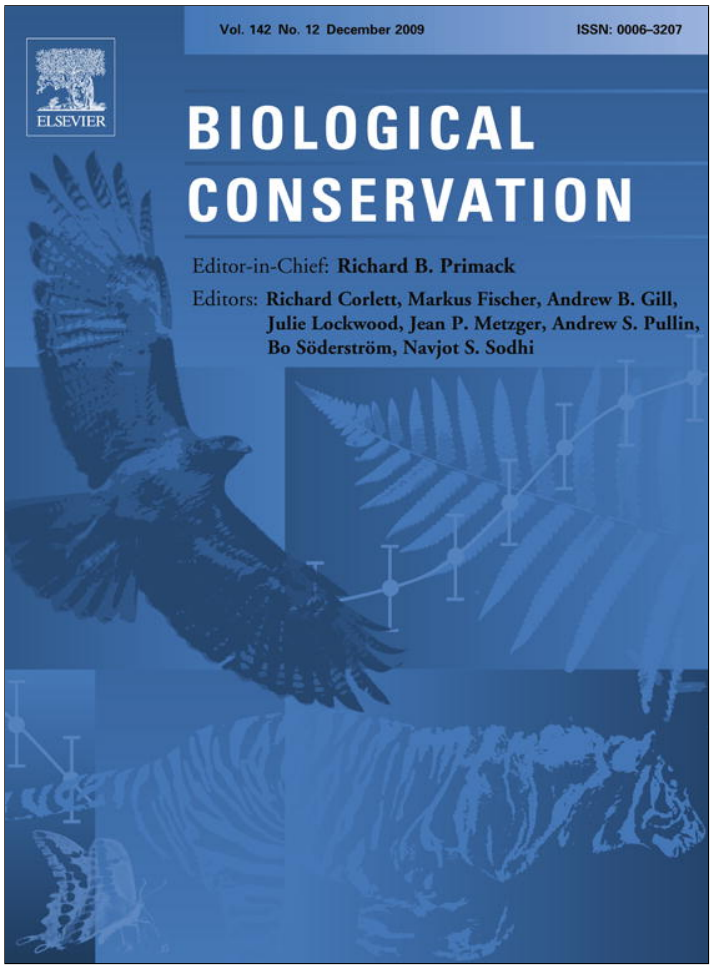

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An unseen foe in arthropod conservation efforts: The case of *Wolbachia* infections in the Karner blue butterfly

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ABSTRACT

Infection by endosymbiotic bacteria is an underappreciated threat to endangered arthropods with serious implications for captive management programs. We examined the nature of *Wolbachia* infection in the North American endangered Karner blue butterfly, *Lycaeides melissa samuelis*, as a case study. Screening for *Wolbachia* across the range of the species confirmed widespread infection in the western half of the Karner blue's range. Multilocus sequence typing using six genes confirmed that the infection in the western populations is attributable to a single strain of *Wolbachia*. This strain was also detected in the closely related Melissa blue butterfly, *L. m. melissa*, the presumed source of the infection. The infection in the Karner blue butterfly was perfectly correlated with the presence of a foreign mitochondrial DNA variant present in the Melissa blue butterfly, consistent with the hypothesis that the mitochondrial introgression was driven by the spread of *Wolbachia*. A single individual out of 71 screened from the eastern portion of the range of the Karner blue butterfly was also infected, however this infection was attributable to a different strain. Simulation models of the demographic effects of the spread of *Wolbachia* infection to uninfected populations and metapopulations suggest that such an infection might further reduce already small population sizes and substantially increase the probability of population extirpation. We discuss threats to other endangered arthropods in the light of this case study and make recommendations for minimizing the impact of endosymbiont infections in conservation plans, especially those including captive propagation and augmentation of endangered arthropod populations.

1. Introduction

Threatened and endangered species face a multitude of complex challenges and conservation biologists and natural resource managers must find effective ways to ameliorate the risks associated with these challenges. Arthropod species face an underappreciated additional threat in the form of heritable endosymbiotic bacteria. These intracellular microorganisms are maternally inherited, and often manipulate and exploit their host's reproductive biology. A wide range of phenotypes are expressed, but these bacteria are commonly considered to be reproductive parasites, adaptively increasing the production of infected female hosts (Moran et al., 2008; Werren et al., 2008). The most well-studied and probably the most frequent endosymbionts of invertebrates are alpha-Proteobacteria, including especially the genus *Wolbachia* (Charlat et al., 2003; Moran et al., 2008; Werren et al., 2008). These intracellular parasites infect a range of invertebrate species, and a recent meta-analysis suggests that up to 65% of insect species might be infected by *Wolbachia* (Hilgenboecker et al., 2008; Jeyaprakash and Hoy, 2000; Werren et al., 2008, 1995; Werren and Windsor, 2000; West et al., 1998).

Wolbachia infections are associated with a variety of phenotypic effects on the host, most that are adaptive from the bacterial perspective and most also have detrimental effects on the population biology of the hosts (Charlat et al., 2003; Moran et al., 2008; Werren et al., 2008). In some instances *Wolbachia* infection might benefit the host. Recent studies indicate that *Wolbachia* infections might confer viral resistance to the host (Hedges et al., 2008; Teixeira et al., 2008) and appear to be beneficial in nematodes (Bandi et al., 1998). However, most *Wolbachia* phenotypes pose unique threats to host populations (Werren et al., 2008). These phenotypes include: (1) those that induce parthenogenesis in the host, (2) feminization of genetic males, (3) killing of sons of infected mothers, and (4) cytoplasmic incompatibility (CI), which is the most frequently observed phenotype (Hoffmann and Turelli,

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1997; Werren et al., 2008). The details of the CI mechanisms vary among strains of *Wolbachia* and are not fully understood (Serbus et al., 2008; Werren, 1997; Werren et al., 2008; Zabalou et al., 2008), however, the overall effect is the same: sperm of infected males are incapable of fertilizing the eggs of uninfected females and females that are infected with a different strain of *Wolbachia*. This apparently involves an alteration of the sperm of infected males, an effect that is only rescued by interaction with an egg infected with the same strain. Thus development of embryos is prevented unless the infected sperm unites with an infected egg. This phenotype can significantly reduce the relative fitness of uninfected females (Jansen et al., 2008). Indeed, the evolutionary consequences of all of these phenotypes include the continued production of infected daughters, which ensures the transmission of the infecting bacteria (Charlat et al., 2003; Hurst et al., 1993).

The biology of *Wolbachia* presents at least two significant problems for the conservation of arthropod species: First, the spread of *Wolbachia* via CI can drive the spread of maternally inherited genetic elements through hitchhiking (Hurst and Jiggins, 2005; Jiggins, 2003; Rasgon et al., 2003; Turelli et al., 1992). For example, specific mitochondrial genotypes surreptitiously associated with a *Wolbachia* CI strain experience linkage disequilibrium with the *Wolbachia* infection and can be "swept" along with the spread of the infection. This amounts to a selective sweep of mtDNA haplotypes through indirect selection and linkage disequilibrium (Hurst and Jiggins, 2005; Jiggins, 2003; Turelli and Hoffmann, 1991; Turelli et al., 1992). Consequently, the distribution of mtDNA variation in infected populations does not conform to the expectations of neutral theory. This has been observed in a variety of invertebrate taxa, including chrysomelid beetles (Keller et al., 2001), fire ants (Shoemaker et al., 2003), mosquitoes (Shaikevich et al., 2005), butterflies (Gompert et al., 2008b; Narita et al., 2006, 2007) and several other insect taxa (Hurst and Jiggins, 2005). *Wolbachia* infections with the CI phenotype can cross species boundaries via hybridization and horizontal transmission. From a conservation perspective, the linkage of mtDNA and *Wolbachia* reduces the efficacy of mtDNA-based identification of taxa, including diagnosis or discovery of population segments that warrant conservation concern. Given the high prevalence of *Wolbachia* infections (perhaps up to 65% of insect species (Hilgenboecker et al., 2008)), this may pose a serious problem for the sole reliance on mtDNA as a marker for conservation purposes (Forister et al., 2008).

The second problem is the potential demographic impact of *Wolbachia* infection on a population. Essentially, the introduction of a CI strain into an uninfected population reduces the effective population size in a manner directly analogous to changes in the effective sex ratio. Such a reduction of population size increases the exposure of populations to demographic stochasticity and raises the probability of extinction for already at-risk populations (Charlat et al., 2003; Dobson et al., 2002; Jiggins et al., 2000). While the reduction in effective population size is a temporary effect, lasting until the infection is fixed or extinct in the population, populations of conservation concern could experience increased risk of extinction above their normally high risk with the introduction of *Wolbachia*. Infection also increases the risk of reducing standing genetic variation within populations (Turelli et al., 1992). Indeed, because of their detrimental demographic effects and the linkage disequilibrium between *Wolbachia* and any deleterious cytoplasmic element, *Wolbachia* inoculation has been proposed as a pest control strategy (Dobson et al., 2002; Jansen et al., 2008; Turelli and Hoffmann, 1991; Zabalou et al., 2004). However, due to the potentially detrimental demographic and genetic effects of *Wolbachia* infections, natural transmission can pose a serious risk for endangered arthropods. Similarly, captive propagation and augmentation or reintroduction programs for endangered invertebrates run the risk of inadvertently introducing *Wolbachia* infections.

Here we focus on these issues using *Wolbachia* infection in the endangered Karner blue butterfly (KBB), *Lycaeides melissa samuelis*, as a case study. The KBB is a North American endangered species whose range formerly included much of the northeastern United States from Minnesota to Maine, and southern Ontario, Canada (Black and Vaughan, 2005). Karner blues are bivoltine, occupy pine-barrens or pine prairies, oak savannas and lakeshore sand dunes habitats, and feed exclusively on *Lupinus perrennis* as larvae. The KBB has declined dramatically throughout its range and now occupies a small fraction of its former range with an overall population size 1% of that a century ago (US Fish and Wildlife Service, 1992; Haack, 1993). The KBB is extinct in Iowa, Illinois, Maine, Pennsylvania, Vermont, Massachusetts and Ontario, and nearly extinct in Minnesota (US Fish and Wildlife Service, 2008). The KBB is also believed to have been extirpated from New Hampshire in 2000 (US Fish and Wildlife Service, 2003) where reintroduction efforts are currently underway (New Hampshire Fish and Game Department, 2005). In fact, captive propagation programs are producing adult butterflies for release and reintroduction in Ohio, Indiana, and New Hampshire (US Fish and Wildlife Service, 2008).

Recently, we detected evidence of mtDNA introgression into the endangered KBB, presumably via hybridization with the Melissa blue butterfly, *L. m. melissa* (Nice et al., 2005). This introgression has apparently replaced KBB mtDNA haplotypes in all sampled KBB populations west of Lake Michigan. All individuals sampled from populations west of Lake Michigan were invariant and fixed for a mitochondrial genotype that was also found in Melissa blue populations. KBB populations east of Lake Michigan (including populations in Indiana, Michigan, New York and New Hampshire) possess a distinctly different haplotype. Gompert et al. (2006) confirmed these observations and demonstrated that this mtDNA introgression was accompanied by little or no nuclear introgression, suggesting that the apparently limited hybridization did not disrupt the integrity of the KBB nuclear genome. Moreover, this examination of nuclear genetic variation demonstrated that the KBB is the most genetically cohesive taxonomic entity we have studied within the North American *Lycaeides* (Gompert et al., 2008b, 2006).

We recently determined that this mtDNA introgression observed in the KBB is part of a more widespread invasion of unique mitochondrial haplotypes among various *Lycaeides* taxa. This mtDNA invasion is associated with *Wolbachia* infection (Gompert et al., 2008a,b). The geographic distribution of mtDNA variation in the KBB, with the sharp boundary between distinctly different haplotypes at Lake Michigan, might indicate that nearly half of the range of this endangered species, and many of the largest KBB populations in Wisconsin that could be sources for augmentation efforts to bolster other KBB populations, contain a *Wolbachia* strain. This is likely a strain conferring CI, given that there have been no reports of sex-ratio distortion characteristic of the other common *Wolbachia* phenotypes, and because CI is the most likely to be associated with a mitochondrial "sweep". This infection, if indeed it has spread throughout the western half of the KBB's range, could pose a serious threat if spread naturally, or inadvertently introduced, into uninfected populations beyond (eastward of) Lake Michigan. In this study, we examined the extent of the *Wolbachia* infection and determined the genotypes infecting KBB populations, and developed demographic models describing the effect of *Wolbachia* infection on KBB population dynamics to answer the following questions:

1. To what extent is the mtDNA introgression in the endangered KBB associated with *Wolbachia* infection? We tested for the presence of *Wolbachia* in KBB individuals to assess the extent

of infection and its correlation with mtDNA variation. Evidence of a near perfect correlation would be consistent with the hypothesis that the mitochondrial introgression is a consequence of the spread of *Wolbachia* infection.

2. Are KBB populations infected with a single strain of *Wolbachia*? The presence of a single strain, as identified by multilocus sequence typing (MLST) (Baldo et al., 2008, 2006), would also provide support for the hypothesis that *Wolbachia* infection is responsible for the mitochondrial "sweep". This hypothesis predicts a homogenous and widespread *Wolbachia* infection in linkage disequilibrium with a mtDNA haplotype, or a closely related group of haplotypes.
3. Does *Wolbachia* infection pose a significant threat to uninfected KBB populations? We employ a modeling approach to explore the demographic consequences of introducing *Wolbachia* into KBB populations or metapopulations by either natural means or as a consequence of population augmentation efforts.

2. Materials and methods

2.1. Population sampling

KBB (*L. m. samuelis*) populations were sampled by collecting only male butterflies from the second flight under USFWS permit PRT842392 (Table 1, Fig. 1), with a few exceptions: two females were included in the sample from Saratoga, NY; the Concord, NH sample consisted of wild-caught individuals that died in captivity or were found dead in the field and included some females; and the samples from Adams, WI and Allegan, MI were collected prior to listing of the KBB as endangered. In total, 212 KBB individuals from 13 populations were sampled. Twenty-nine individuals were also collected from a Melissa blue (*L. m. melissa*) population, the potential source of the *Wolbachia* infection (Gompert et al., 2006, 2008b) (Table 1, Fig. 1). Genomic DNA was extracted from all individuals following the methods of Hillis et al. (1996) and Brookes et al. (1997). Genomic DNA preparations specifically did not include reproductive tissues; male abdomens were removed for morphological analysis of genitalia (Lucas et al., 2008; Nice et al., 2005; Nice and Shapiro, 1999), and female abdomens were removed to prevent potential contamination by mitochondria associated with any spermatophores. Consequently, *Wolbachia* screening assumed that the infecting strain was not confined to reproductive tissues (Dobson et al., 1999; Espino et al., 2009; Frydman et al., 2006; Serbus et al., 2008).

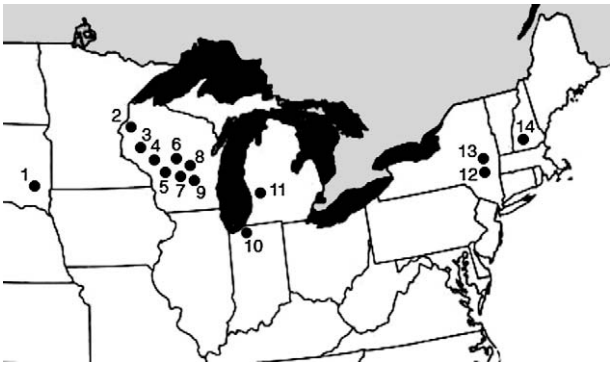

Fig. 1. Map of *Lycaeides* sampling localities. Population numbers correspond to Table 1.

2.2. Wolbachia screening

Screening for *Wolbachia* in genomic DNA samples of butterflies required two PCR reactions: primers for the amplification of *Wolbachia* 16S rDNA were used to detect *Wolbachia*, and universal arthropod primers for 28S rDNA were used to verify negative results of the *Wolbachia* PCR. In other words, a negative *Wolbachia* reaction combined with a positive second reaction for arthropod 28S rDNA indicated that PCR inhibitors were not present in the reactions. The *Wolbachia* 16S fragment was amplified with standard PCR protocols with the following primers: 16S forward primer WSpecF 5′ CAT ACC TAT TCG AAG GGA TAG 3′(*Escherichia coli* position 1004); 16S reverse primer WSpecR 5′ AGC TTC GAG TGA AAC CAA TTC 3′ (*E. coli* position 1442) (Werren and Windsor, 2000). The arthropod 28S fragment was amplified with the following primers: 28sF3633 forward primer 5′ TACCGTGAGGGAAAGTTGAAA 3′; 28sR4076 reverse primer 5′ AGACTCCTTGGTCCGTGTTT 3′ (Morse et al., 2009). PCR products were visualized on 1% agarose gels and scored for the presence of *Wolbachia* infection. Any individuals that failed to produce a PCR band for the arthropod 28S rDNA reaction were subjected to a dilution series from 1 part genomic DNA in 10 parts water (1/10) to 1 part in 200 (1/200). (Dilution is thought to remove the effects of PCR-inhibiting substances (Werren and Windsor, 2000).) Any sample dilution that produced a positive 28S rDNA band was then tested at that concentration with the *Wolbachia* primers. A haphazardly chosen subset of 40 (17% of the total number of individuals sampled) were rerun for both reactions to confirm results.

Table 1
Sampling locality data, distribution of mitochondrial DNA variation and details of the frequency and strain of *Wolbachia* infection.

Pop. #	Taxon	Locality	Sample date	mtDNA genotype group[a]	*Wolbachia n* (# infected)[b]	Strain[c]
1	*L. m. melissa*	Brandon, SD	2004	A	29 (26)	162
2	*L. m. samuelis*	Fish Lake, WI	1996	A	20 (20)	162
3	*L. m. samuelis*	Eau Claire, WI	1996	A	22 (22)	162
4	*L. m. samuelis*	Black River Falls, WI	1996	A	17 (17)	162
5	*L. m. samuelis*	Fort McCoy, WI	1998	A	25 (25)	162
6	*L. m. samuelis*	Sandhill, WI	1996	A	21 (21)	162
7	*L. m. samuelis*	Necedah, WI	1996	A	22 (22)	162
8	*L. m. samuelis*	Welch/Hartman, WI	1996	A	10 (10)	162
9	*L. m. samuelis*	Adams, WI	1978	A	4 (4)	Not genotyped
10	*L. m. samuelis*	Indiana Dunes, IN	1999	C	22 (0)	
11	*L. m. samuelis*	Allegan, MI	1980	C	1 (0)	
12	*L. m. samuelis*	Pine Bush/ Albany, NY	1999	C	5 (0)	
13	*L. m. samuelis*	Saratoga, NY	1998	C	30 (1)	SS8
14	*L. m. samuelis*	Concord, NH	1991–1996	C	13 (0)	

[a] Data from Nice et al. (2005) and Gompert et al. (2006, 2008a).
[b] Number of individuals tested using the *Wolbachia* specific 16S rDNA primers (see text for details).
[c] Determined using the multilocus sequence typing protocols of Baldo et al. (2006, 2008) (see text for details).

2.3. Multilocus sequence typing

Identification of *Wolbachia* strains present in populations was performed by sequencing multiple loci recommended by the *Wolbachia* MLST database (http://pubmlst.org/wolbachia) (Baldo et al., 2008, 2006). This included sequencing fragments from six *Wolbachia* genes: *gatB*, *coax*, *hcpA*, *ftsZ*, *fbpA*, and the *Wolbachia* surface protein, *wsp*. Two arbitrarily chosen individuals from each population showing infection were sequenced, except for the Adams, WI population which was omitted because of the age of the samples and the proximity of this locality to other KBB populations. The Saratoga, NY population had only one individual that tested positive for *Wolbachia* (see Section 3), and this individual was sequenced for the six markers. Primer sequences and PCR protocols are available from the *Wolbachia* MLST database (http://pubmlst.org/wolbachia). Sequencing was performed at the Nevada Genomics Center at the University of Nevada, Reno using Applied BioSystems (Foster City, CA) protocols. Sequences were edited and aligned using Sequencher 4.2.2 and uncorrected sequence distances were calculated using PAUP* version 4.0b10 (Swofford, 2002). Sequence chromatograms were submitted to the *Wolbachia* MLST database for identification. Identification is based on comparison of genotypes for all loci against the database (Baldo et al., 2008, 2006).

2.4. Demographic models of Wolbachia infection

We developed a simple stage-structured demographic model to assess the effect of the spread of *Wolbachia* infection on KBB demography. The model did not include density-dependence and four KBB developmental stages were modeled: egg, larva, pupa, and adult. Stage specific survival proportions and fecundity were assumed to vary stochastically among generations (survival proportions) and individuals (fecundity). Survival proportions and fecundity for specific generations and individuals were sampled from rescaled beta distributions ($\alpha = 2$, $\beta = 3$), which are continuous unimodal right-skewed distributions defined over discrete intervals. The minimum and maximum values for these beta distributions are provided in Table 2. Two sets of minimum and maximum values were modeled for the survival proportion of eggs to the larval stage, as these proportions are thought to vary between the first and second KBB brood each year, resulting in a higher adult population size for the second brood (Lane and Andow, 2003; US Fish and Wildlife, 2003). The adult to egg fecundity corresponds to the output of a mated pair and thus reflects the fecundity of two butterflies. Survival proportions and fecundity were based on estimates from the literature (e.g. Lane and Andow, 2003; Pickens and Root, 2008) and the experience of the authors in rearing *Lycaeides* butterflies. Minor changes to these values did not qualitatively affect the model results, however, lowering these values substantially caused KBB populations to go rapidly extinct (results not shown).

We assumed a carrying capacity (K) for each KBB population. This carrying capacity was an abstraction representing the amount of host plant available for simulated populations, and thus, the number of eggs that could be supported with a non-zero probability of survival. Simulations were run with low (12,500), intermediate (25,000) and high (125,000) egg carrying capacities, which resulted in adult KBB population sizes within the range of population sizes observed in the wild (i.e. fewer than 100 to thousands of adults; US Fish and Wildlife Service, 2003).

Table 2
Transition matrix for demographic models of *Wolbachia* infection. Values are stage specific minimum and maximum survival proportions and fecundity schedules which are sampled from a rescaled beta distribution (see text for details).

Egg	0	0	0	0–180
Larva	0.05–0.35[a] 0.15–0.65[b]	0	0	0
Pupa	0	0.1–0.55	0	0
Adult	0	0	0.35–0.8	0

[a] Survival proportion for first generation (see text for details).
[b] Survival proportion for second generation.

The model was run in the absence *Wolbachia* infection and with CI *Wolbachia* at an initial infection frequency (w) of 0.05. This value was chosen to represent the early stage of the spread of *Wolbachia* into a previously uninfected population. *Wolbachia* infection transmittance from infected females to their progeny was assumed to be perfect. This assumption seems reasonable as many KBB populations are fixed for *Wolbachia* infection (see results). We assumed that all offspring produced by mating between infected males and uninfected females suffered embryonic mortality. The extent of mortality incurred by CI *Wolbachia* varies by strain and host, but is often high and can be 100% (Hoffmann and Turelli, 1997; Zabalou et al., 2004). Thus, this assumption represents a possible, albeit worst-case scenario, for the effect of *Wolbachia* infection on KBB demography. Mating between individuals with and without *Wolbachia* infection occurred proportional to the frequency of *Wolbachia* infection in the population (i.e. we assumed random mating with respect to infection status), and all individuals were assumed to mate only once. There is little information on mating frequency for KBB females, however, this assumption is supported by indirect evidence from other lycaenid butterflies (Pierce and Nash, 1999) in which females rarely mate more than once, and by sperm precedence observed in swallowtail butterflies (Clarke and Sheppard, 1962).

We ran 1000 model replicates of 100-generations (corresponding to 50 years as KBB are bivoltine) for each model combination (i.e. low, intermediate, and high carrying capacity with and without *Wolbachia* infection). Simulations were initiated with an egg population arbitrarily set at 75% of the carrying capacity (varying the initial population size did not qualitatively change the model outcomes, results not presented). Population size and *Wolbachia* infection frequency through time were monitored. A permutation test was conducted to test for differences in adult KBB population size through time between simulations with and without *Wolbachia* infection for each egg carrying capacity. This was done by first calculating the difference between the summed adult population size across all replicates and generations for simulations with and without *Wolbachia* (i.e. $\Sigma_{\text{diff}} = \Sigma w^{-} - \Sigma w^{+}$, where w^{-} denotes the adult population size for simulations without *Wolbachia* and w^{+} denotes the adult population size for simulations with *Wolbachia*). We then permuted simulation replicates between the two *Wolbachia* treatment levels while keeping individual replicates intact and calculated test statistics as described above. Permuting replicates in this manner results in a statistical test analogous to repeated-measures ANOVA. We then calculated P-values by calculating the proportion of permuted data sets that had a greater summed difference than the original data sets (i.e. this was a 1-tailed test). A second set of permutation tests was conducted to test whether the number of replicates resulting in KBB population extirpation was greater for simulations with *Wolbachia* than for simulations without *Wolbachia*. We produced 1000 permuted data sets for each test. The KBB demographic model and permutation tests were conducted in R (R Core Development Team 2008) with code written by the authors.

We developed a metapopulation model as an extension of the single population KBB demographic model described above to assess the effect of *Wolbachia* infection on metapopulation site occupancy. This extension is important as KBB display metapopulation dynamics (US Fish and Wildlife, 2003). We modeled a KBB metapopulation with 10 sites of equal size (i.e. equal carrying capacity)

connected by migration. Migration occurred between the adult and egg stages. Migration probabilities were set equal among all pairs of sub-populations (i.e. as an island model). Specifically, there was a 0.05 probability of migration occurring from each occupied site to an occupied or unoccupied site each generation. The destination site for migrants was determined randomly (if the selected destination site was the same as the source site migration did not occur). The proportion of the source population emigrating was determined stochastically by sampling a rescaled beta distribution ($\alpha = 2$, $\beta = 3$, minimum = 0.01, maximum = 0.2). We assumed migrant dependent mortality such that a proportion of emigrants died during migration, this proportion was determined stochastically and assumed to be beta distributed ($\alpha = 2$, $\beta = 2$). These parameters were selected to roughly approximate dispersal and migration estimates for KBB from the literature assuming sub-populations separated by a few kilometers (Welch, 1993; King, 1998; US Fish and Wildlife, 2003). Migration to an unoccupied site resulted in colonization, whereas migration to an occupied site augmented the size of the local sub-population. Stochastic local extirpation of sub-populations was also modeled. Each generation there was 0.05 probability that each sub-population with an adult population of less than 1000 individuals was extirpated with a probability (P_D) where $P_D = 1 - (\log(\text{adult population size})/\log(1000))$. The value of 1000 adult individuals was selected because the KBB population at Ontario Port Franks was extirpated despite a second brood adult population size of approximately 900 individuals (Packer, 1994; Schweitzer, 1994). Survival proportions and fecundity for the metapopulation were determined in an identical manner to the single population model (Table 2). As with the single population model, 1000 replicate 100-generation simulations were conducted for each of three sub-populations egg carrying capacities (low = 12,500; intermediate = 25,000; high 125,000) with and without *Wolbachia* infection. For simulations involving *Wolbachia*, the initial *Wolbachia* infection frequency for all sub-populations was set to 0.05 and migrants were assumed to be infected with *Wolbachia* at the same frequency as their source population. All simulations were initiated with five occupied sites with an egg population size 75% of carrying capacity.

The number of sites occupied through time and the frequency of *Wolbachia* infection in each sub-population were tracked. We used permutation tests to determine if the number of sites occupied through time was greater for simulations without *Wolbachia* than simulations with *Wolbachia* for each carrying capacity. Permutation tests were conducted as described above for tests of differences in adult population size for the single population models (i.e. entire replicates were permuted, however the difference in the number of occupied sites was assessed instead of the difference in adult population size). As with the single population model simulations, KBB metapopulation model simulations and associated permutation tests were conducted in R (R Core Development Team 2008) using code written by the authors.

3. Results

3.1. Wolbachia screening

Two hundred and forty-one individuals were screened for *Wolbachia* (Table 1). Twenty six of 29 individuals from the Brandon, SD *L. m. melissa* population tested positive for *Wolbachia*. All 120 individuals from KBB populations in Wisconsin tested positive for infection, thus, 100% of KBBs sampled from west of Lake Michigan were infected with *Wolbachia* (Table 1). Of the 71 KBB samples from the five localities sampled from east of Lake Michigan, only one individual from Saratoga Springs, NY tested positive for *Wolbachia*. In terms of mtDNA association with *Wolbachia*, the presence of mtDNA haplotypes from *L. m. melissa* populations (the A-group of haplotypes; see Gompert et al., 2006, 2008a,b) in KBB populations was perfectly correlated with our detection of *Wolbachia* infection, with the exception of the infected Saratoga Springs individual (Table 1). This single infected individual from east of Lake Michigan possessed a distinctly different mtDNA genotype (the C-group of mtDNA haplotypes) (see details in Gompert et al., 2008a,b).

3.2. Multilocus sequence typing

Screening of the five MLST loci and the *wsp* locus from two individuals from seven western KBB populations and the *L. m. melissa* population from South Dakota indicated the presence of a single strain. All 16 individuals possessed the same sequence for all six loci. Sequence typing of these individuals produced new alleles (i.e. not already present in the database) for the *gatB* and *ftsZ* loci, with alleles at the other four loci matching existing alleles in the database. This strain was identified by the curator of the *Wolbachia* MLST database as new strain 162. Sequencing these six loci for the infected individual from Saratoga Springs, NY produced sequence genotypes that were substantially different from strain 162 for all six loci. New alleles in the Saratoga Springs individual were observed for four loci: *gatB*, *hcpA*, *ftsZ*, and *wsp*. However, the sequences for *gatB*, *hcpA*, and *wsp* were not long enough for official strain designation due to difficulty in sequencing, though they were sufficiently long to recognize them as new alleles. We refer to this new strain as "strain SS8". Uncorrected sequence divergence between the two strains ranged from 9% (for *ftsZ*) - 12% (for *gatB*, *coxA* and *hcpA*) up to 21% (*fbpA*) and 25% (*wsp*).

3.3. Demographic models of Wolbachia infection

Mean adult KBB population sizes (averaged across 100-generations and 1000 replicates) for the six single population model parameter combinations were 259.4 (low *K* without *Wolbachia*), 176.7 (low *K* with *Wolbachia*), 557.4 (intermediate *K* without *Wolbachia*), 410 (intermediate *K* with *Wolbachia*), 2935.1 (high K without *Wolbachia*), and 2457.1 (high K with *Wolbachia*) (Fig. 2). Permutation tests indicated that single population model simulations conducted without *Wolbachia* resulted in significantly higher adult population sizes through time than model simulations with *Wolbachia* infection for all carrying capacities (low *K*: $\Sigma_{\text{diff}} = 8.28 \times 10^6$, $P < 0.001$; intermediate *K*: $\Sigma_{\text{diff}} = 1.473 \times 10^7$, $P < 0.001$; high *K*: $\Sigma_{\text{diff}} = 4.779 \times 10^7$, $P < 0.001$). These lower adult population sizes associated with *Wolbachia* infection resulted in significantly more replicate simulations ending with population extirpation than occurred during model simulations without *Wolbachia* infection for each carrying capacity (low *K*: $\Sigma_{\text{diff}} = 302$, $P < 0.001$; intermediate *K*: $\Sigma_{\text{diff}} = 201$, $P < 0.001$; high *K*: $\Sigma_{\text{diff}} = 59$, $P < 0.001$). Specifically, *Wolbachia* infection increased the number of replicate simulations (out of 1000) that resulted in extirpation from 218, 113, and 16 without *Wolbachia* to 520, 314, and 75 with *Wolbachia* for low, intermediate, and high carrying capacities, respectively. The negative demographic effects of CI *Wolbachia* infection on simulated KBB populations appeared to be most pronounced in the low carrying capacity populations, perhaps due in part to the increased mean time that *Wolbachia* infection segregated in these populations, which resulted from increased demographic stochasticity (Fig. 2).

For the metapopulation models the number of sites occupied throughout the 100-generation simulations by KBB was significantly greater without *Wolbachia* than with CI *Wolbachia* infection (low *K*: $\Sigma_{\text{diff}} = 1.336 \times 10^5$, $P < 0.001$; intermediate *K*: $\Sigma_{\text{diff}} = 1.311 \times 10^5$, $P < 0.001$; high *K*: $\Sigma_{\text{diff}} = 7.695 \times 10^4$, $P < 0.001$) (Fig. 3). Specifically, *Wolbachia* infection decreased the

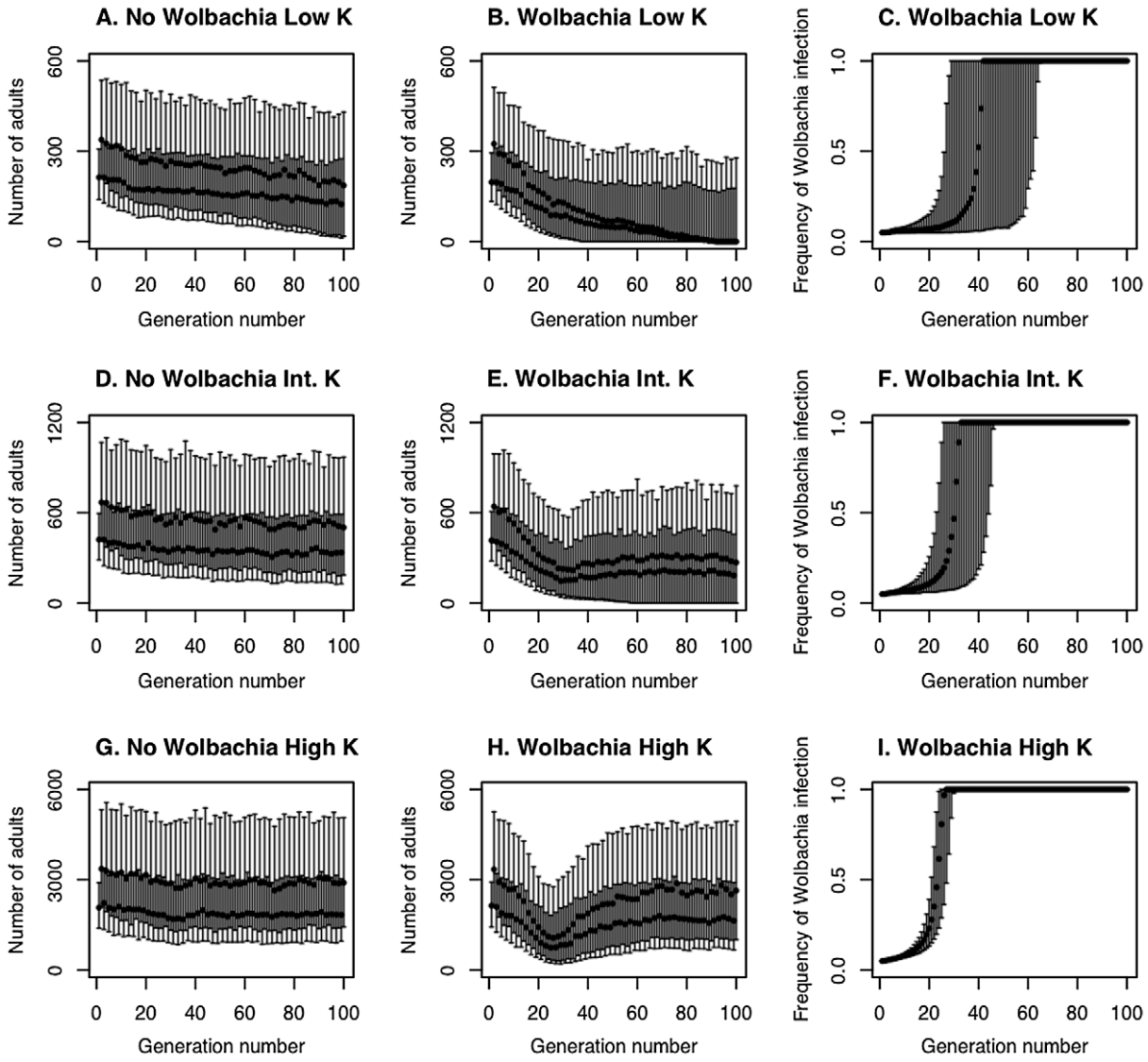

Fig. 2. Simulation results from single population models. Plots depict median (circles) and 1st and 3rd quartiles (bars) adult populations size for: (A) no *Wolbachia* with low carrying capacity, (B) *Wolbachia* with low carrying capacity, (D) no *Wolbachia* with intermediate carrying capacity, (E) *Wolbachia* with intermediate carrying capacity, (G) no *Wolbachia* with high carrying capacity, (H) *Wolbachia* with high carrying capacity and the frequency of *Wolbachia* infection for (C) low, (F) intermediate, and (I) high carrying capacities. Adult population size oscillations between consecutive generations reflect different egg to larva survival probabilities for each year's first and second brood. *Wolbachia* infection leads to a dip in adult population size while segregating in populations, which is recovered with large carrying capacity. However, this leads to local extirpation for many populations with small carrying capacity. Additional model details are described in the text.

mean number of sites occupied (out of 10) from 4.33, 5.76, and 7.82 to 2.99, 4.45, and 7.05 for low, intermediate, and high carrying capacity simulations, respectively.

4. Discussion

4.1. Wolbachia in Karner blue butterfly populations

Screening for *Wolbachia* demonstrated that the whole of the range of the KBB west of Lake Michigan appears to be infected, consistent with the findings of Gompert et al. (2008b). This is a widespread infection that includes many of the largest and least-impacted populations of this endangered species. This is significant not because the *Wolbachia* infection currently poses a threat to these infected populations (once a population is fixed for *Wolbachia* there is no reduction in effective population size), rather, these largest populations seem to be likely candidates from which captive propagation efforts would draw individuals. Thus, the chance of inadvertently introducing *Wolbachia* into uninfected populations appears high. It should be noted, however, that none of the current KBB augmentation programs involve individuals from the infected western populations.

The KBB infection west of Lake Michigan is attributable to a single strain of *Wolbachia* and is perfectly correlated with the presence of *L. m. melissa* mitochondrial haplotypes. These results are consistent with the hypothesis that the mitochondrial introgression was facilitated, or driven, by the spread of a CI *Wolbachia* infection from *L. m. melissa* to the KBB, as suggested by Gompert et al. (2008b). This would have likely involved hybridization between *L. m. melissa* and KBBs and the transmission of the CI strain to KBB females. This hybrid event appears to have been limited to mtDNA exchange, with very little nuclear gene introgression (Gompert et al., 2006). It should be noted that the phenotype of the strain cannot be determined from its multilocus sequence type. Phenotypes are distinctly not monophyletic (Baldo et al., 2006; Zhou et al., 1998) and the manifestation of a strain's phe-

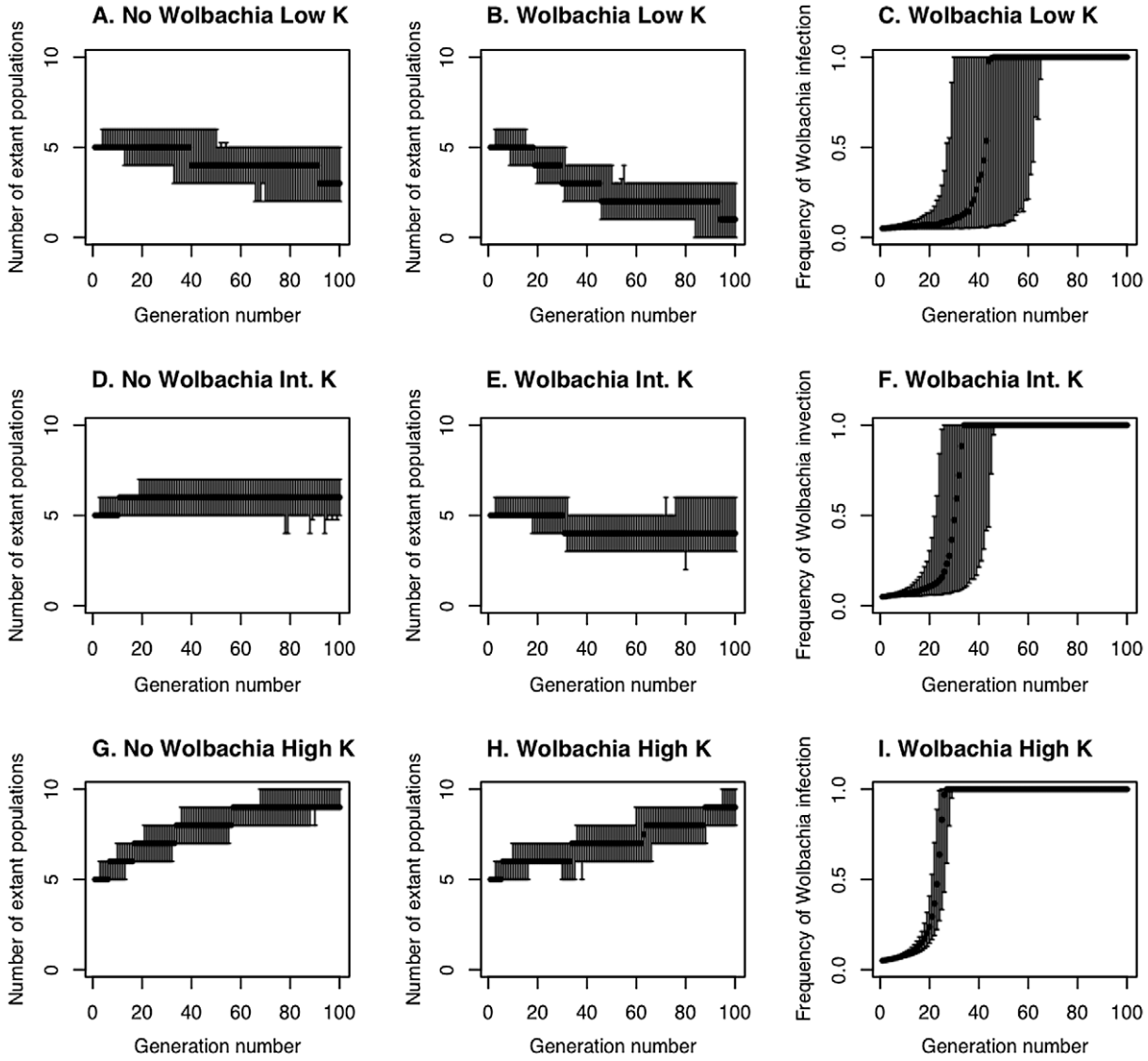

Fig. 3. Simulation results from metapopulation models. Plots depict median (circles) and 1st and 3rd quartiles (bars) number of extant sub-populations (out of 10): (A) no *Wolbachia* with low carrying capacity, (B) *Wolbachia* with low carrying capacity, (D) no *Wolbachia* with intermediate carrying capacity, (E) *Wolbachia* with intermediate carrying capacity, (G) no *Wolbachia* with high carrying capacity, (H) *Wolbachia* with high carrying capacity and the frequency of *Wolbachia* infection for (C) low, (F) intermediate, and (I) high carrying capacities. Additional model details are described in the text.

notype can vary with the genome of its hosts or a strain might be capable of producing multiple phenotypes (Hornett et al., 2006, 2008; Jaenike, 2007; Zabalou et al., 2008). CI seems most likely in this case because it is the most frequently observed phenotype, the one most associated with mtDNA sweeps, and there is no evidence of sex-ratio distortion in the KBB. (Confirming the CI phenotype often requires experiments using individuals that are cured of their infection and crosses with infected individuals, an endeavor that should be undertaken with KBB populations in the future.)

The only other *Wolbachia* infection detected was in the Saratoga Springs, NY population. This individual was infected by a markedly different *Wolbachia* strain that exhibits 9–25% uncorrected sequence divergence across the six loci used here for sequence typing compared to the strain 162 found west of Lake Michigan. This infection is not associated with foreign mtDNA, as far as we can tell. Again, its phenotype will remain unknown until experiments and further analyses can be performed. Nevertheless, the detection of *Wolbachia* east of the mitochondrial phylogeographic boundary at Lake Michigan was unexpected. New York populations have been the source for captive propagation and reintroduction efforts, particularly in New Hampshire (US Fish and Wildlife Service, 2008). It is difficult to gauge the potential threat posed by this low level infection because of lack of information on the strain's phenotype. Plus, this single case of infection might be attributable to infection of a parasitoid or parasite rather than the butterfly itself. We also do not know the current distribution of this strain in eastern populations. Our screening was performed on individuals that were collected in 1999 or earlier and thus does not represent a contemporary estimate of the infection frequency in this region. The infection that we have detected may have declined to extinction or increased and spread. However, to our knowledge, no dramatic declines in KBB populations in New York have been reported over the last 10 years, leading us to hypothesize that the infection by this strain has not negatively affected these populations. It should also be noted that *Wolbachia* infections are not always detrimental to the host. Beneficial effects of *Wolbachia* infection, such as resistance to viral attack and other phenomena have been

documented (Bandi et al., 1998; Hedges et al., 2008; Teixeira et al., 2008).

The results of our single population model simulations suggest that the spread of CI *Wolbachia* to previously uninfected KBB populations can negatively affect population demography by reducing adult population sizes and increasing the probability of local extirpation. These adverse demographic effects resulted in reduced site occupancy in our KBB metapopulation models. Local extirpation was most prevalent for simulations of low egg carrying capacities (i.e. populations with relatively few larval host plants), which are the populations most likely to be augmented by captive propagation. Thus, the KBB populations most likely to be artificially augmented are the same populations that are most likely to be driven to extinction if augmentation introduces CI inducing *Wolbachia*. Thus, we urge extreme caution regarding the use of captive propagation followed by translocation to augment KBB populations as a management strategy as this could have consequences counter to those intended. At minimum we suggest that a subset of KBB butterflies from each source population reared in captivity be screened for *Wolbachia* to verify that they are free of infection (or have the same strain of *Wolbachia* as the recipient population) or that captive reared butterflies are only reintroduced into their source population.

While our modeling results are consistent with the hypothesis that *Wolbachia* spread might negatively affect KBB demography, these findings should be considered preliminary and interpreted with caution, as the validity of many model assumptions is not known. Our models assumed infection of KBB by a CI inducing *Wolbachia* strain, which is likely given the prevalence of this phenotype and its association with mitochondrial sweeps (Hurst and Jiggins, 2005; Jiggins, 2003; Rasgon et al., 2003; Turelli et al., 1992). Moreover, it is unlikely that the KBB populations west of Lake Michigan are infected by *Wolbachia* strains that cause parthenogenesis, feminization of genetic males, or the killing of infected females' sons, as these phenotypes would result in population extirpation or severely female-biased sex ratios as such strains reached fixation; this has not been observed in these KBB populations (US Fish and Wildlife, 2003). However, verification that KBB populations are infected by CI inducing *Wolbachia* requires experimentation with cured individuals from infected populations. Infection by strains with alternative phenotypes could drastically alter model predictions. The use of different demographic parameters might alter specific model results. These parameters were selected based on the available data from the literature and the authors' experiences with *Lycaeides*, however such data are limited particularly regarding variation in survival proportions and fecundity in natural conditions. However, we experimented with different parameter values during model development and found similar results under many different conditions (i.e. a negative effect of CI *Wolbachia* on KBB demography). The exceptions were when very low or very high survival probabilities and fertilities were selected, which resulted in extirpation or the maintenance of populations at carrying capacity, respectively (results not shown). We did not evaluate the assumption that females mate once, although multiple matings might ameliorate some of the effects of infection initially when infected males are rare.

4.2. Conservation recommendations

The spread of *Wolbachia* infection is not only a threat for the KBB, but also for many endangered and threatened arthropods whose management strategies include captive propagation, population augmentation, and translocations. Given the prevalence of *Wolbachia* infection in arthropods (up to 65% of insect species may be infected), this is a threat that should be taken seriously and investigated further (Hilgenboecker et al., 2008; Jeyaprakash and Hoy, 2000; Werren et al., 2008, 1995; Werren and Windsor, 2000; West et al., 1998). Here we documented the extent of *Wolbachia* infection in an endangered butterfly. The existence of both infected and uninfected populations raises the specter of transmission and spread to the uninfected portion of the species' range. Our preliminary modeling of the demographic effects of a CI strain suggests that serious detrimental effects could result from spread of this strain. This danger is all the more problematic for the KBB because it is the uninfected populations in the eastern portion of the range that are most threatened; the western, infected populations include the largest KBB populations which may also be likely candidates for source populations for reintroductions in the future. Spread of this infection, either naturally or through captive propagation and population augmentation efforts, therefore constitutes a serious concern for continued conservation and management efforts. The example of the KBB provides a foundation for examining other endangered arthropods, many of which might experience similar effects of *Wolbachia* infection, and many of which have captive propagation efforts as integral aspects of management and recovery plans.

While conservation managers are regularly aware of the possibility of disease transmission, *Wolbachia* may present an especially difficult problem because the phenotypic effects are often not detectable until after the introduction of infected individuals into an uninfected population. Furthermore, specific *Wolbachia* phenotypes, such as male killing and male feminizing *Wolbachia*, pose an even greater threat to arthropod demography than CI inducing *Wolbachia*, as the negative effects of these strains continue even after *Wolbachia* fixation with the likely effect of arthropod population extirpation. Conservation management plans for threatened and endangered arthropods should therefore include screening for the presence of *Wolbachia*, if not other endosymbionts. This is especially important for reintroduction and/or population augmentation programs. If *Wolbachia* is detected, further identification of strains using the multilocus sequence typing (MLST) protocols (Baldo et al., 2006, 2008) should be pursued to assess the number and geographic distribution of infecting strains. Experimental diagnosis of the *Wolbachia*-induced phenotype will facilitate direct determination of the potential risks associated with the spread of the infection. These efforts should minimize the potentially devastating impacts of *Wolbachia* for endangered arthropods.

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