biology of the host (Table 1).

216

217   *Male-killing*

218   Male-killing (MK) is particularly well known in the Lepidoptera. Here, male offspring are killed

219   early in development (most usually as an egg, but also as first instar larvae) producing a female

220   biased sex ratio within an infected female's offspring (Fig. 1). Should the male-killer infect many

221   females, the host population as a whole may become female-biased. Several hypotheses have

222   been proposed to explain why maternally inherited endosymbionts kill male hosts. If infected

223   females gain a fitness benefit from the death of their male siblings over uninfected females

224   (whose male siblings survive), the infection will invade and spread through the host population.

225   Such benefits may include a reduction in the likelihood of detrimental inbreeding (as there are no

226   brothers with which to mate) or a reduction in competition for resources (as there are half as

227   many siblings with which to compete) (Hurst & Majerus, 1992; Hurst, Hurst & Majerus, 1997).

228   In ladybirds, *Wolbachia*-infected female neonates gain an important first meal by consuming

229   their dead brothers, while uninfected females lack this ready source of nutrients (Elnagdy,

230   Majerus & Handley, 2011). However, in Lepidopteran systems, the relative fitness benefit for

231   infected females remains elusive as many of the species studied lay their eggs singly, thus

232   making the likelihood of inbreeding, sibling egg cannibalism or competition unlikely (e.g.

233   *Danaus chrysippus*: (Jiggins et al., 2000a).

234   Despite the lack of evidence of any fitness benefit being provided to infected females,

235   MK has been recorded numerous times in the Lepidoptera, possibly due to the readily observable

236   phenotype of all-female broods and the long history of Lepidoptera being collected and reared in

237   captivity. Early work recorded the presence of female-biases in wild-caught collections and

238   captive bred broods in both *Acraea encedon* (Poulton, 1914; Owen, 1965; 1970), and

239   *Hypolimnas bolina* (Poulton, 1923; 1926) butterflies. Later, MK *Wolbachia* was identified as the

240   causative agent in both *A. encedon* (Jiggins, Hurst & Majerus, 1998; Hurst et al., 1999) and *H.*

241   *bolina* (Dyson, Kamath & Hurst, 2002). We now know that populations of *Acraea* butterflies

242   carry highly prevalent MK *Wolbachia* infections, with more than 80% and 95% of Ugandan *A.*

243   *encedon* and *A. encedana* females being infected, respectively (Jiggins et al., 2000a; Jiggins,

244   Hurst & Majerus, 2000). The *H. bolina* system has become remarkable due to the extensive

245   spatial and temporal variation in the dynamics of the interaction across the South-east Asian to

246   Eastern Pacific range of the butterfly (Charlat *et al.*, 2005; Hornett *et al.*, 2009). The island of

247   Samoa is particularly notable due to its well-documented history of a highly biased sex ratio of

248   100 females to every male, caused by 99% of female butterflies being infected with a MK

249   *Wolbachia* (Dyson & Hurst, 2004). It appears that male-killers are often found at a particularly

250   high frequency within butterfly populations, contrasting patterns seen in other taxa studied such

251   as the ladybirds, where generally less than 49% of females carry an infection (Hurst & Jiggins,

252   2000). In the lycaenid *Zizina emelina*, at least one of the two *Wolbachia* strains described in

253   Japanese populations is a male-killer that rapidly increased in prevalence from 65% to 86%

254   within a 3 year period (Sakamoto *et al.*, 2011).

255   The consequences of a highly distorted sex ratio are likely to be large (for discussions of

256   evolutionary consequences see (Charlat, Hurst & Mercot, 2003; Engelstädter & Hurst, 2007)).

257   As perhaps can be expected, one direct effect is that a large number of females remain unmated.

258   In Makerere, Uganda, 94% of *Wolbachia*-infected *A. encedon* females were virgins (Jiggins,

259   Hurst & Majerus, 2000). Of Samoan *H. bolina* 50% of infected females were unmated, with the

260   females that did mate showing significant fertility deficiencies, implying sperm limitation

261   (Dyson & Hurst, 2004). However, despite the detrimental impacts of male-killers upon the

262   reproductive biology of their hosts, natural host populations infected with high prevalence

263   infections can persist: the 100:1 female to male sex ratio of the Samoan *H. bolina* population

264   persisted for over 100 years (Dyson & Hurst, 2004). Only recently did the dynamics of this

265   interaction change, with the host evolving resistance of the MK activity (Hornett et al., 2006;

266   Charlat et al., 2007b).

267   *Wolbachia* are not the only endosymbionts that selectively kill male Lepidoptera. In the

268   nymphalid butterfly, *Danaus chrysippus*, a *Spiroplasma* bacteria, related to a MK strain

269   previously found in ladybirds, underlies the observed MK (Jiggins et al., 2000a). Similarly, while

270   *Ostrinia* corn borer moths are especially well-known to harbour MK *Wolbachia* strains (*i.e.* the

271   adzuki bean borer *O. scapulalis* (Kageyama & Traut, 2004), and the Asian corn borer *O.*

272   *furnacalis* (Sakamoto *et al.*, 2007)), a MK *Spiroplasma* related to that found in *D. chrysippus*

273   infects the butterbur borer *O. zaguliaevi* (Tabata *et al.*, 2011). Mirroring the pattern seen in *H.*

274   *bolina*, spatial variation of the MK *Spiroplasma* infection was observed in *D. chrysippus* (Smith

275   et al., 1998; Herren et al., 2007), with 40% of females infected in Uganda *vs*. 4% in East Kenya

276   (Jiggins et al., 2000a). Intriguingly, in this system infection appears to be correlated with a

277   colour pattern allele. Although the forces generating this correlation are unknown, it may be the

278   case that particular host genotypes are more susceptible to, or more efficient at transmitting, the

279   infection than others (Herren *et al.*, 2007).

280   In most study systems the precise mechanisms of MK are unclear, and variation across

281   taxa is expected given that MK occurs in arthropods with widely disparate sex determination

282   systems. Dependent on host context several mechanisms have been proposed including defective

283   male chromatin remodelling (*Wolbachia*-infected *Drosophila*: (Riparbelli et al., 2012)); targeting

284   the dosage compensation complex (*Spiroplasma*-infected *Drosophila*: (Veneti et al., 2005));

285 damaging the host's X chromosome to induce embryonic apoptosis (*Spiroplasma*-infected

286 *Drosophila*: (Harumoto et al., 2016)), and affecting maternally inherited centrosomes

287 (*Arsenophonus*-infected *Nasonia* wasps: (Ferree et al., 2008)). In a *Wolbachia*-infected moth,

288 *Ostrinia scapulalis*, male-killing is unusual in that males (genotype ZZ) selectively die early in

289 development, whereas females (ZW) die if cured of the *Wolbachia* infection following antibiotic

290 treatment (Fig. 2). Studies of this system suggest that MK *Wolbachia* interferes with the sex-

291 specific splicing pattern of the *Ostrinia* homologue of the sex determination gene *doublesex*,

292 *Osdsx* (Sugimoto et al., 2010), producing a mismatch between the genotypic sex and expression

293 of the phenotypic sex and leading to sex-specific death (Sugimoto & Ishikawa, 2012). Later

294 examination of the levels of dosage compensation (Z-linked gene expression) in male and female

295 embryos destined to die, revealed that misdirection of dosage compensation underlies the

296 observed mortality. Males destined to die (from*Wolbachia*-infected females) have higher levels

297 of expression of Z-linked genes than normal; while females destined to die (from females cured

298 of the *Wolbachia* infection) have lower expression levels of Z-linked genes than normal

299 (Sugimoto et al., 2015). In a related moth, *O. furnacalis*, RNA-Seq data of *Wolbachia*-infected

300 embryos demonstrated that MK *Wolbachia* down-regulated a masculinizing gene, *Masc*,

301 essential in controlling both sex determination and dosage compensation in Lepidoptera,

302 compared to uninfected embryos. The decrease in *Masc* mRNA levels is reported to cause the

303 MK phenotype via a failure of dosage compensation, and injection of *in vitro* transcribed *Masc*

304 cRNA into *Wolbachia*-infected embryos rescued male progeny (Fukui et al., 2015).

305   The mechanism of MK in *Ostrinia* moths may be different to that underlying MK in

306 other Lepidoptera. In *H. bolina* butterflies no female specific death is observed following

307 antibiotic treatment to reduce or remove MK *Wolbachia* (Charlat et al. 2007a). It is interesting to

308   note however, that the *doublesex* homologue in *H. bolina* may be involved in male-killing in this

309   butterfly as it resides within the chromosomal region defined as containing a suppressor of male-

310   killing action (Hornett et al., 2014). It would therefore be interesting to compare the *Wolbachia*

311   strains and MK mechanisms of *Ostrinia* and *H. bolina*. Likewise, a comparison between the

312   modes of action of the MK strain of *Wolbachia* in *Ostrinia* moths, which kills males as a

313   consequence of feminising them through alteration of expression of *Osdsx*, and of 'true'

314   feminising *Wolbachia* such as that infecting *Eurema* butterflies (see *Feminisation*) may shed

315   light on how one genus of bacteria can induce multiple reproductive manipulations in their hosts

316   and whether there is a functional link.

317   Finally, the Oriental tea tortrix moth *Homona magnanima* also carries a male-killer

318   (Morimoto *et al.*, 2001), however male death in this case occurs much later in development

319   (termed 'late MK'), and appears to be associated with two novel RNA sequences (Nakanishi *et*

320   *al.*, 2008). Late MK was originally only recorded in mosquitoes, with the causative agent being

321   a microsporidian (Andreadis & Hall, 1979), however subsequent studies have now observed

322   similar phenomena in other taxa including *Drosophila* flies (Jaenike, 2007). The extent of this

323   type of manipulation, and the mechanisms underlying it, is still to be determined in insects,

324   including Lepidoptera.

325

326   *Feminisation*

327   The feminisation of genetic males into functional phenotypic females (Stouthamer, Breeuwer &

328   Hurst, 1999) is another strategy employed by maternally inherited endosymbionts to distort the

329   host sex ratio towards the transmitting sex (females) (Fig. 1). While best known from the work

330   on the association between *Wolbachia* and the terrestrial isopod *Armadillidium vulgare*

331   (Juchault, Rigaud & Mocquard, 1992; Rigaud, Juchault & Mocquard, 1997; Bouchon & Rigaud,

332   1998; Cordaux et al., 2004), feminisation also occurs in other female-heterogametic arthropods

333   such as leafhoppers (XX/X0) (Negri *et al.*, 2006), and Lepidoptera (ZZ/ZW). Other than

334   *Wolbachia*, the Bacteroidetes bacterium *Cardinium* can also feminise males (Chigira & Miura,

335   2005; Groot & Breeuwer, 2006), however *Cardinium* has not yet been reported in butterflies or

336   moths.

337   Observation of female-biased lines of pierid *Eurema* butterflies in Japan (Kato, 2000) led

338   to the identification of a feminising *Wolbachia* in *E. mandarina* (formerly *E. hecabe* Y type)

339   (Hiroki et al., 2002). *Eurema hecabe* (formerly *E. hecabe* B type) was later also discovered to

340   carry a feminising *Wolbachia* indistinguishable from that of *E. mandarina*, thus suggesting that

341   the infection transferred between the allopatric butterfly hosts via a shared predator or parasite,

342   or via hybrid introgression between the species (Narita et al., 2011). When *E. mandarina*

343   infected larvae were fed antibiotics to cure them of the infection, many of the adults emerged

344   displaying sexually intermediate traits in their wings, reproductive organs and genitalia.

345   Moreover, age at which antibiotics were administered was found to be important, with the

346   highest level of intermediate sexual traits being exhibited when first instar larvae were treated.

347   This work demonstrated that endosymbionts might continually influence and interact with their

348   host (Narita *et al.*, 2007) rather than have phenotypes that are effective only at a discrete time

349   point in the lifecycle of the host.

350   The process of feminising in *E. mandarina* is more complex than originally thought.

351   Against expectation, female butterflies infected with the feminising strain of *Wolbachia*, *w*Fem,

352   had only one, paternally derived, Z chromosome. This was proposed to be due to meiotic drive

353   against the maternal Z, preventing the formation of the expected ZZ feminised males. It was also

354   suggested that *w*Fem lines have lost the W chromosome, and rely on *w*Fem for female

355   development as curing the infection with antibiotics results in all-male offspring (Kern et al.,

356   2015). Later work demonstrated that *Wolbachia* itself was responsible for the disruption of

357   maternal Z chromosome inheritance in *w*Fem infected females, as well as the feminisation of

358   female ZO individuals that have lost the female-determining W chromosome (Kageyama et al.,

359   2017).

360

361   *Cytoplasmic Incompatibility (CI)*

362   Perhaps the most commonly observed reproductive manipulation employed by endosymbionts in

363   insects is cytoplasmic incompatibility (CI). Unlike for MK or feminisation, the sex ratio of host

364   populations infected by CI-inducing endosymbionts is generally not altered. Instead the symbiont

365   induces an incompatibility upon mating between infected males and females of a different

366   infection status (*i.e.* uninfected or infected with a different symbiont strain), leading to the death

367   of all or a proportion of the offspring (Yen & Barr, 1971; 1973) (Fig. 1). This incompatibility is

368   proposed to occur due to a modification of the infected male's sperm that can be rescued when

369   the female is similarly infected (mod-res mechanism). This specific rescue function is lacking in

370   uninfected females or females carrying a different infection (Hoffman & Turelli, 1997; Charlat,

371   Calmet & Mercot, 2001; Poinsot, Charlat & Merçot, 2003). Infected females therefore have a

372   reproductive benefit of successfully producing a full complement of progeny (relative to

373   uninfected females), when mated to uninfected or similarly infected males in the population.

374   While *Wolbachia* is often described as the causative agent, *Cardinium* also has this ability, e.g.

375   (Hunter, Perlman & Kelly, 2003; Perlman, Kelly & Hunter, 2008).

376   CI has been observed in a number of Lepidoptera including the Mediterranean flour moth

377   *Ephestia kuehniella* and the almond moth *Cadra cautella* (Sasaki & Ishikawa, 1999).

378   Interestingly, CI *Wolbachia* was discovered to lower the amount of fertile sperm transferred in

379   *Cadra cautella* during second matings. However, no effect was shown on the amount of apyrene

380   (non-fertile) sperm suggesting that *Wolbachia* may only target fertile sperm production (Lewis *et*

381   *al.*, 2011). Further work is required to expand our knowledge of CI mechanisms in Lepidoptera.

382   Similarly to MK, CI *Wolbachia* are often observed at high frequency in Lepidoptera populations.

383   In a study of seven Japanese populations of the pierid butterfly *Colias erate poliographus*, CI

384   *Wolbachia* occurred at 85-100% prevalence. The high infection frequency was ascribed to strong

385   CI (*i.e.* a high proportion of the progeny from an incompatible cross die) and perfect vertical

386   transmission of the bacteria (Tagami & Miura, 2004; Narita, Shimajiri & Nomura, 2008). Where

387   CI reaches very high frequency within a host population, the incompatibility between infected

388   males and uninfected females is rarely observed as few females remain uninfected. However,

389   selection for beneficial effects of infection and an eventual shift towards a mutualistic

390   relationship between host and symbiont would remain.

391   Symbioses can be extraordinarily complex; individuals can carry multiple endosymbionts

392   with differing phenotypes. For instance, *E. hecabe* butterflies carry a feminising *Wolbachia*

393   strain, but also a second strain that causes CI (Hiroki *et al.*, 2004). This was the first indication

394   that different strains of *Wolbachia* could infect a single individual and cause different

395   phenotypes. Host context is important in the expression of endosymbiont-induced phenotypes -

396   one symbiont strain can have the ability to cause more than one phenotype, including

397   reproductive manipulations that were originally assumed to be distinct from each other. This has

398   been exemplified in the butterfly *H. bolina*: in populations where *H. bolina* has evolved

399   suppression of the action of MK, surviving infected males are incompatible with uninfected

400   females in the population *i.e.* expression of the CI phenotype (Hornett *et al.*, 2008). This finding

401   indicates a potential functional or mechanistic link between the two phenotypes. However

402   phenotypic switching between CI and MK through mutations cannot yet be ruled out. An

403   intriguing possibility is whether feminisation is also mechanistically linked to CI and MK. Some

404   evidence that may suggest this latter link is provided in studies of the moth *O. scapularis*. As

405   mentioned above, male moths that die as a result of infection with MK *Wolbachia*, were found to

406   carry the female isoform of a homologue of the sex-determining gene, *doublesex*, and hence

407   were feminised prior to death (Sugimoto & Ishikawa, 2012).

408   Artificial transinfection of *Wolbachia* strains have provided further evidence of the

409   relative importance of endosymbiont or host in determining the nature of the phenotype

410   expressed. While in some cases transfer of *Wolbachia* from the natural host into a novel host did

411   not alter the phenotype expressed (e.g. *Wolbachia* causes MK in the natural host *O. scapulalis*

412   and in the transinfected host *E. kuehniella* (Fujii *et al.*, 2001)), host context is important in

413   others. Transfer of CI *Wolbachia wCau-A* from *C. cautella* to *E. kuehniella* resulted in the

414   expression of MK in the novel host (Sasaki, Kubo & Ishikawa, 2002). The strength of the

415   phenotype may also alter in the novel host: the level of CI induced by *Wolbachia* in the

416   transinfected host *O. scapulalis*, was higher than that in its natural host *E. kuehniella,* indicating

417   that host factors as well as endosymbiont strain are important in determining the phenotype

418   expressed (Sakamoto et al., 2005).

419

420   **Impact upon host fitness**

421 It is becoming increasingly evident that many heritable endosymbionts do not manipulate host

422 reproduction, and yet are still maintained within the host population. Host-associated microbes

423 are now thought to be commonly beneficial to their host. For an inherited endosymbiont, the

424 trade-off between virulence and transmission can lead to a reduction in its pathogenicity towards

425 the host, and evolution towards mutualism (e.g. (Weeks et al., 2007). At the extreme end of the

426 spectrum are the obligatory endosymbionts, which are necessary for host survival or

427 reproduction. The growing number of cases include: *Wolbachia* required for oogenesis in the

428 wasp *Asobara tabida* (Dedeine et al., 2001; Dedeine, Bouletreau & Vavre, 2005);

429 *Wigglesworthia* bacteria acting as an obligate nutritional mutualist in tsetse flies (Aksoy, 1995);

430 and *Buchnera* bacteria providing essential nutrients to aphids (Buchner, 1965).

431   Many more endosymbionts are facultatively (non-essentially) beneficial, with fitness

432 benefits including increasing host survival (e.g. (Fry & Rand, 2002)) or fecundity (e.g. (Vavre,

433 Girin & Boulétreau, 1999; Weeks & Stouthamer, 2004). Studies of the beneficial effects of

434 endosymbiont infection in the Lepidoptera provide an unusual example in *Parnassius apollo*. In

435 one isolated population this near threatened butterfly regularly exhibits deformed or reduced

436 wings, however while 86% of normal winged butterflies are found infected with *Wolbachia*, this

437 percentage drops to 30% in individuals displaying deformed wings, and 0% in individuals with

438 reduced wings. Although this is suggestive of a protective role of *Wolbachia* in the ontogenetic

439 development of the butterfly, further study needs to be carried out to prove causality

440 (Łukasiewicz, Sanak & Węgrzyn, 2016).

441   Microbial endosymbionts can contribute to insect adaptation by providing

442 complementary or novel metabolic capacities, allowing the insect host to exploit host plant

443 nutritional resources. One such instance has been observed in the phytophagous leaf-mining

444   moth *Phyllonorycter blancardella.* In this system, a bacterial endosymbiont, most

445   likely*Wolbachia*, indirectly affects larval nutrition by manipulating the physiology of the host

446   plant to create photosynthetically active green patches in otherwise senescent yellow leaves. The

447   phenotype, termed 'green-island', produces areas of leaf viable for host feeding in a nutritionally

448   constrained stage of the lifecycle. Curing the larvae of endosymbionts resulted in the non-

449   production of 'green-islands', and consequent increased compensatory larval feeding and higher

450   mortality (Kaiser *et al.*, 2010). The mechanism behind green island formation involves increased

451   levels of cytokinins (CKs), plant hormones important in plant senescence and nutrient

452   translocation. *Wolbachia* have been shown to be involved in the release of CKs by the larvae,

453   creating these nutritionally enhanced areas of leaf. Whether the CKs are bacterial-derived or

454   produced by the insect in response to *Wolbachia* infection (or a combination of both) remains to

455   be fully understood (Body *et al.*, 2013; Giron & Glevarec 2014). Several strains of *Wolbachia*

456   from both A- and B-supergroups have been identified in 13 Gracillariidae leaf-mining moth

457   species, while none were found in ancestral Gracillariidae. Acquisition of the green-island

458   phenotype appears to have occurred several times independently across the Gracillariidae in

459   association with different *Wolbachia* infections (Gutzwiller et al., 2015).

460   　　　Generally, vertically inherited endosymbionts are unlikely to be maintained in host

461   populations if they are highly costly. However, direct fitness or physiological costs of infection

462   have been observed where the symbiont also manipulates host reproduction. CI *Wolbachia* are

463   maintained in the host population despite reducing male fertility (Snook *et al.*, 2000) or female

464   fecundity (Hoffman, Turelli & Harshman, 1990) in *Drosophila* flies, or detrimentally affecting

465   fecundity, adult survival and locomotor performance in the parasitoid wasp *Leptopilina*

466   *heterotoma* (Fleury *et al.*, 2000). Among Lepidoptera examples, presence of MK *Spiroplasma* in

467   *D. chrysippus* in Kenya was negatively correlated with forewing length, suggesting that the

468   bacteria may adversely affect development time or the growth rate of larvae (Herren *et al.*,

469   2007). Presumably these physiological costs are counter-balanced by the reproductive

470   manipulations employed by heritable endosymbionts, thus enabling the symbiont to persist.

471   Although not covered in detail here, we note that in contrast, a symbiont that is also (or only)

472   horizontally transmitted, can be highly detrimental to the host yet still be maintained in the host

473   population. Indeed, host death may be its source of transmission to a novel host.

474   The gregarine protozoan infection, *Ophryocystis elektroscirrha,* of the Monarch butterfly

475   (*Danaus plexippus*) is one of the most studied cases of direct fitness costs of symbionts in

476   Lepidoptera. While it is not heritable in the sense of being intracellular, we include it here as it is

477   passed vertically from mother to offspring via the surface of the egg. An infected female

478   inadvertently coats her eggs with protozoan spores that cover the outside of her abdomen during

479   oviposition. Newly hatched larvae ingest these spores while consuming the eggshell

480   (McLaughlin & Myers, 1970). The parasite, which requires the adult host stage for transmission,

481   rarely kills larvae or pupae under natural conditions, however the degree of virulence and

482   transmission trade-off varies depending on the level of infection at the adult stage. Where

483   individual *D. plexippus* butterflies carry high densities of the protozoa, they have both reduced

484   survival and flight capacity compared to individuals with lower density infections (Altizer &

485   Oberhauser, 1999; de Roode, Yates & Altizer, 2008; de Roode & Altizer, 2010).

486

487   **Symbiont-mediated protection**

488   Although understudied in Lepidopteran systems, an exciting avenue of research in arthropods

489   revolves around a symbiont's ability to afford the host some level of resistance to its natural

490 enemies, often through interference with pathogen or parasite replication or transmission

491 (reviewed in insects in (Brownlie & Johnson, 2009)). This may be particularly the case for

492 heritable endosymbionts, where symbiont and host fitness is inextricably linked - competing

493 infections may elicit a response by the endosymbiont to protect the host, and thus simultaneously

494 itself (Haine, 2008). Such symbiont-mediated protection has been documented in numerous taxa,

495 particularly the Diptera, including recent studies demonstrating the ability of *Wolbachia* to

496 supply their *Drosophila* host with anti-viral protection (Hedges et al., 2008; Teixeira, Ferreira &

497 Ashburner, 2008; Martinez et al., 2014). Similarly, maternally transmitted *Spiroplasma* were

498 found to protect *D. neotestacea* against the sterilising effects of a parasitic nematode (Jaenike et

499 al., 2010), and enhance the survival of *D. hydei* parasitized by wasps (Xie, Vilchez & Mateos,

500 2010).

501   Symbiont-mediated protection appears to be extremely diverse. Aphids are host to a

502 range of inherited symbionts, several of which provide protection against parasitoid wasp attacks

503 (Oliver *et al.*, 2003; Ferrari *et al.*, 2004) or fungal infections (Ferrari et al., 2004; Scarborough,

504 Ferrari & Godfray, 2005). In the European beewolf wasp, *Philanthus triangulum, Streptomyces*

505 bacteria are stored in special antennae glands and deposited together with the egg in the

506 oviposition chamber. The bacteria secrete antibiotics protecting the developing wasp larvae

507 against fungal pathogens (Kaltenpoth *et al.*, 2005). There is even some evidence that symbionts

508 can protect their host from predators by producing toxic compounds. For example, a bacterial

509 endosymbiont (that is both vertically and horizontally transmitted) closely related to

510 *Pseudomonas aeruginosa* produces the polyketide toxin pederin, which protects *Paederua* beetle

511 larvae from predatory wolf spiders (Kellner & Dettner, 1996; Kellner, 1999; Piel, Höfer & Hui,

512 2004). Furthermore, endosymbionts may have the ability to inhibit a range of pathogens by

513   priming the host immune system (Braquart-Varnier *et al.*, 2008; Moreira *et al.*, 2009; Hughes *et*

514   *al.*, 2011), suggesting that symbionts can interact with, and alter integral components of, host

515   biology.

516   Conversely, it is important to acknowledge that endosymbiont infection can also increase

517   pathogen load. While *Wolbachia* confers protection against a variety of pathogens and parasites

518   in a wide range of hosts, pathogen or parasite levels can also be enhanced by the presence of an

519   endosymbiont (Hughes, Rivero & Rasgon, 2014): in the moth *Spodoptera exempta, Wolbachia*

520   triggers a higher rate of virus infection and therefore lowers host fitness (Graham & Wilson,

521   2012).

522

523   **Host genetics**

524   *Host population genetics*

525   Sex ratio distorting symbionts are likely to have a severe impact upon host population biology

526   (Engelstädter & Hurst, 2007). If the prevalence of a sex ratio distorter is high, the sex ratio of the

527   population can become severely biased. In consequence, the hosts' effective population size (Ne)

528   will be reduced. Where there is little gene flow into the population (*i.e.* low immigration), a

529   reduction of the effective population size may affect the amount of standing genetic variation and

530   the potential for the host population to respond and adapt to environmental change. In contrast, if

531   gene flow does occur, spatial variation in sex ratio (as seen in the butterfly *H. bolina* (Charlat *et*

532   *al.*, 2005; Hornett *et al.*, 2009)) may result in asymmetric gene flow between populations.

533   Although both sexes typically contribute equally to the gene pool of the next generation,

534   immigration of an individual into a population in which the sex ratio is skewed against it (e.g. a

535   male into a highly female-biased region) can have a much larger genetic impact (*i.e.* contribute

536   more) than if that individual immigrated into an unbiased sex ratio population (Telschow *et al.*,

537   2006). MK symbionts are also thought to hinder the spread of beneficial alleles and facilitate the

538   spread of deleterious alleles, due to constrained gene flow from infected to uninfected

539   individuals within the population (Engelstädter & Hurst, 2007). In a further complication, strains

540   expressing different reproductive manipulations may be incompatible. Although most famous for

541   its MK *Wolbachia* infections, some populations of *H. bolina* also carry a CI-inducing strain of

542   *Wolbachia*. This CI strain is phylogenetically distant from the MK strain, and crosses between

543   MK-infected females and CI-infected males are fully incompatible with no progeny surviving.

544   The incompatibility produced has led to strong competition between the two strains, with the CI-

545   strain being able to not only spread successfully through uninfected populations, but to also resist

546   invasion by the MK-strain carried by butterflies from neighbouring island populations (Charlat et

547   al., 2006). Extending from this model, a study (Zug & Hammerstein, 2017) recently showed that

548   when direct fitness benefits are taken into account in parallel to reproductive costs, the CI-strain

549   is likely to also be able to spread across MK-infected *H. bolina* populations. Taken together, this

550   suggests that successful establishment of particular butterfly genotypes is affected by the

551   endosymbionts they harbour.

552

553   *Linkage with host mitochondrial DNA*

554   Maternally inherited symbionts residing within the cytoplasm of cells can alter the diversity and

555   population genetics of the host's mitochondrial genome (mtDNA). Co-inherited symbionts and

556   mitochondria are in linkage disequilibrium, therefore when a cytoplasmic symbiont invades a

557   population, the initially associated mitochondrial haplotype (mitotype) may 'hitch-hike' and

558   correspondingly increase in frequency. Should such a selective sweep have occurred recently, the

559   effective population size and genetic diversity of mtDNA would be reduced to that of the

560   infected individuals (Johnstone & Hurst, 1996), and the geographic structure of mitochondrial

561   variation lost. The latter has been observed in *Acraea* butterflies (Jiggins, 2003) and the comma

562   butterfly *Polygonia c-album* (Kodandaramaiah et al., 2011). The tight association between

563   endosymbiont and mtDNA can therefore seriously confound the results of any study using

564   mtDNA genes as neutral genetic markers (Hurst & Jiggins, 2005). Reconstruction of

565   phylogenetic trees using mitochondrial markers are hence likely to be misleading, particularly

566   within shallower branches, when the study species is infected. In the Diamondback moth,

567   *Plutella xylostella*, the main correlate of mtDNA variation is presence or absence of the *plutWB1*

568   *Wolbachia* infection (Delgado & Cook, 2009), and the lycaenid butterfly *Lampides boeticus* may

569   have experienced accelerated population differentiation due to *Wolbachia* infection (Lohman *et*

570   *al.*, 2008). Recognition of these processes should lead to an increasing number of Lepidopteran

571   studies interested in using mtDNA markers to systematically screen for maternally inherited

572   symbionts.

573   Where there is perfect transmission of the maternally inherited symbiont from the host to

574   its offspring, infected individuals all carry the same mitotype, while uninfected individuals

575   remain polymorphic. This pattern has been repeatedly observed in natural populations of insects,

576   including in the Lepidoptera. In the butterfly *H. bolina*, a strong association between one specific

577   *Wolbachia* strain and one particular mitotype supported the hypothesis that the MK infection

578   occurred with very high vertical transmission efficiency and rare horizontal transmission. In

579   *H.bolina*, this strain of *Wolbachia* is thought to have undergone a recent selective sweep and was

580   introduced into this butterfly through introgression, potentially from another *Hypolimnas* species,

581   *H. alimena*. Conversely the infection and associated mitotype may have been introgressed from

582   *H. bolina* to *H. alimena* (Charlat et al., 2009; Duplouy et al., 2010; Sahoo et al., 2018). Similarly,

583   in the *Acraea* butterflies, a study of mitochondrial variants demonstrated that a MK *Wolbachia*,

584   together with the associated mitotype, had introgressed from *A. encedana* into *A. encedon* within

585   the last 16,000 years. As female butterflies are heterogametic (ZW), this event could potentially

586   also lead to the introgression of genes on the female W chromosome (Jiggins, 2003). This

587   scenario appears to have occurred in *D. chrysippus* infected with a MK *Spiroplasma*, as all

588   infected females carry the same W chromosome variant (Smith, Gordon & Allen, 2010). This

589   aside, the nuclear DNA is generally less likely to be in linkage with inherited symbionts.

590   Gompert and colleagues studying North American *Lycaeides* butterflies reported that the spread

591   of an endosymbiont (and associated mitotype) through the host population produced substantial

592   mito-nuclear discordance. Therefore, the evolutionary history of an individual's nuclear and

593   mitochondrial genomes may be very different from each other (Gompert *et al.*, 2008). Such

594   discordance may have far-reaching effects on host metabolism and physiology, as coevolution

595   between nuclear and mitochondrial components of essential pathways is broken down.

596

597   *Speciation by symbiosis*

598   The concept that symbionts can be important promoters of speciation and diversity has been

599   around for a long time (Wallin, 1927; Laven, 1959; Thompson, 1987; Breeuwer & Werren,

600   1990; Hurst & Schilthuizen, 1998; Bordenstein, 2003), but has recently been rejuvenated with

601   the development of microbiome analyses (Brucker & Bordenstein, 2012). Contemporary

602   evidence of microbe-assisted speciation involves pre-mating reproductive isolation through

603   behavioural barriers such as mate preference, associated with the microbiome of the potential

604   partners (Koukou et al., 2006; Miller, Ehrman & Schneider, 2010; Sharon et al., 2010; Chafee et

605   al., 2011). The underlying mechanisms may involve alteration of the sex pheromones,

606   interference with sensory organs, or effects upon immune-competence and hence mate

607   attractiveness. Ecological isolation may also be heavily influenced by microbial symbionts.

608   Although the genetic basis of niche or habitat specificity is widely accepted, there is also

609   increasing evidence that symbionts may play a role in determining host resource availability (e.g.

610   (Akman *et al.*, 2002; Hosokawa *et al.*, 2010)), and thus may facilitate niche separation.

611   Additionally, endosymbionts might enable host speciation through post-mating isolation.

612   In particular, strong bi-directional CI may result in reproductive isolation between hosts carrying

613   different CI symbiont strains (Hurst & Schilthuizen, 1998; Werren, 1998; Bordenstein, 2003). In

614   order for speciation to follow CI, a stable infection polymorphism must be maintained across

615   host populations. This has been demonstrated in many systems including the butterfly *H. bolina*

616   (Charlat *et al.*, 2006). Theory predicts that two bi-directional CI-inducing symbionts can be

617   stable for even high migration rates (Telschow, Hammerstein & Werren, 2005). What is more

618   contentious is that for speciation to occur, the CI produced must be very strong (*i.e* no offspring

619   surviving from such crosses), and the symbiont must be maintained at a high transmission rate

620   over time, to allow significant nuclear divergence (Engelstädter & Hurst, 2009).

621   Male-killing has also been linked to speciation in the butterfly *D. chrysippus*. In Kenya

622   two forms exist: *D. c. chrysippus* and *D. c. dorippus*, separated by a hybrid zone. Each

623   subspecies has an individual colour pattern controlled by locus *C*, which is intermediate in the

624   hybrid (*Cc*). The *C* locus lies on an autosome that has fused with the W chromosome within the

625   hybrid zone, physically linking colour pattern with female determination. A locus on this same

626   autosome has also been associated with susceptibility to male-killing by *Spiroplasma*. The

627   hybrid zone is characterised by female-biased sex ratios, caused by MK *Spiroplasma* that infects

628   *D. c. chrysippus* or hybrid females, but rarely *D. c. dorippus* females. As immigrant males into

629   the hybrid zone are predominantly *D. c. dorippus*, gene flow between the two subspecies is

630   restricted: *D. c. chrysippus*/hybrid female x *D. c. dorippus* male crosses produce female-biased

631   broods (Smith et al., 2016).

632

633   *Sex determination*

634   The maternal inheritance of intracellular endosymbionts has led to a great degree of interaction

635   of the symbiont with the sex determination pathways of the host (reviewed in (Cordaux,

636   Bouchon & Grève, 2011; Kageyama, Narita & Watanabe, 2012; Ma, Vavre & Beukeboom,

637   2014) and so not discussed in detail here). Maternally inherited endosymbionts distort the host

638   sex ratio in order to enhance the fitness of the transmitting female sex. The mechanisms

639   underlying these phenotypes often require considerable manipulation of host sex determination.

640   We have seen above that in several cases MK and feminising *Wolbachia* can interfere with

641   central components of the sex determination pathways in Lepidoptera. When a feminising

642   element is highly prevalent in a host population, sex determination may be inextricably linked to

643   the presence or absence of feminising activity (Hiroki et al., 2002), but may also enter into

644   conflict with other genetic elements not under similar maternal inheritance. Furthermore,

645   evolution of host suppressors of feminisation may move the system away from the original

646   ZZ/ZW sex determination system. In *E. mandarina*, *Wolbachia* disrupts the inheritance of

647   maternal Z chromosomes in *Wolbachia*-infected females, and feminises the resulting Z0

648   individuals that have lost the female-determining W chromosome (Kageyama et al., 2017). The

649   host may then be prompted to evolve a strategy to counteract the feminising effects of the

650   symbiont. It has been speculated that in the pillbug *A. vulgare*, a masculinising factor in the form

651 of a dominant autosomal *M* gene has evolved in the host to counter the effect of the feminising

652 endosymbiont (Rigaud & Juchault, 1993; Caubet *et al.*, 2000).

653

654 *Evolution of host resistance*

655 Co-evolution between a host and a detrimental symbiont can result in the evolution of host

656 genetic modifiers of symbiont presence or action. Despite this, and considering the wide array of

657 costly effects that endosymbionts can impose on their hosts, it is perhaps surprising that there are

658 relatively few well documented examples of the host having evolved genetic resistance to an

659 endosymbiont. Indeed no suppression of the detrimental phenotype is observed in several studies

660 where it may have been expected (Hurst, Jiggins & Robinson, 2001; Veneti, Toda & Hurst,

661 2004; Dyer & Jaenike, 2005). However, artificial transinfection experiments have provided an

662 indirect method of discovering whether a host has evolved resistance to an endosymbiont, and

663 have suggested that suppression of reproductive manipulation phenotypes may actually be

664 common. In the moth *C. cautella*, which is naturally infected with two *Wolbachia* strains

665 (*w*CauA and *w*CauB), artificial transinfection of CI-inducing *w*CauA to a sister host species, *E.*

666 *kuehniella*, resulted in the transferred bacteria inducing MK instead of CI in the novel host

667 (Sasaki, Kubo & Ishikawa, 2002; Sasaki, Massaki & Kubo, 2005). By interpreting these data in

668 the light of the hidden MK theory (where MK is masked by the presence of a fixed suppressor),

669 this switch in phenotype between species could be interpreted as the 'unmasking' of MK when

670 released into a background devoid of host suppression genes. More generally, resistance may

671 also underlie the loss of infections from populations or host species, however this is obviously

672 hard to document in nature.

673   The selective pressure for host resistance is particularly strong when the sex ratio is

674   severely biased (Düsing, 1884; Fisher, 1930; Hamilton, 1967), and therefore one would expect

675   the evolution of resistance particularly in cases of highly prevalent sex ratio distorters. As

676   mentioned above, the Samoan population of the butterfly *H. bolina* had an extraordinarily

677   female-biased sex ratio of 100 females per male, caused by 99% of the females being infected

678   with MK *Wolbachia* (Dyson & Hurst, 2004). However between 2001 and 2006 the dynamics of

679   the interaction changed dramatically when *H. bolina* evolved suppression of the MK trait,

680   allowing infected males to survive and rapidly re-establishing a 1:1 sex ratio within

681   approximately 10 generations of the host butterfly (Charlat et al., 2007b). The presence of a

682   zygotically acting dominant suppressor locus had previously been documented in SE Asian *H.*

683   *bolina* populations (Hornett *et al.*, 2006).

684   Sex ratio distorting endosymbionts can also have much wider implications upon host

685   genetics. Recent work on the same Samoan population of *H. bolina* investigating the genomic

686   impact of the rapid spread of suppression revealed that a substantial selective sweep had taken

687   place, covering at least 25cM of the chromosome carrying the suppressor locus. In addition to

688   large changes in the frequency of genetic variants across this broad region, the sweep was

689   associated with the appearance of several novel alleles. This suggests that the suppressor spread

690   following migration of butterflies carrying the locus, potentially from SE Asia, rather than from a

691   *de novo* mutation occurring within the population. It is also interesting to note that the suppressor

692   of MK has been located to the chromosome containing *doublesex* (Hornett et al., 2014)– a sex

693   determination gene demonstrated to be involved in *Wolbachia*-induced MK in *Ostrinia* moths.

694

695   *Horizontal transfer of genetic material*

696   While the extent of horizontal (lateral) gene transfer (HGT) between eukaryotes and prokaryotes

697   remains uncertain, technological advances in genomics followed by an accumulation of

698   microbial and host genomic data, have revealed that endosymbionts, particularly those that are

699   vertically inherited, may readily exchange genetic material with their host. HGT from a

700   prokaryote symbiont to its eukaryote host has been reported in many insects including beetles,

701   flies, parasitoid wasps, mosquitoes and butterflies (e.g. (Hotopp et al., 2007; Nikoh et al., 2008;

702   Klasson et al., 2009; Werren et al., 2010) and has recently been reviewed in detail (Husnik &

703   McCutcheon, 2017). Such movement of genes can afford the receiving organism important

704   benefits. For instance, horizontally transferred bacterial DNA that is involved in the

705   detoxification of cyanide has been identified in several moths and butterflies, allowing these

706   insects to utilise otherwise noxious plants (Wybouw et al., 2014). However, the discovery of

707   bacterial DNA within the host's genome does not necessarily imply functionality, and definitive

708   proof of function is difficult to obtain, indeed many transferred *Wolbachia* genes are not

709   expressed at a significant level in the host (Hotopp et al., 2007; Nikoh et al., 2008). To date, the

710   identification of a 350bp long *Wolbachia* gene insert in the genome of the butterfly *Melitaea*

711   *cinxia*, is the only reported example of an HGT from an endosymbiont to a Lepidoptera species

712   (Ahmed et al. 2016), its origin and functionality have yet to be demonstrated.

713   Horizontal gene transfer is also known to occur in the opposite direction, from eukaryote

714   host to symbiont. *Wolbachia* genome projects have indicated that genome fragments have been

715   transferred from host to the bacteria, including in the *H. bolina* system. The MK *Wolbachia*

716   strain sequenced appears to be extremely receptive to exogenous genetic material (Duplouy *et*

717   *al.*, 2013). In addition to cross-level transfer of genes, bacteria within a host may also exchange

718   genetic material. Bacteria are known to be promiscuous with regard to DNA, with movement of

719   bacteriophages between co-infecting symbiont species providing a convenient method of transfer

720   of genes. Some endosymbiont traits are associated with phage presence (Oliver *et al.*, 2009) and

721   thus this movement offers the potential for transfer of traits between co-infecting symbiont

722   strains (Duron & Hurst, 2013). Indeed extensive HGT involving the bacteriophage *WO* has been

723   reported between several *Wolbachia* strains infecting diverse hosts including within the

724   Lepidoptera, Diptera and Hymenoptera (Masui *et al.*, 2000; Bordenstein & Wernegreen, 2004).

725

726   **Behavioural modification**

727   The transmission of many parasites is facilitated by their ability to manipulate the behaviour of

728   their hosts (Lefevre *et al.*, 2009). Reported cases are often restricted to viral and fungal

729   pathogens, for instance, some baculoviruses and fungi cause summit disease – a syndrome that

730   induce caterpillars to climb to high vegetation prior to being killed so that any spores released are

731   carried further by the wind (Maitland, 1994; Yamazaki & Sugiura, 2004). Behavioural

732   modification of arthropod hosts by heritable endosymbionts is less evident, and where observed

733   are perhaps more attributable to indirect effects of infection. *Rickettsia* bacteria have been

734   associated with limiting long distance dispersal in a spider (Goodacre *et al.*, 2009), and

735   *Wolbachia* has been demonstrated to reduce wasp locomotor performance (Fleury *et al.*, 2000).

736   Models of MK endosymbionts in metapopulations have suggested that male-killers can increase

737   host dispersal rates (Bonte, Hovestadt & Poethke, 2008). These patterns may be attributed to the

738   evolution of adaptive modifications by the symbiont to promote its own transmission. However

739   another explanation is that these behavioural changes are merely side effects of physiological

740   alterations without any adaptive causality.

741   In the butterfly, *D. plexippus*, the protozoan *O. elektroscirrha* has attracted much

742   attention because of its potential involvement in the famous migratory behaviour of its host. This

743   parasite is known to reduce the flight capacity of the host (Altizer & Oberhauser, 1999; Bradley

744   & Altizer, 2005) – a trait that creates an important trade-off as the butterflies' dispersive

745   behaviour allows the spread of the protozoa across the species range, and thus increases the

746   chance of it infecting naive populations. For the butterfly, migration offers an opportunity of

747   escaping highly infected habitats where they may risk reduced fitness (Altizer, Bartel & Han,

748   2011). Altizer and colleagues demonstrated that variation in protozoa prevalence correlates with

749   host movement - non-migratory populations have high infection prevalence whereas populations

750   that migrate long distances show less than 10% prevalence of infection (Altizer, Oberhauser &

751   Brower, 2000). More recently it was found that where migratory behaviour has been lost, the risk

752   of infection is increased (Satterfield, Maerz & Altizer, 2015). Thus in part the presence of the

753   protozoa may have led to Monarch butterflies forming both resident and migratory populations.

754   Further indirect behavioural consequences of microbial infection are also possible. In

755   order to escape the fecundity and physiological costs of mating with an incompatible mate,

756   individuals may evolve new adaptive mating strategies, including increased polyandry or mate

757   discrimination (reviewed in (Miller & Schneider, 2012). *Wolbachia* influences mate-choice in

758   the two-spotted spider mite, where uninfected females preferentially mate with uninfected males

759   (Vala *et al.*, 2004), while in *Drosophila paulistorum*, *Wolbachia* titer and mate discrimination

760   are positively correlated (Miller, Ehrman & Schneider, 2010). In *Acraea* butterfly populations

761   harbouring high frequency MK bacteria (thereby having highly female-biased sex ratios),

762   infected females more often remained unmated than uninfected females (Jiggins, Hurst &

763   Majerus, 2000). While this is suggestive of preferential mating by the male, further work needs

764   to be carried out to test this. However, *Acraea* butterflies afford another example: in butterflies,

765   males are often the competing sex and court the females. When the butterfly population is

766   strongly female biased due to the presence of a highly prevalent sex ratio distorting

767   endosymbiont, the roles of the sexes may reverse. Such sex-role reversal was observed in *Acraea*

768   butterflies infected with MK *Wolbachia*. Although male 'hill-topping' (swarming at the tops of

769   hills) is common throughout the genus (Jiggins, 2002), in *A. encedon* the lack of males induced

770   females to swarm instead, and to exhibit behaviours soliciting the males' attention (Jiggins,

771   Hurst & Majerus, 2000).

772   MK endosymbionts may also result in female reproduction becoming sperm limited. In a

773   comparison of *H. bolina* populations varying in MK *Wolbachia* prevalence, the prediction that

774   female mating rates would decline with increasing MK infection prevalence as males became

775   increasingly rare was not borne out. Unexpectedly the opposite occurs – as the population sex-

776   ratio becomes more biased, the female mating rate increased until a point at which the lack of

777   males makes it impossible for females to find a mate. It was suggested that female promiscuity

778   increased in response to increasing male 'fatigue'. Males from more highly female-biased

779   populations produced smaller spermatophores thus necessitating females to become more

780   solicitous (Charlat et al., 2007c).

781

782   **Outstanding questions and future directions**

783   We here are promoting the Lepidoptera as important models in the study of endosymbiont

784   induced reproductive manipulations, with MK, feminisation and CI all being evident in

785   butterflies and moths. Current research is uncovering the genetic and functional basis underlying

786   these phenotypes but many outstanding questions remain: Are all three reproductive

787　manipulations found in Lepidoptera functionally linked? How commonly can a single

788　endosymbiont strain confer more than one phenotype? How do different endosymbiont genera

789　confer similar phenotypes in their host (e.g. both *Wolbachia* and *Spiroplasma* cause MK in

790　Lepidoptera), and are the mechanisms related? How does MK, feminisation and CI in

791　Lepidoptera differ from that expressed in taxa with divergent sex determination systems? Also,

792　how do sex-ratio distorting endosymbionts affect the long-term evolution of the host. Given

793　recent advances in genomics this now can include investigations of the genomic impact of a

794　sustained population sex ratio bias. Sex-linked traits in particular may be expected to be affected.

795　　　　More questions are provoked when research into heritable endosymbionts associated with

796　other arthropod taxa is considered. Of particular interest is the evidence accruing that symbionts

797　often afford the host some level of protection against pathogens and parasitoids. But how

798　frequent is this phenomenon in butterflies and moths? Also, can we see these effects in

799　combination with reproductive manipulations, producing a trade-off between detrimental and

800　mutualistic effects of infection? Conversely, where we see highly prevalent and persistent

801　endosymbiont infections in host populations that do not induce reproductive manipulations, do

802　these symbionts offer the host protection? While there are clearly many outstanding questions to

803　examine in the Lepidoptera, in this next section we focus upon four further areas of research that

804　will move Lepidoptera-heritable endosymbiont research forward.

805

806　*Comparative endosymbiont genomics*

807　The genomes of many arthropod heritable endosymbionts have now been assembled, however

808　very few of those sequenced are associated with Lepidoptera hosts. A comparative genomics

809　approach can be used to elucidate endosymbiont evolution and function in its host including

810   identifying candidate genes involved in reproductive manipulations such as CI (as in *Drosophila*

811   (Sutton et al., 2014; LePage et al., 2017)), and parthenogenesis induction (in parasitoid wasps

812   (Newton et al., 2016; Lindsey et al., 2016)). A recent comparison of 16 *Wolbachia* genomes

813   identified a core *Wolbachia* genome of 496 sets of orthologous genes, 14 of which were unique

814   to *Wolbachia* among the Rickettsiales bacteria, of which it is a member (Lindsey et al., 2016).

815   This study included the MK *Wolbachia* strain *w*Bol1b from *H. bolina* butterflies, which was

816   revealed to be closely related to a CI *Wolbachia* infecting *Culex pipiens* mosquitoes, *w*Pip. A

817   comparison of the two strains identified a number of genes specific to *w*Bol1b that could be

818   potential candidates involved in the induction of MK (Duplouy et al., 2013). An interesting

819   future research direction that may inform on the diversity and genetic basis of MK, would be to

820   expand this line of enquiry by comparing the genome of *w*Bol1b with other MK and non-MK

821   *Wolbachia* genomes. Candidate loci could also be investigated in other MK-inducing symbiont

822   genomes such as *Spiroplasma*. While the genomes of many *Spiroplasma* bacteria have been

823   chracterized from various arthropods (see (Bolaños, Servín-Garcidueñas & Martínez-Romero,

824   2015) for a minireview), including the MK *Spiroplasma* endosymbiont MSRO found in *D.*

825   *melanogaster* (Paredes et al., 2015), to our knowledge none have as yet been published that

826   specifically associate with Lepidoptera.

827   As high-throughput sequencing costs reduce, the genomes of increasing numbers of

828   Lepidoptera are being sequenced (for a review of the current status see (Triant, Cinel &

829   Kawahara, 2018)). A happy indirect consequence of this is that endosymbiont genome sequences

830   can be retrieved as a by-product of host genome sequencing. This is a particularly useful tool

831   when studying intracellular endosymbionts that are not readily culturable, and hence difficult to

832   directly isolate and sequence (such as *Wolbachia*). This approach has been used to reconstruct

833   the genome of *Wolbachia* infecting the moth *Operophtera brumata* (Derks et al., 2015), and that

834   of *Wolbachia*, *w*Aus, associated with the moth *Plutella australiana* (Ward & Baxter, 2017).

835   Interestingly, and similarly to *w*Bol1b from *H. bolina*, both strains were most closely related to

836   the CI *Wolbachia w*Pip from the mosquito *C. pipiens* (Derks et al., 2015; Ward & Baxter, 2017),

837   however in the case of *w*Aus, two genes previously determined to be involved in CI caused by

838   *Wolbachia* from *Drosophila melanogaster* were not found in the genome of *w*Aus (Ward &

839   Baxter, 2017). Further work needs to be conducted to characterize the nature of the interaction

840   between *Wolbachia* and host before more insight can be gained through genomic comparisons.

841

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

843   This review has revealed a marked bias in Lepidopteran heritable endosymbiont research –

844   *Wolbachia* is by far the most studied endosymbiont in butterflies and moths. While the incidence

845   of *Wolbachia* is undoubtedly high in Lepidoptera and its effects upon its hosts important, the

846   development of routine PCR assays and resources specific to this one genus of bacteria may have

847   inflated its significance relative to other endosymbionts. Thus a practical limitation of the current

848   methodology in the study of heritable endosymbionts in Lepidoptera is the lack of an unbiased

849   approach to determine what microbes butterflies and moths carry. This is changing with the

850   development of culture-independent methods of ascertaining what microbes, particularly

851   bacteria, are present within an organism. High-throughput sequencing of the hypervariable

852   bacterial 16S rRNA gene, and metagenomics allow the characterisation of whole bacterial

853   communities of hosts. Particular to heritable endosymbiont research, attention should be given to

854   the tissue from which DNA is sourced, as heritable bacteria are not necessarily found in the

855   commonly sequenced gut tissue or lumen. Amplifying bacterial DNA from whole insects or the

856   reproductive tracts may yield a clearer idea of the vertically inherited symbionts present. We also

857   have to consider what constitutes a heritable endosymbiont; many Lepidopteran gut bacteria are

858   transitory and/or environmentally acquired for example via the food plant as larvae (Mason &

859   Raffa, 2014; Hammer et al., 2017), or nectar as adults and as such may not evolve symbiotically

860   with the host. However, gut bacteria may be transmitted by the female to the progeny via for

861   example the egg coating, which neonates often consume upon hatching. One challenge will be to

862   distinguish which of the microbes present in a community are symbiotic, and further, which are

863   vertically transferred. Therefore close behind microbiome characterisations of Lepidoptera will

864   be experimental manipulations of the microbiome and the sequencing of progeny to ascertain

865   heritability.

866   Revealing the microbiome of Lepidoptera will open up a new set of questions such as do

867   gut microbes and heritable endosymbionts interact? Can endosymbionts affect the composition

868   of the microbiome? Do their effects interact? One promising avenue of research is the

869   antimicrobial activity of gut symbionts. The moth *Spodoptera littoralis* habours a gut bacterium

870   *Enterococcus mundtii* that secretes an antimicrobial peptide (mundticin KS) against invading

871   bacteria, but not against other resident gut bacteria. This antimicrobial activity directly inhibits

872   competitors, but also potential pathogens, from the gut of its host. In *S. littoralis*, this

873   extracellular symbiont persists across host developmental stages and is a major constituent of the

874   microbiome across generations, suggesting that it can be vertically inherited, and that it may

875   form a long-term symbiotic association with its host (Shao et al., 2017).

876   A further avenue for future research is the presence and impact of non-bacterial heritable

877   endosymbionts. In particular there is increasing recognition that viruses may be vertically

878   inherited and can have dynamic interactions with their host (reviewed in insects in (Longdon &

879   Jiggins, 2012)). The moth *Helicoverpa armigera*, a crop pest, is infected with a vertically (and

880   horizontally) inherited densovirus (HaDNV-1) that appears to be mutualistic. In wild larvae a

881   negative interaction exists between the symbiotic densovirus and the presence of a

882   nucleopolyhedrovirus (HaNPV) that is widely used as a pesticide against *H. armigera*.

883   Laboratory work confirmed that larvae carrying HaDNV-1 had significantly higher resistance to

884   the HaNPV pesticide, and also to low doses of *Bacillus thuringiensis* (Bt) toxin. Additionally,

885   HaDNV-1 infected individuals have a higher developmental rate and higher fecundity than that

886   of their uninfected counterparts (Xu et al., 2014). In contrast, in the moth *Homona magnanima* a

887   novel RNA virus appears to be responsible for 'late' male-killing while being benign to female

888   moths, thus acting as a reproductive manipulator (Nakanishi et al., 2008). Metagenomic

889   sequencing has identified viruses across diverse arthropods (e.g. (Li et al., 2015)), and while

890   often pathogenic a recent study identified a vertically inherited sigma virus in the nymphalid

891   butterfly *Pararge aegeria*, that may have a more symbiotic role. In this species transmission of

892   the virus was predominantly maternal (through eggs), with paternal (through sperm) transmission

893   rates being much lower. Wild populations of *P. aegeria* experience high levels of infection, with

894   a mean viral prevalence of 74%, and marked population structure in the genetic diversity of the

895   virus (PAegRV). The nature of the relationship between *P. aegeria* and PAegRV remains to be

896   determined (Longdon et al., 2017).

897

898   *Global environmental change: can endosymbionts facilitate or constrain adaptation?*

899   Predicting if or how organisms adapt to environmental change is a critical and timely question.

900   Every organism interacts with a multitude of abiotic and biotic factors, including heritable

901   endosymbionts, and knowledge of how these influence each other is imperative in understanding

902 an organism's adaptive potential. Global environmental change is likely to alter the level and

903 direction of natural selection in host/symbiont co-evolution (Wolinska & King, 2009). In one

904 direction, endosymbionts may increase the host's potential repertoire for responding to

905 environmental changes such as temperature, while we also recognise that the destabilisation of

906 often finely tuned host-symbiont interactions may be severely detrimental for natural

907 populations.

908   As poikilotherms - organisms that do not maintain internal thermal homeostasis -

909 butterflies and moths are very susceptible to extreme temperatures (Denlinger & Yocum, 1998).

910 While they utilise a range of mechanisms, including behavioural and physiological responses, to

911 regulate temperature, every species is defined by thermal limits. Recent work has indicated that

912 microbial symbionts of insects can often facilitate or constrain adaptation to environmental

913 changes, including temperature. For instance, aphids carry symbionts that proffer heat stress

914 protection (Montllor, Maxmen & Purcell, 2002; Russell & Moran, 2006), including a point

915 mutation (a change in a single nucleotide), which governs host thermal tolerance (Dunbar *et al.*,

916 2007). The temperature insects are exposed to during development is also important in the

917 maintenance of symbionts (Anbutsu, Goto & Fukatsu, 2008), or to the phenotype expressed by

918 the symbiont in the host (Hurst et al., 2000). With global environmental change, de-stabilisation

919 of the host-symbiont interaction may become more frequent and have severe consequences for

920 many species. The sudden loss of an obligatory mutualistic symbiont, for example, would almost

921 certainly lead to a host population decline (for further discussion of host-symbiont interactions

922 and temperature see (Wernegreen, 2012; Corbin et al., 2016; Moran, 2016).

923   Furthermore, the nature of the relationship between host and symbiont may be indirectly

924 affected by the changing climate. A few degrees rise in temperature can alter the geographic

925   range of Lepidoptera (Parmesan *et al.*, 1999). For many species, such range shifts and

926   colonisation events should only be possible if the plants they utilise were following a similar

927   expansion, such as in the host-limited butterfly *Gonepteryx rhamni* (Gutiérrez & Thomas, 2000).

928   Additionally, range shifts may lead to a switch in host plant species or increased generalisation

929   (Braschler & Hill, 2007), bringing subsequent repercussions for Lepidopteran-endosymbiont

930   interactions. For example, in the moth *P. blancardella*, where endosymbionts nutritionally

931   benefit the host by creating photosynthetically active green patches in otherwise senescent leaves

932   of the host plant (Kaiser *et al.*, 2010), a shift in host plant use could make this "green-island"

933   strategy ineffective in a novel plant with a different chemical makeup. In contrast, novel host

934   plant utilisation may also be facilitated by endosymbionts, including through enhanced

935   provisioning of nutrients, or detoxification (reviewed in (Hansen & Moran, 2014)).

936   Finally, habitat degradation and fragmentation is likely to have several implications for

937   natural host-symbiont dynamics. Habitat destruction has the effect of crowding insect

938   populations into smaller patches, and through fragmentation and subsequent isolation, the

939   amount of gene flow between populations becomes reduced. These factors may increase disease

940   transmission within a population, and alter geographical variance in endosymbiont presence and

941   prevalence.

942

943   *Screening butterflies and moths of conservation concern for endosymbionts*

944   The Lepidoptera are model organisms in the fields of conservation and climate change research.

945   However, despite the high occurrence of endosymbionts in Lepidoptera, current conservation

946   planning rarely includes data on endosymbiont infections of the species under consideration, a

947   deficit that may profoundly influence the outcome of any management undertaken. For effective

948   conservation, or to understand how species will respond and adapt to environmental and

949   anthropogenic changes, it is important that we try to understand the intricate relationships that

950   microbes have with the hosts in which they reside. Fortunately there is increasing recognition of

951   this importance with several recent studies reporting endosymbiont infections in populations of

952   endangered or near threatened Lepidoptera (Nice et al., 2009; Sakamoto et al., 2011; Patricelli et

953   al., 2013; McHugh et al., 2013; Łukasiewicz, Sanak & Węgrzyn, 2016; Fenner et al., 2017). One

954   study surveying 22 species of conservation concern (comprising members of the Lycaenidae,

955   Nymphalidae, Hesperidae and Noctuidae) for *Wolbachia* found 19 to be infected (Hamm *et al.*,

956   2014). Nice and colleagues examined the nature of a *Wolbachia* infection in the North American

957   endangered Karner blue butterfly, *Lycaeides melissa samuelis*. Screening for endosymbionts

958   revealed that across the western edge of this butterfly's range there was a widespread *Wolbachia*

959   infection. They went on to simulate demographic effects of the spread of *Wolbachia* into

960   uninfected populations and suggested that the spread of such an infection might further reduce

961   already small population sizes. The authors show concern that the *Wolbachia* infection was

962   prevalent in many of the largest and least impacted populations of this butterfly. This is

963   significant as these populations are likely candidates from whom captive propagation efforts

964   would draw individuals, and so the chance of inadvertently infecting a naturally uninfected

965   population is high (Nice *et al.*, 2009).

966   Release of wild individuals or of those reared in captivity, either as part of conservation

967   management schemes or for commercial purposes (birthdays or weddings), might have

968   unexpected and undesirable impacts if not monitored correctly. Rearing Lepidoptera, which

969   often occurs at high densities, can allow the accumulation of pathogens. Releasing these

970   individuals back into the field may therefore alter the parasite load and consequent fitness of the

971 receiving population. Movement of individuals between populations may also affect the natural

972 spatial pattern of endosymbiont diversity and prevalence: novel microbes may be introduced, or

973 symbionts that have locally adapted in the donor population may affect the host in dramatically

974 different ways in the novel population or environment. Further consequences with regard to

975 endosymbiont infection are likely to be numerous, for example competition between native and

976 novel infections may result in a shift in the natural equilibrium between the host and its native

977 microbes or the introduction of cytoplasmic endosymbionts may also introduce linked variants

978 such as host mtDNA haplotypes or female-linked nuclear DNA. Furthermore, as we have seen in

979 the butterfly *H. bolina*, movement of individuals could also introduce host resistance loci that

980 irrevocably alter the dynamics of host-symbiont interaction, and may have a wider impact upon

981 the host genome. In general, if a novel association does form and/or spread, there follows rapid

982 evolution of both host and symbiont, with phenotypic alterations that alter or optimise the new

983 symbiosis (for an example see (Weeks et al., 2007).

984

985 **Conclusion**

986 The Lepidoptera have emerged as important models in the study of the genetic and functional

987 basis of the reproductive manipulations heritable endosymbionts employ, particularly with

988 regard to *Wolbachia* bacteria. The results of this cumulative work is suggestive of the role of

989 endosymbionts in the evolution of host sex determination itself. We have no doubt Lepidopteran

990 endosymbiont research will continue to highlight the omnipresence and importance of

991 *Wolbachia* but we suggest that more attention should now be given to the presence and

992 interaction of other heritable endosymbionts Lepidoptera carry. Metagenomic approaches enable

993 an unbiased view of the microbial community residing within moths and butterflies, while

994 comparative endosymbiont genomics may illuminate the genetic mechanisms underlying the

995 phenotypes endosymbionts induce in their host. Finally, given the importance of Lepidoptera as

996 key indicators of climate change and the growing numbers of species listed as endangered, the

997 study of heritable microbial endosymbiont in the Lepidoptera should transition from being a pure

998 science filled with interesting curiosities, to a necessity that will contribute to the preservation of

999 natural biodiversity and inform conservation management.

1000

1004

1005 **Figure and Table legends**

1006 **Figure 1. Endosymbiont-induced manipulation of Lepidoptera reproduction.** In the

1007 Lepidoptera, endosymbionts are currently known to manipulate host reproduction in three ways

1008 in order to increase their transmission to the next generation. Male-killing: female hosts infected

1009 with male-killing endosymbionts only give rise to infected female offspring, with male offspring

1010 dying early in development. Feminisation: female hosts infected with feminising endosymbionts

1011 only give rise to infected female offspring, with male offspring having been feminised so that

1012 they are genetically male (ZZ) but phenotypically female. Uninfected males may arise through

1013 inefficient transmission of the infection. Cytoplasmic incompatibility (CI): crosses between

1014 uninfected females and infected males result in few or no viable offspring, as the result of an

1015 incompatibility induced by the endosymbiont in the male. Infected females are able to rescue this

1016 incompatibility and hence are able to produce viable (infected) offspring when mated with

1017   infected males. For male-killing and feminisation the endosymbiont acts as a sex-ratio distorter,

1018   creating a female-bias in the offspring, and potentially in the population if the infection is highly

1019   prevalent.

1020

1021   **Figure 2. *Wolbachia*-induced male-killing and interference of sex determination in *Ostrinia***

1022   ***scapulalis* moths**. A) Uninfected females gives rise to a normal 1:1 sex ratio in progeny: female

1023   offspring have ZW sex chromosomes and express the female isoform of the *Ostrinia* homologue

1024   of a gene in the sex determination cascade, *doublesex (dsx),* called $Osdsx^F$; male offspring have

1025   two Z sex chromosomes and express the male *dsx* isoform $Osdsx^M$. B) *Wolbachia* infected

1026   females only give rise to infected female progeny. Male offspring die early in development due

1027   to a mismatch between the genotypic sex (ZZ) and phenotypic sex ($Osdsx^F$). C) *Wolbachia*-

1028   infected females cured of the infection as larvae by antibiotic treatment only give rise to

1029   uninfected males. Female offspring die early in development due to a mismatch between their

1030   genotypic sex (ZW) and phenotypic sex ($Osdsx^M$). D) *Wolbachia*-infected females cured of the

1031   infection as adults prior to oviposition by antibiotic treatment give rise to sexual mosaics which

1032   have the male ZZ genotype but both $Osdsx^F$ and $Osdsx^M$. Note: there are two female isoforms of

1033   *dsx in Ostrinia scapulalis*: $Osdsx^{FL}$ and $Osdsx^{FS}$; these are simplified to $Osdsx^F$ in this schematic.

1034   White circles: uninfected individual; Red circles: *Wolbachia*-infected individual; Dark grey

1035   circle: *Wolbachia*-infected female cured as larva; Light grey circle: *Wolbachia*-infected female

1036   cured as adult.

1037

1038   **Table 1. Butterfly and moth species recorded as carrying heritable endosymbionts that**

1039   **manipulate the reproduction of the host.** Endosymbiont induced phenotypes are given as MK:

1040   Male-killing, Late MK: Male-killing occurring late in development; CI: Cytoplasmic

1041   Incompatibility; Feminisation; or Sex-ratio distortion (where further investigation is needed to

1042   determine the nature of the sex-ratio bias).

1043

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Table 1(on next page)

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 encedana	*Wolbachia*	MK	Jiggins *et al.* 2000a
	Acraea encedon	*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*	MK	Jiggins *et al.* 2000b
	Hypolimnas bolina	*Wolbachia*	MK &/or CI	Dyson *et al.* 2002; Charlat *et al.* 2006; Hornett *et al.* 2008
	Pieridae			
	Colias erate poliographus	*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 zaguliaevi	*Wolbachia, Spiroplasma ixodetis*	MK	Kageyama *et al.* 2004; Tabata *et al.* 2011
	Ostrinia zealis	Undefined agent	Sex-ratio distortion	Kageyama *et al.* 2004
	Erebidae			
	Lymantria 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	MK	Brimacombe 1980
	Plutellidae			
	Plutella xylostella	*Wolbachia*	Sex-ratio distortion	Delgado & Cook 2009
	Pyrallidae			
	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

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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 distorter, creating a female-bias in the offspring, and potentially in the population if the infection is highly prevalent.
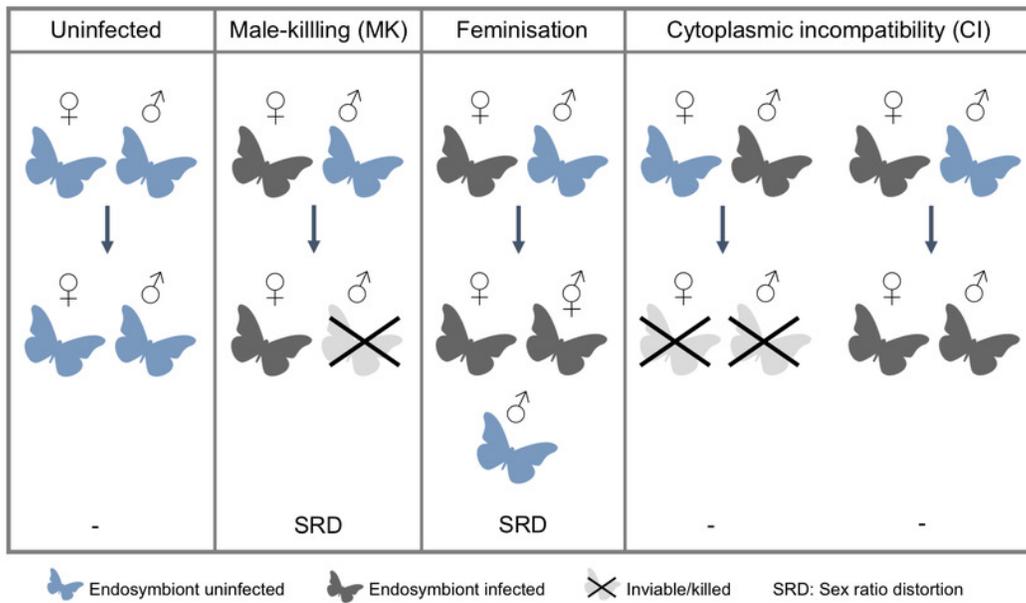
Figure 2

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 *Osdsx*^{F}; male offspring have two Z sex chromosomes and express the male *dsx* isoform *Osdsx*^{M}. 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 (*Osdsx*^{F}). 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 (*Osdsx*^{M}). 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 *Osdsx*^{F} and *Osdsx*^{M}. Note: there are two female isoforms of *dsx in Ostrinia scapulalis*: *Osdsx*^{FL} and *Osdsx*^{FS}; these are simplified to *Osdsx*^{F} 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.