V ab a b N Z a a a *Hemideina* . (O a: A a a)

NATASHA E. MCKEAN[1], STEVEN A.

Ab a

Natural hybridization between species provides an opportunity to

Richards 2005), and they now have broadly parapatric distribu tions (Trewick and Morgan-Richards 1995, Bulgarella et al. 2014). Hybridisation in the wild between the New Zealand tree wētā *H* (White) and both *H* (Blanchard) and *H* Morgan-Richards has recently been confirmed with genetic data, but so far only F_1 hybrids have been confirmed (Mckean 2014, Mckean et al. 2016). These three *H* 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.* [illegible] and *H.* [illegible] in a large area of sympatry 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 prolateral hind tibia (typically between the three spines seen in *H.* [illegible] and the four in *H.* [illegible]/*H.* [illegible], 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 sympatry between *H.* [illegible] and *H.* [illegible], 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.* [illegible] × *H.* [illegible] hybrids), were heterozygous for these markers (Mckean et al. 2016). Whether hybridization occurs between *H.* [illegible] and *H.* [illegible] 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.* [illegible] × *H.* [illegible] hybrids, which are found at a frequency of 1% of wētā in sympatry, are infertile, but genetic and morphological data suggest a low, but potentially significant, level of introgression between *H.* [illegible] and *H.* [illegible], where hybrid frequency is ~3 in every 100 wētā (Mckean et al. 2016).

Introgression is the signal of past hybridization, and an abil-

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.* and 19 *H.* ; Mckean et al. 2016), and to a separate sample of adult males from both species (25 *H.* and 22 *H.*), 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.

M .—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 running 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 F_1 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.

E .—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 F_1 hybrid adult females and 18 *H.* 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 unlaid 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).

M .—Two adult F_1 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).

Table 2. Average number of eggs +/- standard deviation for *H.* females vs. F_1 hybrid females.

	Sample size	Age since maturity (days)	Eggs (unlaid)	Eggs (laid)	Eggs (total)
H.	18	201 +/- 70.7	26 +/- 30.9	65 +/- 32	91 +/- 26.5
F_1 Hybrids	5	139 +/- 47.8	0	0	0

Table 3.

.—Two methods were used to obtain evidence of infection by the bacteria : amplification of DNA sequences using specific Polymerase Chain Reactions (PCR) primers, and whole genome sequencing and alignment to a reference genome. For amplification of specific DNA sequences, DNA was extracted from three tree wētā specimens representing each of the three North Island species (*H.* , *H.* and *H.*). 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). -specific primers (Appendix 1) were used in PCR with wētā DNA, and DNA from an introduced gregarious parasitoid wasp (*N*) known to be infected with 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 fragment from the CoxA 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 6.1.7 (Biomatters LTD; Kearse et al. 2012) software. The resulting 269 bp sequence was compared 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.* male collected from the Kahutawera Valley and an *H.* 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.* specimen and 17,434,429 100 bp paired-end sequences for the *H.* specimen. An annotated reference genome was obtained from New England Biolabs (http://tools.neb.com/wolbachia, originating from infection of *B*; Foster et al. 2005). Reads were trimmed to remove index sequences using solexaQA (Cox et al. 2010) before mapping to the genome using the default settings with Bowtie 2 (Langmead and Salzberg 2012). Results were visualised with Tablet v1.7.0_35 (Milne et al. 2010). Sequences that matched parts of the genome were compared with published data using the NCBI (National Library of Medicine) GenBank BLAST search algorithm to determine their similarity to DNA sequences from other hosts. This enabled us to determine whether the sequences came from the genome or another related bacterial species, which could be determined by sequence similarity.

R

F.—Hybrids were identified by genetic markers and intermediate phenotypes, and no morphologically cryptic hybrids were identified (Mckean et al. 2016). The sex ratio of F_1 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 F_1 hybrid females and adult females of the two parent species from the same location with ANOVA; F = 2.575, = 0.09 (Fig. 2A), however male F_1 hybrids were significantly larger than males of either parent species (ANOVA; F = 8.969, = 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 *H.* (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).

M.—All three F_1 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.* 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.* male, and then resisted all subsequent mating attempts, and was the only hybrid female wētā that was observed to accept a spermatophore.

F.—None of the five female F_1 hybrids contained eggs in any stage of development when killed and dissected as adults. This contrasts with 18 *H.* 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.* 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.* female mate 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 F_2 generation look the same as F_1, or resemble the wētā of the parent species). No other eggs hatched during the study, including the eggs produced by the control wētā

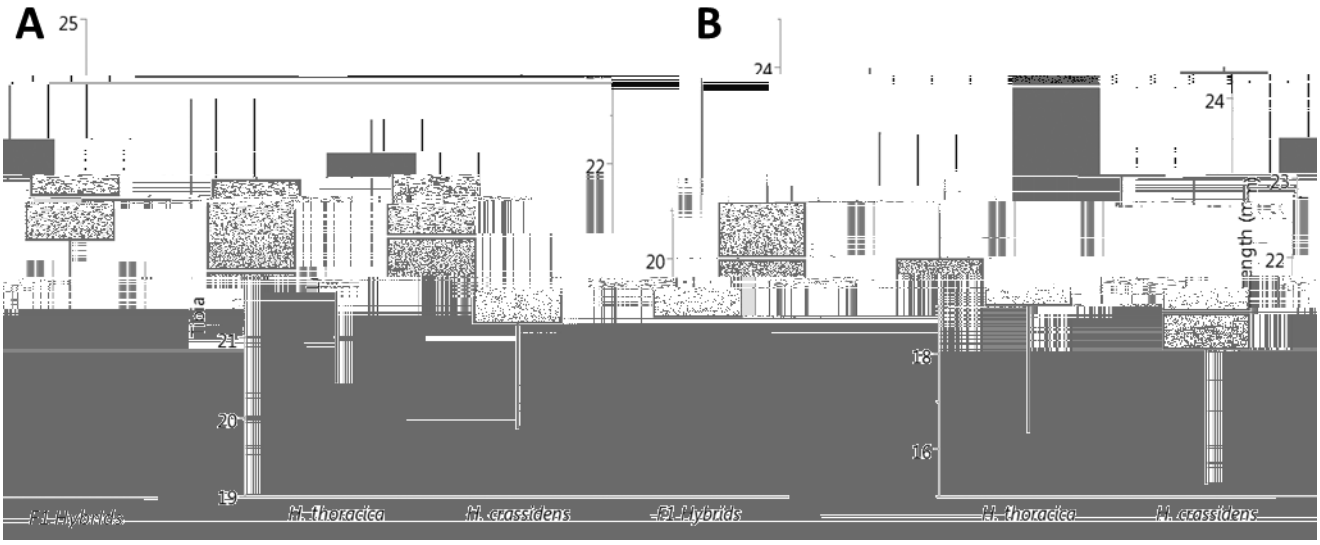

Fig. 2. A. Tibia length of adult female F_1 hybrids compared with adult females from the two parent species, showing no significant difference; **B.** Tibia length of F_1 hybrid males compared with males of the two parent species, showing a significant difference: p-value = 0.0001.

No close sequence match was found when compared to DNA sequences on the database Genbank, including sequences.

None of the > 17 million *H.* *E.* next-generation short read DNA sequences mapped to the genome. Howev-

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

Becker M, Gruenheit N, Steel M, Voelckel C, Deusch O, Heenan PB, McLenachan PA, Kardailsky O, Leigh JW, Lockhart PJ (2013) Hybridization may facilitate in situ survival of endemic species through periods of climate change. Nature Climate Change 3: 1039–43. https://doi.org/10.1038/nclimate2027

Mallet J, McMillan WO, Jiggins CD (1998) Mimicry and warning color at the boundary between races and species. In: Howard D, Berlochers S (Eds) Endless Forms: Species and Speciation. Oxford University Press, Oxford, 390–403.