Viability and fertility of hybrid New Zealand tree wētā Hemideina spp. (Orthoptera: Anostostomatidae)

Natasha E. Mckean1, Steven A. Trewick1, Melissa J. Griffin1, Eddy J. Dowle1, Mary Morgan-Richards1

1 Ecology Group, Institute of Agriculture and Environment, Massey University, Palmerston North 4442, New Zealand.

Key words
Haldane’s rule, Hybridization, introgression, sexual exclusion, Wolbachia

Introduction
Natural hybridization between species provides an opportunity to study the mechanisms that maintain independent lineages and may help us understand the process of speciation. The New Zealand tree wētā species Hemideina thoracica produces F1 hybrids where it lives in sympathy with two closely related species: Hemideina crassident and Hemideina tewicki. This study looked at the viability and fertility of F1 hybrid wētā between H. thoracica and H. crassident that were collected from the wild and kept in captivity. The hybrids appeared to have normal viability from the late juvenile stage, with all male wētā maturing at a late instar. Male F1 hybrids displayed normal mating behavior and one male produced offspring in captivity. In contrast to Haldane’s rule, female F1 hybrids appeared to be infertile; they refused to mate and did not produce eggs. No evidence of Wolbachia infection was identified in any of the three North Island Hemideina species.

Abstract
Natural hybridization between species provides an opportunity to study the mechanisms that maintain independent lineages and may help us understand the process of speciation (Butlin 1987, Barton and Gale 1993). Hybrid animals often have lower fitness than individuals of their parent species (Harrison 1993 and references therein) as a result of incompatible genetic combinations, such as Dobzhansky-Muller incompatibilities (Dobzhansky 1937, Muller 1942, Bolnick and Near 2005), mismatched chromosomes (Shaw and Wilkinson 1980, Searle 1993), incompatible cellular organelles (e.g. mitochondria: Ellison and Burton 2008) and symbiotic bacteria (e.g. intracellular Wolbachia infections: Bordenstein et al. 2001; gut bacteria: Brucker and Bordenstein 2013). However, low hybrid fitness also arises through natural and sexual selection when an intermediate phenotype is a disadvantage (Svedin et al. 2008).

If hybrids are viable they might nevertheless have limited fertility. If fertility of F1 hybrids is very low, fertility levels usually improve in subsequent generations of backcross hybrids (Mallet et al. 1998, Mallet 2005, Descimon and Mallet 2009), and even limited fertility provides the possibility for introgression that can have important consequences for species interactions. Adaptive alleles that arise in one population/species can be transferred to another through hybridization, known as adaptive introgression, as seen in wing coloration in Heliconius butterflies (Pardo-Diaz et al. 2012). In some cases, when hybridization reduces fertility a unimodal hybrid (or tension) zone forms. Unimodal hybrid zones are geographically constrained, with most individuals in the zone having mixed ancestry, and the width of the hybrid zone depending on hybrid disadvantage and dispersal of the species (Jiggins and Mallet 2000). If hybridization is more limited, a bimodal hybrid zone may result, where parental forms overlap and predominate with a few individuals of mixed ancestry amongst them. Bimodal hybrid zones are typically associated with assortative mating, so, in tree weta, reproductive character displacement is a likely outcome that increases assortative mating (Dieckmann and Doebeli 1999, Jiggins and Mallet 2000). Where two species compete for the same resources, hybridization may prevent one from successfully out-competing the other. Alternatively sexual exclusion, where one species (often the males of that species) out-competes the other for mates, can limit fitness through reproductive interference (Gröning and Hochkirch 2008), as observed between the tetrigids: Tetrix ceperoi and Tetrix subulata (Hochkirch et al. 2007).

Tree wētā (Orthoptera: Anostostomatidae: Hemideina) are a genus of seven nocturnal arboreal insects, with high morphological and ecological similarity (Field and Bigelow 2001, Dewhurst 2012, Bulgarella et al. 2014). They are hypothesized to have speciated in allopatry during the Pliocene or earlier (Trewick and Morgan-Richards 2005), and they now have broadly parapatric distributions (Trewick and Morgan-Richards 1995, Bulgarella et al. 2014). Hybridisation in the wild between the New Zealand tree wētā Hemideina thoracica (White) and both Hemideina crassident (Blanchard) and Hemideina tewicki Morgan-Richards has recently been confirmed with genetic data, but so far only F1 hybrids have been confirmed (Mckean 2014, Mckean et al. 2016). These three Hemideina species each have distinct karyotypes (i.e. different numbers of
chromosomes with some differing in size and shape; Morgan-Richards 1995, 1997, 2000, Mckean et al. 2015). Karyotype differences are generally seen as presenting barriers to gene flow by disrupting meiosis and rendering F$_1$ hybrids infertile. However, some tree wētā species naturally comprise multiple chromosome races that are capable of interbreeding in the wild (Morgan-Richards 1997, 2000, Morgan-Richards et al. 2000, Morgan-Richards and Wallis 2003). The apparent tolerance of chromosome rearrangements displayed in this orthopteran lineage might influence fertility of interspecies hybrids. Karyotype, mtDNA haplotypes, and alleles at four nuclear DNA loci were found to differentiate parent populations of H. tho-racica and H. trewicki in a large area of sympathy in Hawke’s Bay. These markers (except mtDNA, which is maternally inherited) were heterozygous in individuals who were phenotypically intermediate in abdominal coloration (orange rather than yellow or brown), abdominal bands (faint rather than striking or non-existent), abdominal stripe (a series of spots rather than a stripe or the absence of a stripe) and the number of spines on the protalrard hind tibia (typically between the three spines seen in H. thoracica and the four in H. crassidens/H. trewicki, with a half-sized medial spine on each leg being common, or three spines on one leg and four on the other). A similar situation was seen in the Manawatu area of sympathy between H. thoracica and H. crassidens, where karyotype, mtDNA and three nuclear DNA markers were found to differentiate the two species (with some introgression detected relative to allopatric populations). All individuals, which had an intermediate phenotype (the same phenotype as for H. thoracica × H. trewicki hybrids), were heterozygous for these markers (Mckean et al. 2016). Whether hybridization occurs between H. crassidens and H. trewicki is currently unknown due to the morphological similarities of these two species, and unknown distribution boundaries due in part to clearance of native forest where the two are hypothesized to have historically met (Trewick and Morgan-Richards 1995). A lack of gene flow suggests that H. thoracica × H. trewicki hybrids, which are found at a frequency of 1% of wētā in sympathy, are infertile, but genetic and morphological data suggest a low, but potentially significant, level of introgression between H. thoracica and H. crassidens, where hybrid frequency is ~3 in every 100 wētā (Mckean et al. 2016).

Introgression is the signal of past hybridization, and an ability to successfully hybridize might be of fundamental importance to the future of a species, while climates and environments continue to change (Grant and Grant 1993, Allendorf et al. 2001, Becker et al. 2013, Taylor et al. 2015, Sivyer et al. 2018). There is evidence that H. crassidens formerly occupied much of central North Island that is now the range of H. thoracica (Bulgarella et al. 2014). Isolated populations of H. crassidens remain in regions of high elevation, which suggests they are adapted to colder environments. With global warming, H. crassidens might continue to be displaced, but this depends on the ecological and sexual interaction between species. Adult tree wētā often form harems in tree cavities during the summer and autumn (Wehi et al. 2013), and mixed species harems in areas of overlap suggest that species recognition is not complete (Trewick and Morgan-Richards 1995, Wehi et al. 2017). Of F$_1$ hybrids collected in the wild, the majority had an H. thoracica father and H. crassidens mother (Mckean et al. 2016), which suggests sexual exclusion by H. thoracica males; as in Hemideina spp. the females do not appear to actively choose their mates. Although females will sometimes resist mating, resistance times are similar whether mating occurs or not (Field and Jarman 2001). This gives rise to the possibility that hybridization may be an important factor in the coexistence/exclusion of these species.

Haldane (1922) observed that where one sex is absent, rare, or infertile in F$_1$ hybrids, it is usually the heterogamic sex. In tree wētā this is the male, as tree wētā, like most Orthoptera, have an XO sex determination system where females have two copies of the sex chromosome and males one (White 1940, Morgan-Richards 1997, Morgan-Richards and Wallis 2003). Based on this, if there is a difference between the sexes, we would expect male F$_1$ hybrids to have lower viability and/or fertility than female F$_1$ hybrids.

Given their apparent tolerance of karyotype variation, the high degree of infertility in wētā might have another source. Wolbachia is an endosymbiotic intracellular bacteria that infects a large proportion of the arthropod and nematode phyla (Werren et al. 2008). In arthropods, Wolbachia is estimated to infect about 65% of species (Hilgenboecker et al. 2008) including many grasshoppers and crickets (Werren and Windsor 2000, Mandel et al. 2001, Bella et al. 2010). Wolbachia is known to manipulate the reproductive biology of many of its hosts to its own advantage (Werren et al. 2008). Some of the currently known host-reproductive manipulations include male killing, induction of parthenogenesis, feminization of genetic males, forced production of haploid individuals in haplodiploid systems, and cytoplasmic incompatibility (Werren 1997). Wolbachia is hypothesized to have a role in arthropod speciation via induction of cytoplasmic incompatibility (Werren 1998 and references therein). Wolbachia infections appear to be responsible for maintaining hybrid zones between the well-studied grasshopper subspecies Chorthippus parallelus parallelus and Chor-thippus parallelus erythropus via two different forms of cytoplasmic incompatibility (Bella et al. 2010). Although recently detected in New Zealand insects (Bridgeman et al. 2018), it is not known whether the tree wētā lineage (with many hybrid zones; Morgan-Richards and Wallis 2003) contains this intracellular parasite.

Here, we describe the viability and fertility of hybrids between Hemideina thoracica and H. crassidens, using F$_1$ hybrids collected in the wild and held in captivity. We sought evidence of Wolbachia infections to assess whether this common intracellular parasite has potential to limit reproductive compatibility among these wētā species.

Methods

Sampling and captive conditions.—Eleven F$_1$ hybrid tree wētā were captured from native forest in Turitea Valley (S40.47184, E175.60943) and Kahutawera Valley (S40.431725, E175.674595), Manawatu, New Zealand (Fig. 1, Table 1). Hybrid identity was tested and confirmed using genetic markers for eight of the 11 individuals which died or were euthanized during the course of this study, and were preserved as specimens in alcohol (Table 1; F$_1$ hybrids; Morgan-Richards 1995, Mckean et al. 2016). The other three putative hybrid individuals were assumed to be F$_1$ hybrids as their phenotypes were completely consistent with the F$_1$ hybrids that had been genetically identified. No cryptic hybrids were identified in previous studies of tree wētā from this population (Trewick and Morgan-Richards 1995, Morgan-Richards and Gibbs 2001, Bulgarella et al. 2014, Mckean et al. 2016). Live wētā were held in individual containers at a constant temperature of 14 °C. They were given a suitable daytime roost cavity made from harakeke (Phormium tenax) flower stalk, and were fed palatable leaves from at least three native plant species each week and 80% soy protein pellets (Griffin et al. 2011).

Body size of F$_1$ hybrids.—No significant difference in body size between adult females of the two parent species has been found in
Table 1. Sampling information, size and results for mating behavior in both sexes and egg production in hybrid females.

<table>
<thead>
<tr>
<th>Wētā</th>
<th>Sample</th>
<th>Location</th>
<th>Genetically Confirmed Hybrid</th>
<th>Sex</th>
<th>Age</th>
<th>Tibia Length (mm)</th>
<th>Instar at Maturity</th>
<th>Behavior</th>
<th>Age since Maturity</th>
<th>Eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybrid 1</td>
<td>Live</td>
<td>Kahutawera valley</td>
<td>Yes</td>
<td>M</td>
<td>Adult</td>
<td>23.63</td>
<td>10</td>
<td>Normal; mated*</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Hybrid 2</td>
<td>Live</td>
<td>Turitea valley</td>
<td>Yes</td>
<td>M</td>
<td>Adult</td>
<td>24.01</td>
<td>10</td>
<td>Normal; mated*</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Hybrid 3</td>
<td>Live</td>
<td>Turitea valley</td>
<td>Yes</td>
<td>M</td>
<td>Adult</td>
<td>23.62</td>
<td>10</td>
<td>Normal; mated*</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Hybrid 4</td>
<td>Live</td>
<td>Kahutawera valley</td>
<td>Yes</td>
<td>F</td>
<td>Adult</td>
<td>22.92</td>
<td>10</td>
<td>Resisted Mating+</td>
<td>6 months</td>
<td>No</td>
</tr>
<tr>
<td>Hybrid 5</td>
<td>Live</td>
<td>Kahutawera valley</td>
<td>No</td>
<td>F</td>
<td>Adult</td>
<td>21.26</td>
<td>10</td>
<td>Resisted Mating+</td>
<td>4 months</td>
<td>No</td>
</tr>
<tr>
<td>Hybrid 6</td>
<td>Live</td>
<td>Kahutawera valley</td>
<td>No</td>
<td>F</td>
<td>Adult</td>
<td>23.76</td>
<td>10</td>
<td>Partial Resistance+</td>
<td>3 months</td>
<td>No</td>
</tr>
<tr>
<td>Hybrid 7</td>
<td>Preserved</td>
<td>Kahutawera valley</td>
<td>Yes</td>
<td>F</td>
<td>Adult</td>
<td>21.26</td>
<td>10</td>
<td>NA</td>
<td>6 months</td>
<td>No</td>
</tr>
<tr>
<td>Hybrid 8</td>
<td>Preserved</td>
<td>Kahutawera valley</td>
<td>No</td>
<td>F</td>
<td>Adult</td>
<td>22.29</td>
<td>10</td>
<td>NA</td>
<td>3 months</td>
<td>No</td>
</tr>
<tr>
<td>Hybrid 9</td>
<td>Preserved</td>
<td>Kahutawera valley</td>
<td>Yes</td>
<td>M</td>
<td>Juvenile</td>
<td>16</td>
<td>10</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Hybrid 10</td>
<td>Live</td>
<td>Kahutawera valley</td>
<td>Yes</td>
<td>M</td>
<td>Sub-adult</td>
<td>18.42</td>
<td>10</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Hybrid 11</td>
<td>Preserved</td>
<td>Kahutawera valley</td>
<td>Yes</td>
<td>M</td>
<td>Adult</td>
<td>21.11</td>
<td>10</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Legend: *See Table 3, + See section 'Mating Behavior' in methods.
this zone of sympatry (McKean et al. 2016). To detect signs of impaired growth (hybrid inviability) the hind tibia length of both dead and living adult hybrids was measured with electronic calipers and compared via ANOVA to wild adult females of both parent species measured in a previous study (15 H. thoracica and 19 H. crassidens; McKean et al. 2016), and to a separate sample of adult males from both species (25 H. thoracica and 22 H. crassidens), that were sampled from the same locations as the hybrids. Hind tibia length is a reliable proxy for body size in tree wētā (Minards et al. 2014, Bulgarella et al. 2014.) The sex of hybrids and instar at maturity for male wētā were both recorded. Maturity is determined in tree wētā by the shape and size of the cerci or ovipositor. Tibia length data for each sex were compared via ANOVA.

Mating behavior.—Six hybrid wētā (three males, three females) were provided with one potential mate of each parent species, on different nights, in a Perspex tank (60 cm × 60 cm × 60 cm) (Table 1). Mating trials were observed for 30 min in the evening when tree wētā are most active (Kelly 2006a). For male wētā, successful transfer of spermatophores was recorded as well as attempts to mate, defined as curling the abdomen to position for mating. Other mating behavior prior to this, such as following the female or rapid twitching of the palps that indicated the male had scented the female, and scurrying the palps over the female’s abdomen, were recorded (Field and Jarman 2001 and references therein). As male mating behavior has been well described elsewhere, the male F1 hybrids’ behavior was compared to what is known from previous work which details the parental species’ behavior. Female tree wētā do not appear to actively choose or approach male wētā (Field and Jarman 2001 and references therein), so their acceptance or active resistance to mating was recorded. Resistance was defined as any behavior that appeared to obstruct mating attempts by the male including moving away, stridulating (a defensive/aggressive gesture in tree wētā; Field 2001, Field and Glasgow 2001), and biting and kicking the male to dislodge him. Acceptance was defined as the female staying still and allowing copulation to be initiated and completed, as evidenced by the successful transfer of a spermatophore.

Egg production.—Females of both parent species begin producing eggs as soon as they reach maturity (N.E.M. personal observation, >50 females 2012–2013). Eggs inside the ovarioles of mature females typically vary in developmental stage and range from very small undeveloped yellow eggs through to large black mature eggs with a thick outer casing (Griffin 2011). After laying, the embryo case expands and turns from black to brown and eventually yellow (Stringer 2001). Four F1 hybrid adult females and 18 H. crassidens females were given soil slightly deeper than the length of the ovipositor to lay eggs in (Table 2). Conditions were otherwise the same as detailed in captive conditions above. After approximately 100 days (StDev = 35.9) the eggs laid were removed and counted. Each wētā was euthanized, dissected and the number of un laid mature eggs counted under a dissecting microscope. Additional data were obtained from a preserved hybrid female euthanased before she laid eggs (n=5 in Table 2).

Male fertility.—Two adult F1 hybrid males, which were adults at the time of the study, were each provided with virgin females of both parent species, as above (Table 3). They were observed until a mating occurred and then left together in the tank overnight. Female wētā were removed the next morning and placed in a container with a layer of soil slightly deeper than the length of the ovipositor. After a period of oviposition the female was removed, the eggs counted and placed back into the soil. As little is known about triggers for embryo growth and hatching in wētā, the eggs were stored outside, exposed to the ambient winter temperature fluctuations experienced by the wild population from which they were derived. Expansion and hatching were recorded the following summer (approximately 9 months after laying).

**Wolbachia detection.**—Two methods were used to obtain evidence of infection by the bacteria *Wolbachia*: amplification of DNA sequences using *Wolbachia* specific Polymerase Chain Reactions (PCR) primers, and whole genome sequencing and alignment to a reference *Wolbachia* genome. For amplification of specific *Wolbachia* DNA sequences, DNA was extracted from three tree wētā specimens representing each of the three North Island species (*H. thoracica, H. crassidens* and *H. trevickii*). Tissue was taken from the hind femur and testes or ovariole of each tree wētā specimen and DNA isolated using a salting out method (Trewick and Morgan-Richards 2005). *Wolbachia*-specific primers (Appendix 1) were used in PCR with *w*DNA, and DNA from an introduced gregarious parasitoid wasp (*Nasonia vitripennis*) known to be infected with *Wolbachia* as a positive control. Standard PCR conditions for these primers were followed (Braig et al. 1998, Heddi et al. 1999, Baldo et al. 2006) (Appendix 1). PCRs were repeated to rule out problems with reaction conditions. One PCR product longer than the expected *Wolbachia* fragment from the CoA primer pair was amplified. This long DNA fragment was sequenced at the Massey Genome Service with a capillary AB13730 Genetic Analyzer (Applied Biosystems Inc.), and then visualized and trimmed in Geneious Pro 10.8.5 and aligned to known *Wolbachia* genomes using BLAST. The resulting 269 bp sequence was aligned to public databases using the Basic Local Alignment Search Tool (BLAST) algorithm on the NCBI website.

Total genomic DNA from two tree wētā specimens (an *H. thoracica* male collected from the Kahutawera Valley and an *H. crassidens* male collected from a South Island population) were separately processed through parallel, high-throughput sequencing (Illumina HiSeq 2500) for a separate phylogenetic study (Dowle 2013). Briefly, DNA was extracted from a single male individual (testes tissue), fragmented, prepared using the ThruPLEX DNA-seq Kit (Rubicon Genomics) and used to generate 100 bp paired-end
sequence on a Hi-Seq 2000 (BGI). This resulted in 5,191,884 100 bp-paired-end sequences 200 bp apart for the *H. thoracica* specimen and 17,434,429 100 bp-paired-end sequences for the *H. crassidens* specimen. An annotated reference Wolbachia genome was obtained from New England Biolabs (http://tools.neb.com/wolbachia, originating from infection of *Brugia malayi*; Foster et al. 2005). Reads were trimmed to remove index sequences using sol 

The results were visualised with Tablet v1.7.0_35 (Milne et al. 2010). Sequences that matched parts of the Wolbachia genome were compared with published data using the NCBI (National Library of Medicine) GenBank BLAST search algorithm to determine their similarity to Wolbachia DNA sequences from other hosts. This enabled us to determine whether the sequences came from the Wolbachia genome or another related bacterial species, which could be determined by sequence similarity.

**Results**

**Phenotype F₁ hybrids.**—Hybrids were identified by genetic markers and intermediate phenotypes, and no morphologically cryptic hybrids were identified (McKean et al. 2016). The sex ratio of F₁ hybrids in our small sample was even (five females, six males). All but two hybrid wētā examined were adults (or reached adulthood in captivity – two wētā) providing no evidence of reduced hybrid viability. There was no significant size difference between adult F₁ hybrid females and adult females of the two parent species from the same location with ANOVA; F = 2.575, P = 0.09 (Fig. 2A), however male F₁ hybrids were significantly larger than males of either parent species (ANOVA; F = 8.969, P = 0.00049; Fig. 2B). The five adult male hybrids matured at the tenth instar as determined by comparing their hind tibia lengths to data of wētā trimorphism in *Hemideina crassidens* (Kelly and Adams 2010, Bulgarella et al. 2015). Although one male did not reach maturity (Hybrid 10; Table 1), as a ninth instar sub-adult he would have been an adult at the tenth instar, as determined by growth/size charts from previous studies (Spencer 1995, Kelly and Adams 2010).

**Mating behavior.**—All three F₁ hybrid males mated with females of both species (Table 1). Each male exhibited normal and similar mating behavior to females of both species he was housed with (Field and Jarman 2001 and references therein), and was accepted by females of both species. In contrast, two of three hybrid females actively resisted mating. The third allowed the *H. thoracica* male to begin copulation several times, but then dislodged him and proceeded to bite him and display other resistance behaviors. She allowed mating to occur once with the *H. crassidens* male, and then resisted all subsequent mating attempts, and was the only hybrid female wētā that was observed to accept a spermatophore.

**Fertility.**—None of the five female F₁ hybrids contained eggs in any stage of development when killed and dissected as adults. This contrasts with 18 *H. crassidens* females that each laid and/or contained an average of 91 eggs (Table 2). Females that were mated to the hybrid males laid 35–111 eggs (except one *H. crassidens* female that died soon after mating with Hybrid 2). Some eggs from every female showed signs of expansion after 6–8 months, with many eggs increasing in size and changing color from black to light brown or yellow (Table 3). Four eggs by male Hybrid 2 and his *H. thoracica* female expanded and then hatched to produce offspring. The nymphs were inferred to be phenotypically normal, as no obvious morphological differences were seen under a dissecting microscope. The color of nymphs is uniformly grey (dorsal) and yellowish white (ventral) at this stage regardless of species, so no inferences could be drawn about eventual color phenotype (whether the F₂ generation look the same as F₁, or resemble the wētā of the parent species). No other eggs hatched during the study, including the eggs produced by the control wētā (Table 3).

**Wolbachia.**—The fbpA and Wol16S primers failed to amplify a DNA fragment when used with tree wētā DNA, but produced a DNA fragment with the positive control (a wasp know to be infected with *Wolbachia*). The Wsp and CoxA primers gave a series of weakly amplified DNA fragments longer than that expected from the Wolbachia genome. A consistent DNA fragment amplified with the CoxA primers was 200 bp longer than the positive control.

![Fig. 2. A. Tibia length of adult female F₁ hybrids compared with adult females from the two parent species, showing no significant difference; B. Tibia length of F₁ hybrid males compared with males of the two parent species, showing a significant difference: p-value = 0.0001.](image-url)
No close sequence match was found when compared to DNA sequences on the database Genbank, including Wolbachia sequences. None of the > 17 million H. crassidens next-generation short read DNA sequences mapped to the Wolbachia genome. However, eight 100 bp DNA sequences from genomic H. thoracica DNA shared similarity with Wolbachia. Six identical DNA sequence reads mapped to one location, all with the same ten mismatches. The other two reads mapped to a different location on the Wolbachia genome, differing at nine sites (mismatches). However, the paired-end for all eight of these sequence reads (100–300 bp downstream from the putative-bacteria DNA sequence) did not map to the Wolbachia genome sequence. Comparing the putative Wolbachia sequences to the Genbank database identified these sequences as: 1) 93% similarity with the 16S rRNA gene from various members of the Chlamydiae phylum, with six of these matches belonging to the Rhabdochlamydia genus, and 2) 93% match for three 28S gene fragments from Simkania negenerisis, which also belongs to the Chlamydiae phylum. As similarity with Wolbachia sequences was lower (90–91%), it is likely that the H. thoracica wētā was infected with a bacteria species from the chlamydia family, not closely related to Wolbachia. Both the 16S and 28S rRNA genes are highly conserved among bacteria, and of the > 22 million DNA short-sequences from the wētā none mapped to Wolbachia-specific regions of the Wolbachia genome. A separate study of other Orthoptera confirmed that this level of data was sufficient for detection of Wolbachia infections (Bridgeman et al. 2018).

**Discussion**

The size of H. thoracica × H. crassidens hybrids fell within the normal range expected for the parent species (with males at the larger end), and many hybrids were found as adults in the wild, therefore we have no positive evidence of hybrid inviability or abnormal development. There could be some inviability early in development, during the pre-hatching or early instar phases, but it appears that at least by the time F1 hybrids have reached the larger instars (5th to 7th), they are as successful as a typical wētā of either parent species. Female tree wētā all mature at the tenth instar but males can mature at the eighth, ninth or tenth instar (Spencer 1995, Kelly and Adams 2010), resulting in a wide size range of adult males. All F1 hybrid males in this study matured at the tenth instar, which may be important in understanding their reproductive success (if any). Male tree wētā compete for females via competition for tree cavities (resources) that females use as refuges during the day (Spencer 1995, Field 2001). Tenth instar males have much larger mandibles than eighth instar adult males, which are used during male-male competition, but which also limit their mobility, leading to the hypothesis that smaller males actively search for and mate with females which are away from their tree cavities foraging at night (Field 2001, Kelly 2004, Kelly 2006a, Kelly and Adams 2010). If male F1 hybrids all mature at a later instar, it is unclear whether this is an advantage or disadvantage for reproductive success. As the ratios of other male wētā mating at different instars may play a part in determining success in controlling harems, more research into reproductive success among males in this location is needed to determine the outcome of these 10th instar hybrid males.

Our observations of mating were limited to experimental pairs (rather than harems, which are common in the wild; Wehi et al. 2013) and this might have influenced the behaviors exhibited and observed. Female wētā were not given a choice of mate, which could have also influenced mating behavior. However, mating behavior appeared to be normal for our limited sample of F1 hybrid males when paired with adult H. thoracica and H. crassidens females. One of the hybrid males had been found in the wild with a harem consisting of two adult H. crassidens females. This male produced offspring in captivity, hence it is likely that this male, along with at least some others, are behaving in the wild in a manner typical of males from the parent species. One significant limitation of this study is the lack of control mating crosses and mating behavior comparisons for parental species from the same populations, as previous mating studies were conducted with Hemideina crassidens from southern populations (Field and Jarman 2001, Kelly 2006b, c). There have been few studies of H. thoracica mating behavior (Wehi et al. 2013) and there is a general lack of understanding of mating outcomes in this lineage (Field and Jarman 2001). Unfortunately, the triggers for embryo development and hatching are unknown for tree wētā, making laboratory crosses difficult and prone to failure, as evidenced by the control wētā embryos failing to hatch. Therefore no inferences can be drawn about the success of these crosses relative to parental crosses, but given that laboratory crosses are sometimes successful when virgin females are mated to single males, it does not appear that sperm competition has to take place in these species to induce fertility in females (Morgan-Richards 2000, Stringer 2001, present study), and can probably be ruled out as an explanation for lack of fertility.

In contrast to the males, the female F1 hybrids did not show typical mating behavior, but this may be irrelevant to fertility if they cannot produce eggs. The lack of egg production in all five F1 females is probably biologically important, despite the small sample, because it contrasts with that observed in adult H. crassidens females kept in the same conditions (Table 2). A lack of eggs was also never observed in more than 50 mature parental females of both species that were dissected (N.E.M. personal observation, 2012–2013). The absence of eggs suggests that F1 females may typically be infertile, whereas at least some F1 male hybrids are fertile, as was evident from the offspring produced in captivity.

Male F1 hybrids being partially fertile while females are infertile contrasts with the usual variation between the sexes in reduced fertility (Haldane’s rule) and may be of interest for future research. Haldane’s rule applies across many animal taxa, including others with a XO sex determination system (Haldane 1922, and one analysis found that it applied in 99% of 223 cases of sex-specific hybrid sterility and 90% of 115 cases of sex-specific hybrid inviability (Laurie 1997). Infertility as opposed to inviability appears to be the most normal sex-skewed outcome, as heterogametic infertility is known to outnumber heterogametic infertility about 10:1 in Drosophila and mammals (Wu and Davis 1993). The mechanism behind Haldane’s rule is still unclear although two main hypotheses are X chromosome to autosome imbalance, and incompatibilities between the sex chromosomes. It is also possible that there are multiple causes underlying this phenomenon, but with so few sex determination systems for comparison a conclusive inference is elusive (Coyne 1985, Wu and Davis 1993, Turelli 1998). There is also evidence that the genetic basis of inviability in heterogametic hybrids differs from the genetic basis for infertility (Coyne 1985). Exceptions such as this may eventually shed light on why this rule applies so well to the majority of species. There are some contradictions to Haldane’s rule in other XO systems, such as the field crickets Teleogryllus oceanicus and Teleogryllus commodus (Moran et al. 2017), although
whether contradictions to Haldane’s rule are more common in XO systems is unknown.

One question remaining unanswered in the present study is where the barriers to reproduction are. As bimodal hybrid zones are typically associated with pre-mating rather than post-mating barriers (Jiggins and Mallet 2000 and references therein), the situation here is somewhat unusual. As there does not appear to be assortative mating between these species pairs (Field and Jarman 2001, Morgan-Richards et al. 2001, Wehi et al. 2017), it suggests that barriers are more likely to be the result of genetic constraints. It is not known at which stage the production of F₁ hybrids is limited, but as intermediate forms are far less common than expected if the species were freely interbreeding (Mckean et al. 2016) some reproductive constraint must operate. Barriers are hypothesised to be at the post-mating pre-zygotic stage or early in development, and it is possible that the wētā use unknown behavioral mechanisms to limit interbreeding. A bimodal hybrid zone in two species of chrysomelid beetles (Chrysocochus cobaltinus and C. auratus) also involves stronger post-zygotic barriers than pre-zygotic barriers (Peterson et al. 2005), so the association of assortative mating and bimodal hybrid zones has exceptions. A later study of these same beetles also showed a significant sex-bias in the production of offspring (most had mtDNA haplotypes and hence mothers from one species), despite mating occurring in both directions in the wild, and offspring in both sex-pairings being produced in equal numbers and with equal viability in laboratory crosses at the first instar (Monsen et al. 2007). The proposed explanation was asymmetric post-mating pre-zygotic barriers, or possible asymmetric inviability later in development. As H. thoracica and H. crassidens appear to exhibit both a bimodal hybrid zone in the apparent absence of pre-mating barriers, and also a sex-bias production of F₁ offspring, there may be some similarities in the mechanisms causing reproductive isolation in these disparate species pairs. Examples such as these may give insights into how bimodal hybrid zones are typically formed and maintained.

Female infertility would prevent mtDNA passing the species boundary (introgressing), and this may explain why no evidence of mtDNA introgression has been seen in previous studies (Burligella et al. 2014, Mckean et al. 2016), despite evidence of a low level nuclear DNA and possible phenotypic introgression (Mckean et al. 2016). The low number of F₁ hybrids seen in the wild suggests that these wētā are forming a bimodal hybrid zone (Mckean et al. 2016), and with reduction in fertility of at least a 50% (due to female infertility), production of hybrids is probably costly. The most likely outcome in this scenario would appear to be reproductive character displacement or niche divergence limiting hybridization and loss of reproductive potential, as loss of reproductive compatibility allowing merging of the species would be unlikely with such a significant reduction in fertility (Dieckmann and Doebeli 1999, Jiggins and Mallet 2000). However, if one species uses sexual exclusion to monopolize mates of the other, it could enable for the range expansion of this species. Given that most F₁ hybrids (8/9) were shown to have a H. thoracica father, this may in part explain how H. thoracica has been able to replace H. crassidens from much of its former range as climate has warmed since the last glacial maximum (Burligella et al. 2014). Introgresion of adaptive alleles (e.g. cold tolerance) could enable continued expansion of H. thoracica, otherwise the hybrid zone is likely to settle where environmental selection and mate competition are at equilibrium. Further work to determine if male F₁ hybrids are more likely to successfully reproduce with H. thoracica or H. crassidens females would be valuable, because a bias at this point could influence introgressive asymmetry.

Neither of the two methods employed here provided evidence of Wolbachia infection in Hemideina. The primer pairs that amplify DNA from the common Wolbachia supergroups that infect arthropods (Simoes et al. 2011), and the mapping software used with NGS were sensitive enough to detect infection by another bacterial parasite, so it is highly likely that these wētā did not contain Wolbachia although other New Zealand Orthoptera do (Bridgeman et al. 2018). A Chlamydia-like infection was detected in one of the wētā in this study however, and as this bacteria also functions as an intracellular parasite (Wyrick 2000), it may be of interest. Wētā make good candidates for sexually transmitted diseases, as they generally have some level of promiscuity and have overlapping adult generations (Knell and Webberley 2004).

Our sample of hybrid individuals was small, due to the low frequency of hybrids in the wild (Mckean et al. 2016), however, the results are biologically significant (i.e. F₁ males having at least some fertility, the infertility of female F₁ hybrids, thus being an exception to Haldane’s Rule), and raise questions about future interaction and survival of these species. In summary, both male and female F₁ hybrids are capable of reaching maturity, and although Wolbachia is not involved in limiting hybridization, there is at least a 50% (probably higher) reduction in F₁ hybrid fertility due to female infertility, which might have a strong limiting effect on introgression in the wild. There appears to be a contrast between complete failure by female F₁ hybrids to produce eggs and partial fertility of some male hybrids, suggesting this system provides another exception to Haldane’s rule that in interspecific hybrids the heterogametic sex (in this case males; XO) will have lower fertility than the homogametic sex (female Hemideina XX).

Tree wētā are an interesting group for evolutionary studies, in part because they appear to have a high tolerance for chromosome rearrangement that leads to many intraspecific hybrid zones. Much remains unknown about wētā biology, particularly with regard to species coexistence and production of hybrids where these wētā meet in sympathy lends an extra layer of complexity to the situation. Given that these species meet in different zones of sympathy across the country (and in different species combinations), there is the possibility that different mechanisms have, or will, evolve in different areas, which could be another promising area for further study.

Acknowledgements

We thank Mariana Bulgarella, Emily Koot, Shaun Neilon, Anne Kim and Priscilla Wehi for help collecting hybrid wētā. This work was supported by the Massey University Research Fund (M. Morgan-Richards; MURF2013: Can wētā coexist?). The paper was much improved by constructive comments from Mike Ritchie and two anonymous reviewers.

References


Griffin MJ (2011) Wellington Tree Wētā (Hemideina crassidens) diet and the effect of some of their dietary choices. MSc Thesis, Massey University, Palmerston North.


Laurie CC (1997) The weaker sex is heterogametic: 75 years of Haldane’s rule. Genetics 147: 937–951.


McKeen NE (2014) Hybridization of North Island Tree Wētā. MSc Thesis, Massey University, Palmerston North.


Wehi PM, Monsk A, Morgan-Richards M (2017) Male tree wētā are attracted to cuticular scent cues but do not discriminate according to sex or among two closely related species. Ethology 123: 825–834. https://doi.org/10.1111/eth.12652


### Appendix 1

**Table 4.** Information for wētā DNA amplification with *Wolbachia*-specific PCR primers.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Source</th>
<th>Forward / Reverse Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Wolbachia</em> surface protein (<em>wsp</em>)</td>
<td>Braig et al. 1998</td>
<td>Wsp81F / Wsp691R</td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase (<em>fbpA</em>)</td>
<td>Baldo et al. 2006</td>
<td>fbpAF1 / fbpAR1</td>
</tr>
<tr>
<td>Cytochrome c oxidase, subunit I (<em>coxA</em>)</td>
<td>Baldo et al. 2006</td>
<td>CoxAF1 / CoxAR1</td>
</tr>
<tr>
<td><em>Wolbachia</em> specific portion of 16S ribosomal RNA gene (<em>wol16S</em>)</td>
<td>Heddi et al. 1999</td>
<td>Wol16SF / Wol16SR</td>
</tr>
</tbody>
</table>