Title: Effects of *Wolbachia* on butterfly life history and ecology.

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ABSTRACT
Wolbachia are intracellular bacteria found in the cytoplasm of a wide range of arthropods. They are geographically ubiquitous and estimated to infect 20-70% of all insect species. They have been in the limelight due to their fascinating manipulations of the reproductive ecology of their hosts, all of which confer a selective advantage to the bacteria by enhancing their vertical transmission efficiency. In butterflies, they induce cytoplasmic incompatibility (CI) and feminization of genetic males. They have also been known to skew sex ratios in favour of females through direct male killing. CI is caused when sperm from infected males cannot produce viable offspring with eggs of females that are not infected by the same *Wolbachia* strain. Some species are infected by more than one strain, resulting in complex interactions between the hosts and *Wolbachia*. In this chapter, I present a synthesis of current knowledge about the extent of infestation and strain diversity of *Wolbachia* in butterflies. I also review experimental work on male-killing, feminization and CI in different butterfly species. I discuss further challenges and future avenues of research in this field.

INTRODUCTION
Bacteria in the genus *Wolbachia* (Rickettsiales) are intracellular symbionts found in a remarkably wide range of invertebrate species across the world including insects, crustaceans, arachnids and nematodes (Werren et al., 2008). They are especially common in insects, with recent estimates suggesting that up to a third of all species, or perhaps even more, harbour these symbionts (Jeyaprakash & Hoy, 2000; Hilgenboecker et al., 2008). The rapid rise in the number of species reported to be infected is in large part due to relatively simple PCR (polymerase chain reaction) based molecular assays that can be used to detect the presence of the bacterium in host tissue (Zhou et al., 1998). Molecular methods have also identified several 'supergroups' and strains along with their distribution across different taxonomic groups. Predominantly found in the reproductive organs of their hosts, they also known in other parts such as haemolyph, brain, gut, salivary glands, muscles, etc (Saridaki & Bourtzis, 2010)

The ubiquitous presence of *Wolbachia* as well as their intriguing effects on host life history and ecology has brought them into the limelight. They can rapidly spread among host populations through vertical lineages (i.e., vertical transmission from mothers to daughters). Although much less frequently, they have repeatedly transferred between different host species (i.e., horizontal transmission) in the evolutionary timescale. Understanding the mechanisms of the latter mode is an interesting endeavour *per se*, but the strategies they have evolved to rapidly spread within a host species are wonderfully fascinating and have attracted much attention. These include reproductive manipulations such as feminization (where a male acquires female traits), induction of parthenogenesis (where female offspring are produced without fertilization), embryonic male-killing (MK) and cytoplasmic incompatibility (CI) (Werren et al., 2008). Of these, the most widespread and best known phenotypic effect is CI, wherein sperm from infected males cannot

produce viable offspring with females that do not harbour the same bacterial strain. Since the bacteria are maternally inherited through the cytoplasm, their fitness in males is effectively zero. Therefore, the four strategies mentioned above – referred to as reproductive parasitism – are thought to enhance the survival and spread of the bacterium in host populations even at the expense of host fitness.

WOLBACHIA IN BUTTERFLIES

This chapter introduces readers to the ecology of interactions between butterflies and *Wolbachia*. Systematic screenings for the presence of *Wolbachia* in butterflies are currently under way. Thus far, they have been found in five (Nymphalidae, Papilionidae, Pieridae, Lycaenidae and Hesperiidae) out of the six butterfly families, and it is very likely that they will also be discovered in Riodinidae. Our work (U. Kodandaramaiah & N. Wahlberg, unpublished data) indicates that at least 50 % of all nymphalid species (Fam: Nymphalidae) are infected. *Wolbachia*-butterfly interactions have been studied extensively in the widespread nymphalid species *Hypolimnas bolina.* This species is distributed in the tropical parts of South and South-East Asia, New Guinea, Australia, Saudi Arabia and Madagascar. All-female broods were discovered in the Fiji islands in the 1920's by H. W. Simmonds, who wrote that the all-female trait was inherited by their daughters. (Clarke et al., 1975) found that mortality occurred in the pre-adult stages and suspected that the all-female trait was passed on cytoplasmically. However, it was not until 2002 that the *Wolbachia* strain *wBol1*was identified as the causal agent using PCR based screening (Dyson et al., 2002)

(Dyson & Hurst, 2004) concluded that extreme female-biased sex-ratios, of the order of 1 male per 100 females, have persevered for at least 75 years in Independent (Western) Samoa. They deduced this by comparing records from the early 20th Century to data from their own resurveys in 2001. Thus, the male-killer appears to be amazingly persistent in nature, especially in light of the drastic effect expected on host populations. Exactly how the symbiont has persisted without the population going extinct or the host evolving mechanisms to counter the sex-ratio distorting effects, is an interesting question. (Dyson & Hurst, 2004) found that male spermatophores were much smaller in the Independent Samoan population compared to that in males of neighbouring islands. They surmise that the persistence of the sex-ratio distorter is related to males having evolved the capacity to mate > 50 times, as also high intrinsic rates of demographic increase in the population.

(Charlat et al., 2007) studied the effects of sex-ratio distortion in several *H. bolina* populations, including Independent Samoa. They found that male spermatophore sizes decreased with increasing female bias, corroborating findings in (Dyson & Hurst, 2004). Interestingly, female promiscuity was higher in female-biased populations, inconsistent with expectations that female mating frequency should be lower when fewer males are available. They conclude that males in populations with skewed ratios are depleted of mating resources, which leads to females mating more frequently in accordance with decreasing male investment per copulation. Thus, *Wolbachia* engenders a domino effect of increasing female promiscuity and decreasing male productivity.

The impact on reproductive ecology is even more profound in another nymphalid species *Acraea encedon*. (Jiggins et al., 1998) first showed that all-female broods in this African species were caused by *Wolbachia*. In uninfected populations with normal sex-ratios, males typically seek out and compete for matings from females who fly in the vicinity of their larval host plants. Such female sexual selection is typical in most animals since females invest more per offspring. However, in female biased populations of *A. encedon*, females flock together in dense lekking swarms in grassy patches nearby prominent landmarks, and exhibit a range of behaviours to attract males (Jiggins et al., 2000). More than 350 females have been recorded in as small an area as 10x20m! Mark recapture experiments show that mated females are more likely to remain in swarms compared to virgin females, which reinforces the idea that the female lekking behaviour is intended for mating. Uninfected females are more likely to be mated, indicating that males might

preferentially mate with uninfected individuals. Male lekking, where males exhibit lekking behaviour and females choose amongst them, is a common mating system found in other animals. *Wolbachia* infestation has hence effected a fascinating reversal of sex roles in this species. If males indeed discriminate against infected females, uninfected females are at a selective advantage, but only when infection rates in the population are high. This eventually results in frequency dependent selection that prevents the population from going extinct (Jiggins et al., 2000).

In both *H. bolina* and *A. encedon*, prevalence rates of *Wolbachia* vary considerably across populations (Jiggins et al., 2000; Charlat et al., 2005). It must also be noted that embryonic mortality of males is not necessarily 100%, although male offspring from females infected with MK strains are always rare. Some SE Asian and Japanese populations of *H. bolina* have in fact evolved mechanisms that counter the male killing effects of *wBol1* (Charlat et al., 2005; Mitsuhashi et al., 2004). This is not highly surprising considering that selection to counter the pernicious effects of extreme sex-ratios is expected to be strong and relentless. Given the widespread presence of *Wolbachia* in a diversity of taxa, very few of which have been reported to have distorted sex-ratios, it is likely that such suppression has evolved repeatedly. What is interesting is that in *H. bolina* populations where the MK phenotype is suppressed, the same *wBol1* strain induces CI (Hornett et al., 2008) Hornett and colleagues argue, with good reason, that the expression of CI follows immediately after MK is inhibited. The CI phenotype in *H. bolina* therefore appears to be a backup strategy when the primary mechanism of MK fails.

CI in this case is of the simplest form – unidirectional incompatibility – wherein mating between infected males and uninfected females produce fewer viable offspring. Infected females, though, are fully compatible with all males. It is easy to see, unlike in the case of MK, how a CI-inducing parasite can quickly spread through a panmictic population. It is somewhat perplexing as to why CI is merely plan B for *wBol1* and not the primary one. Nevertheless, a novel CI-only strain does not possess any selective advantage over an already established MK+CI strain. Hence, although a CI-only strain can potentially gain higher prevalence in a completely uninfected population, it is unable to achieve this in a population where the MK+CI strain is already well-entrenched.

The mechanistic basis of CI has not been deciphered completely. Based on earlier theories, Werren (Werren, 1997) presented a synthesis of the '*mod-res*' model, which is now the most widely accepted explanation. Accordingly, sperm of infected males are modified by *Wolbachia* rendering them incompatible with normal eggs. However, the same strain when present in eggs can rescue the sperm modification, making the crosses completely compatible. Interested readers are referred to (Poinsot et al., 2003) for a discussion of theories seeking to explain the molecular mechanism of CI. Patterns of CI are more complex in the case of infections with more than one strain, or 'super-infection'. Double infections are common whereas triple or higher level infections are rare in nature (Jamnongluk et al., 2002). Sperm from singly infected males can be rescued by multiply infected females whereas singly infected females cannot rescue sperm from multiply infected males. This is because rescue and modification takes place independently among the strains. This form of CI is termed additive incompatibility.

Hiroki and colleagues (Hiroki et al., 2002; Hiroki et al., 2004) have studied additive incompatibility within *Eurema mandarina* (Fam Pieridae; formerly *Eurema hecabe*, yellow type) in Japan. On Okinawa-jima Island, two *Wolbachia* induced phenotypes are found. The first is CI induced by a strain very similar to those found in *H. bolina* and *A. encedon* (note that more than one MK strain is known in *A. encedon*). The second phenotype is feminization, where genetic males express female morphological traits and are also reproductively competent. It is unknown specifically how *Wolbachia* achieve this sex change in this species, but it has been shown in isopods that the bacterium acts by suppressing an androgenic gland during development (Rigaud et al., 1991). (Narita & Kageyama, 2008) provide a good discussion about the mechanistic bases of MK and

feminization in Lepidoptera, and conclude that these symbionts closely interact with the sex determination system to bring out these changes. Experiments carried out by (Narita et al., 2007) indicate that suppression of the male phenotype in *E. mandarina* occurs continuously over larval development, rather than a 'developmental switch' been triggered at some point. (Hiroki et al., 2004) discovered that *E. mandarina* individuals affected by feminization were in fact infected by two strains, one of which was the CI-inducing strain in the species and the second, restricted to feminized matrilines. Experiments confirmed that feminization-affected females could rescue sperm from CI males. To date, this is the only species among insects where complete sex-reversal by an endosymbiont has been reported (Narita & Kageyama, 2008).

In addition to unidirectional and additive incompatibility, a third type of CI - bidirectional incompatibility - occurs when a male and female harbour mutually incompatible strains. (Charlat et al., 2007) discovered this incompatibility between *wBol1* and a second strain, *wBol2*, in *H. bolina*. *wBol2* is widespread in populations without the MK strain *wBol1*, but rare in populations where the latter prevails. This type of CI raises the prospects of some interesting scenarios of competition and co-evolution among different *Wolbachia* strains.

WHY KILL MALES AT ALL?

Although the fitness of *Wolbachia* in males is nil, specifically how the bacterium benefits from MK is unknown. One hypothesis is that the clonal relatives in the dead males' sisters benefit through cannibalization of their dead brothers (Hurst & Majerus, 1993). However, there is little experimental evidence to support this suggestion. Generally speaking, MK is beneficial to the bacterium only when females can take advantage of the resources for which they would have otherwise have had to compete with their brothers (Hurst & Majerus, 1993). Further work is needed to understand this phenomenon.

OTHER EFFECTS ON HOST BIOLOGY

The effects of *Wolbachia* on host biology apart from reproductive parasitism are much less understood. Given their phenomenal abundance and omnipresence, one can envisage a diverse array of parasitic, beneficial and mutualistic interactions with their hosts. For instance, CI inducing *Wolbachia* in the pierid butterfly *Colias erate* enhance survival rates in relation to *Wolbachia*-free individuals under laboratory conditions (Narita et al., 2009). Similarly, infected females of *Aedes albopictus* mosquitoes have higher longevity, egg hatch rate and fecundity compared to uninfected females (Dobson et al., 2004). In *Drosophila* flies, infection by the bacterium decreases mortality against a range of RNA viruses (Hedges et al., 2008; Dobson et al., 2004). These examples illustrate mutualism where both the host and bacteria are benefited. On the other end of the spectrum, they also produce drastically negative effects on their hosts. An excellent example is the well known life shortening strain that can reduce longevity by half or even more in *Drosophila* (Min & Benzer, 1997). (Fry et al., 2004) have demonstrated that fecundity and survival effects in *Drosophila melanogaster* vary among fly strains, ranging from positive to negative. Findings from such studies that have investigated the influence of *Wolbachia* on life history traits have probably merely scratched the surface. Given the ubiquity of the parasite, further studies are likely to reveal other intriguing effects on host life history.

CONCLUSION

The effects of *Wolbachia* on their hosts are profound, multifarious and arguably as fascinating as any other aspect of ecology. There is good reason to believe that at least half of all butterflies harbour this bacterium, and hence the effect of the symbiont on butterfly life history and ecology are likely to be significant. Butterflies have been hailed as a model taxon in the study of ecology and evolution (Boggs et al., 2003) and have indeed been the subject of some of the most interesting *Wolbachia* related studies. There is enormous scope for further research into understanding the fascinating biology of *Wolbachia*-butterfly interactions. Molecular techniques for rapid detection of

Wolbachia are a huge boon for such work. An important step will be to characterize the prevalence of different bacterial strains across various phylogenetic groups and geographic regions. Nonetheless, field and lab-based experimental work on selected species will likely unearth the most spectacular findings.

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