

1 Genetic rescue attempt in a small, inbred population of a wild endangered passerine

2 Abstract

3 Translocations can facilitate gene flow between fragmented populations, introducing new genetic
4 variants that may mask deleterious alleles and relieve inbreeding depression (i.e., ‘genetic rescue’).
5 Genetic rescue attempts in threatened populations are underutilised due to uncertainties
6 surrounding outbreeding depression, appropriateness of source and recipient populations, and the
7 duration of beneficial effects. The Aotearoa New Zealand hihi (*Notiomystis cincta*) is a threatened
8 passerine hampered by disease outbreaks and reproductive failure. We assessed the long-term
9 outcomes of a genetic rescue attempt, ten years after 20 immigrant hihi were translocated from Te
10 Hauturu-o-Toi Island, the last natural population, to a reintroduced population on Tiritiri Mātangi
11 Island. There was marginally higher multilocus heterozygosity in the source individuals, and low
12 genetic differentiation, based on F_{ST} , between the populations. Six immigrants successfully fledged
13 offspring, but only three left descendants. Following translocation, we observed short- and long-
14 term increases in microsatellite-based neutral genetic diversity and increases in functional diversity
15 across three Toll-like receptor loci. Survival to hatch decreased with proportion of immigrant
16 ancestry, but individuals with immigrant ancestry were more likely to fledge and recruit into the
17 breeding population. F1 immigrant offspring were less likely to hatch, but more likely to fledge
18 and recruit indicating potential heterosis at later life stages. However, differences in early-life
19 survival between individuals with local and immigrant ancestry declined in the F2 onwards.
20 Inbreeding increased following translocation, likely because one immigrant contributed a
21 disproportionate number of descendants. Benefits of translocating individuals may be limited by
22 the high degree of stochasticity in immigrant establishment. In species like the hihi with high levels
23 male-male competition, establishment may be improved by translocating females or pre-
24 reproductive young into recipient populations.

25

26 **Introduction**

27 Translocations can facilitate gene flow between small, isolated populations when natural migration
28 processes are curtailed due to habitat loss and fragmentation. Therefore, translocations may be an
29 effective method to mitigate genetic threats such as loss of genetic diversity and high inbreeding
30 levels, which can lead to inbreeding depression (Frankham 2015; Tallmon et al. 2004). Establishing
31 gene flow through translocation can be difficult and dependent on the migrant genetic profile,
32 survival, and mating success. However, if successful, the addition of new immigrant alleles may
33 relieve inbreeding depression by masking deleterious recessive alleles and promote heterosis
34 (Atsumi et al. 2021). Further, benefits of translocating individuals may include increased
35 population size, thereby reducing Allee effects and demographic stochasticity, i.e. demographic
36 rescue and increased additive genetic variance and adaptive potential, i.e. evolutionary rescue,
37 particularly in larger recipient populations (Hufbauer et al. 2015). Genetic changes combined with
38 the management of non-genetic drivers of decline (Bell et al. 2019), may subsequently translate
39 into ‘genetic rescue’ effects if increases in population fitness, population growth rate and genetic
40 diversity occur (Grueber et al. 2017). However, the efficacy of such translocations and ability to
41 achieve long-term genetic rescue in threatened populations in the wild remains uncertain (Ralls et
42 al. 2018).

43 Genetic rescue has been empirically tested in livestock, laboratory model species (Spielman and
44 Frankham 1992; Wade and Goodnight 1991) and non-threatened wild populations (Fitzpatrick et
45 al. 2020; Robinson et al. 2017). In threatened populations, translocations for assisting gene flow
46 have successfully resulted in genetic rescue in species such as the Florida panther *Puma concolor*
47 (Johnson et al. 2010), the rocky mountain bighorn sheep *Ovis canadensis* (Hogg et al. 2006; Poirier
48 et al. 2019) and the South Island robin *Petroica australis* (Heber et al. 2013). These studies have

49 shown evidence of changes in inbreeding and molecular diversity at neutral as well as functional
50 regions of the genome. For example, Grueber et al. (2017) found increased diversity in the Toll-
51 like receptor (TLRs) loci. TLRs are functional genes that regulate innate and adaptive immune
52 response and impact disease susceptibility in threatened populations. Despite examples of
53 successful genetic rescue and frequent calls for its uptake, its use as a management tool for the
54 conservation of threatened, wild populations remains low (Frankham 2015; Ralls et al. 2018).
55 Translocations can be logistically difficult, costly, and have associated risks of disease transmission
56 (Weeks et al. 2011).

57 Conservation managers may also have concerns about whether assisted gene flow will lead to
58 outbreeding depression. After the initial increase in fitness in the first generation due to heterosis,
59 subsequent generations may experience a decline in fitness as gene flow can also introduce
60 maladapted genes and breakup co-adapted gene complexes that enable the recipient population to
61 be adapted to local conditions (Frankham et al. 2011; Grummer et al. 2022; Hwang et al. 2011;
62 Mitchell et al. 2019; Templeton et al. 1986; Whitlock et al. 2013). However, empirical evidence of
63 outbreeding depression in wild and threatened populations is limited (but see Templeton et al. 1986
64 and Whitlock et al. 2013), and more commonly observed in experimental, laboratory and
65 domesticated species (Grummer et al. 2022; Whitlock et al. 2013). Regardless, uncertainties remain
66 about the duration of genetic rescue effects in wild and threatened populations, the conditions that
67 lead to a successful genetic rescue and what entails a suitable source of individuals for genetic
68 rescue.

69

70 The hihi is an endangered forest passerine endemic to Aotearoa, New Zealand. In 1995, the only
71 remaining natural population of hihi on Te Hauturu-o-Toi (Little Barrier Island: 36°12'S,
72 175°05'E; 3083 ha; est. population size 3000 individuals, Brekke et al. 2011) was used to establish

73 a new population on the island of Tiritiri Mātangi (36°36'S, 174°53'E; 220 ha) which has
74 subsequently been intensively monitored for the last 24 years and has grown to around 200
75 individuals (Parlato et al. 2021). Following the initial reintroduction, only 21 of the 51 translocated
76 individuals survived to breed, but the population has grown consistently since (Armstrong et al.
77 2002; Parlato et al. 2021). However, hihi on Tiritiri Mātangi have experienced multiple disease
78 outbreaks which have reduced the population size (Ewen et al. 2007). For example, coccidiosis
79 (Schoener et al. 2013), aspergillosis (Alley et al. 1999) and salmonellosis (Alley et al. 2012; Ewen
80 et al. 2007; van Andel et al. 2015). These diseases are known to elicit immunogenetic TLR
81 responses in other bird species (Takeda et al. 2003), which suggests immune gene diversity at these
82 sites may be compromised on Tiritiri Mātangi, potentially affecting disease susceptibility (Grueber
83 et al. 2017). A lack of natural dispersal between hihi populations not only led to the loss of
84 functional diversity (de Villemereuil et al. 2019a; de Villemereuil et al. 2019b), but also neutral
85 diversity (Brekke et al. 2011a), reduced additive genetic variance and adaptive potential (de
86 Villemereuil et al. 2018 (de Villemereuil et al. 2019b) in the recipient population on Tiritiri
87 Mātangi. This population also shows evidence of inbreeding depression in reproduction and early-
88 life survival (Brekke et al. 2010; Duntsch et al. 2023), all of which suggest the Tiritiri Mātangi
89 population is genetically imperilled.

90 These genetic risks led to the conservation management decision to attempt genetic rescue
91 following the guidelines outlined by Frankham et al. 2011 and Frankham 2015. In short, these
92 stipulate that the recipient population should show evidence of genetic erosion and/or inbreeding
93 depression and the source population should be of the same species, larger and less inbred, with no
94 fixed chromosomal differences. Furthermore, gene-flow between recipient and source should have
95 occurred within the last 500 years or less than 20 generations. Finally, there should be no substantial
96 biotic and abiotic differences between the populations (Frankham et al. 2011).

97 In hihi, the population on Te Hauturu-o-Toi Island was regarded as the only suitable source
98 population for attempting a genetic rescue: it is the largest and most genetically diverse hihi
99 population (Brekke et al. 2011a; de Villemereuil et al. 2019b), despite also undergoing bottlenecks
100 in the last century (Duntsch et al. 2023; Rasch et al. 1996). Te Hauturu-o-Toi is also geographically
101 close to Tiritiri Mātangi Island, and within the same ecological region (Armstrong et al. 2007). The
102 populations have only recently been isolated (in 2010 by 15 years or 3-4 generations), therefore
103 differences in local adaptation are unlikely, although the populations have shown some genetic
104 differentiation (Brekke et al. 2011a; de Villemereuil et al. 2019b). However, lack of population
105 monitoring on Te Hauturu-o-Toi Island due to its remoteness and inaccessibility, made it difficult
106 to assess the direct impact of removing individuals for translocation. Further, the population is the
107 only natural, self-sustaining population of hihi. Therefore, a conservative approach was adopted
108 when deciding on the number of individuals to remove for translocation, aiming to minimise
109 unforeseen impacts of reducing the source population size.

110 Here, we assess whether the translocation of 20 hihi from Te Hauturu-o-Toi Island to Tiritiri
111 Mātangi Island in 2010, in an attempt to establish gene flow, led to an improvement in the recipient
112 population's genetic health. We predict that (i) Te Hauturu-o-Toi will be a suitable source of
113 immigrants to translocate to Tiritiri Mātangi, as the source population is larger, less inbred and
114 more genetically diverse at neutral and functional sites as well as sharing biotic and abiotic
115 conditions. (ii) That translocating 20 immigrants from Te Hauturu-o-Toi will be sufficient to
116 facilitate gene-flow as previous hihi translocation attempts using similar numbers of individuals
117 have led to immigrant establishment, survival, and reproduction post-translocation. (iii) The
118 likelihood of outbreeding or genetic swamping is very low as they are the same species, the number
119 of immigrants used is small relative to the local population size (20:150) and gene-flow has
120 occurred within 20 the last generations. Furthermore, if gene-flow can be achieved, it will lead to
121 (iv) higher neutral (based on microsatellite loci) and functional (based on TLR loci) genetic

122 diversity, lower inbreeding levels and higher early-life survival in the offspring with immigrant
123 ancestry, particularly in the first generation due to heterosis. Finally, (v) these improvements in the
124 genetic health of individuals from the first generation with immigrant ancestry are unlikely to
125 decline in subsequent generations because of outbreeding depression, as the recipient and donor
126 populations have not been separated for long enough for substantial local adaptation to occur.

127

128 **Methods**

129 Study species

130 The hihi breeding season takes place between September and February (the Austral summer). Hihi
131 are highly territorial, form social pair bonds but have high levels of extra-pair paternity through
132 solicited and forced copulations (Brekke et al. 2013; Ewen et al. 2004). Hihi are able to reproduce
133 within their first year of life (Low et al. 2007). A single female can successfully rear a maximum
134 of two broods per reproductive season (Ewen et al. 2004). The first clutch is generally laid between
135 October and December while the second clutch is laid between January and February. Each clutch
136 comprises between three to five eggs (Taylor et al. 2005). Females carry out nest building and
137 incubation, which lasts approximately 14 days. Females also provision nestlings. Fledging occurs
138 approximately 30 days after hatching (Castro et al. 2003). An individual is considered to have
139 recruited if they are alive and entered the breeding population at any point during their lifetime.

140

141 Study sites

142 Te Hauturu-o-Toi Island translocation: In March 2010, 20 adult and juvenile hihi (7 females, 13
143 males) were captured using mist nets from two sites, Te Waikohare and Tirikakawa valleys, on Te

144 Hauturu-o-Toi Island and translocated to Tiritiri Mātangi, following standard translocation
145 protocols developed for hihi (Ewen et al. 2011).

146 Tiritiri Mātangi population monitoring: Since 1995, every breeding event has been monitored daily
147 from nest building to the fledging of chicks, as the vast majority of females nest in one of the two
148 nest boxes provided in each territory. Natural nest building has been observed in the population but
149 is rare and estimated to happen less than once per season. At 21 days after hatching, each offspring
150 is measured, weighed and colour-banded with a unique combination. Two surveys are conducted
151 annually, pre-breeding in September and post-breeding in February. Adult resighting probabilities
152 based on these surveys are extremely high and are consistent between the sexes (September survey
153 = 1; February survey = 0.83; Low and Pärt 2009).

154

155 Sampling

156 Individuals caught in the source population on Te Hauturu-o-Toi were uniquely colour-banded and
157 blood sampled using brachial venepuncture. Blood samples were stored in 95% ethanol. On Tiritiri
158 Mātangi blood samples were collected during banding at the nest via brachial venepuncture and
159 stored in 95% ethanol ($n = 4559$ as at March 2019). Unhatched eggs were collected 3 days after
160 the expected hatching date and dead nestlings are recovered during daily nest checks ($n = 1730$).
161 Tissue samples taken from unhatched embryos and dead nestlings were also preserved in 95%
162 ethanol. Any birds not sampled in the nest were captured using mist nets or traps and blood-sampled
163 via brachial venepuncture as adults.

164

165 Neutral loci genotyping and pedigree building

166 All individuals sampled were genotyped at 18 neutral and highly polymorphic microsatellite loci
167 and two sex-linked loci (Brekke et al. 2009). Microsatellite genotyping was used to estimate
168 relatedness, genetic diversity metrics (see below) and genetically resolve paternity (>99%
169 combined exclusion probability; COLONY V.2; Wang & Santure 2009), while maternity and
170 sibship can be assigned from behavioural observation to build a genetically resolved pedigree (see
171 Brekke et al. 2015; de Villemereuil et al. 2019b; Duntsch et al. 2020 for details on pedigree
172 construction).

173

174 Functional loci sequencing

175 A total of 340 individuals were sequenced at three functional TLR immune gene sites, TLR1LA,
176 TLR1LB and TLR5 where amplification was consistent and variation present. Of these individuals,
177 164 were born in the 2009-2010 cohort, prior to the genetic rescue event, 17 were the immigrant
178 individuals from Te Hauturu-o-Toi, and 159 were the individuals born on Tiritiri Mātangi two
179 breeding seasons after the genetic rescue attempt in 2012-2013 (immigrant ancestry, $n = 40$; local
180 ancestry, $n = 119$). The samples were Sanger sequenced at Microsynth (Microsynth, Switzerland).
181 SNP phasing to obtain haplotype alleles was carried out according to Grueber et al. (2012).

182

183 Data analysis

184 Genetic differentiation between source and recipient population

185 All data analysis was carried out in R version 4.3.0. To assess genetic differentiation between the
186 20 immigrant individuals and the 144 individuals that bred in 2009 on Tiritiri Mātangi prior to the
187 genetic rescue attempt, we calculated F_{ST} with 97.5% confidence intervals (CI), estimated via 1000

188 bootstrap samples in the hierfstat package version 0.5.11 (Goudet et al. 2015) as well as testing the
189 difference in expected heterozygosity using a Monte-Carlo test with 1000 simulations in the
190 Adegenet package version 2.1.9 (Jombart and Ahmed 2011). Finally, a principal component
191 analysis (PCA) using the microsatellite genotypes was used to visualize the amount of genetic
192 differentiation between the two groups using the Adegenet package (Jombart and Ahmed 2011).
193 Immigrants are commonly assumed to be outbred and unrelated to the local population. However,
194 this assumption is unlikely to be met in this instance as the same source population was used for
195 both the initial founding of the recipient population and genetic-rescue attempt. We estimated
196 average pair-wise relatedness among immigrants ($n = 20$), among the local Tiritiri Mātangi
197 individuals ($n = 144$) in 2010 and average pair-wise relatedness between immigrant and local
198 individuals in COANCESTRY V1.0.1.10 using the TrioML estimator and microsatellite genotype
199 data (Pew et al. 2015).

200

201 Changes in genetic diversity and inbreeding due to immigration

202 To assess changes in genetic diversity prior to and following the translocation, genetic diversity
203 measures were quantified for each breeding season using all fledged individuals genotyped at 18
204 microsatellite loci between 2004-2010 ($n = 920$) and from 2010-2019 ($n = 1491$). The mean
205 observed total number of alleles per locus, the number of effective alleles, the number of observed
206 private alleles, observed heterozygosity, expected heterozygosity, unbiased expected
207 heterozygosity and the fixation index (the proportion of the variance in the subpopulation contained
208 in an individual), were calculated using GenAlEx V6.501 (Smouse and Peakall 2012). Individual
209 multilocus heterozygosity (MLH) at microsatellite loci was calculated for all individuals fledged
210 on Tiritiri Mātangi from 2004 to 2019 using inbreedR version 0.3.3 (Stoffel et al. 2016). Individual
211 inbreeding coefficients (f) were calculated from the pedigree using the Optisel package version

212 2.0.6 (Wellmann 2019) for all individuals fledged on Tiritiri Mātangi from 2004 to 2019. For
213 individuals with immigrant ancestry, inbreeding was calculated by assuming that immigrants were
214 unrelated to each other or the local population. However, this assumption is unlikely to be met here,
215 as outlined above, due to both translocations utilizing same source population. Mean MLH and f
216 were estimated across all fledglings per breeding season to assess changes following the
217 translocation. In addition, for 2010 onwards, MLH and f were aggregated separately for individuals
218 with immigrant ancestry (at least one immigrant ancestor) compared to those with only local
219 ancestry. Finally, we used pedigree data to calculate a continuous proportional measure of ancestry
220 between 0 (“pure” local/recipient genotype) and 1 (“pure” immigrant/source genotype) for all
221 genotyped individuals fledged from 2010-2019 ($n = 2943$). This was calculated using the Optisel
222 package version 2.0.6 (Wellmann 2019) to determine pedigree-based relatedness between each
223 individual and any of the 20 immigrant individuals. If an individual was related to more than one
224 immigrant, relatedness to each immigrant was summed to obtain a total proportion of immigrant
225 ancestry per descendant individual. For example, if an individual had one parent of local descent
226 while the other was one of the 20 immigrants, the immigrant ancestry would be 0.5, if both parents
227 were from the group of 20 immigrants, immigrant ancestry would be 1, and if an individual had
228 one immigrant and three local grandparents, the immigrant ancestry would be 0.25.

229 To test the impact of translocation on genetic diversity and inbreeding, the average MLH and f of
230 fledglings in 2009 was compared to the average in 2010 using a two-sided t test with 10,000
231 bootstraps with the boot package version 1.3.28 (Canty 2002; Davison and Hinkley 1997). Finally,
232 to test the impact of immigrant ancestry on genetic diversity, the correlation between microsatellites
233 and immigrant ancestry, and between f and immigrant ancestry was calculated for all fledglings
234 across breeding seasons 2010-2019.

235 To assess changes in functional genetic diversity, individuals with at least two successfully
236 genotyped TLR loci from 2009-2010 ($n = 134$, 71% of fledged individuals that year) and 2012-

237 2013 (n = 116, 63% of the fledged individuals that year) were analysed. Genetic diversity at these
238 functional sites was assessed by estimating the levels of haplotype diversity (nucleotide and
239 haplotype diversity, Tajima's D, number of effective and private alleles, observed and expected
240 heterozygosity and fixation) across TLR sequences in Pegas version 1.1 (Paradis 2010) and
241 GenAlEx version 6.501 (Smouse and Peakall 2012). To test whether the level of TLR
242 heterozygosity increased following the translocation, the multilocus heterozygosity (MLH) was
243 calculated for individuals where at least two TLR loci had successfully amplified using inbreedR
244 version 0.3.3 (Stoffel et al. 2016) before and after the translocation. The difference between MLH
245 was tested using a two-sided t test with 10,000 bootstraps using the boot package version 1.3.28
246 (Canty 2002; Davison and Hinkley 1997). The TLR MLH was correlated to the individual's
247 pedigree-based proportion of immigrant ancestry.

248

249 Immigrant establishment, survival, and reproduction

250 Each individual was tracked using the bi-annual survey, breeding monitoring and the pedigree to
251 assess survival and reproduction until they died (not seen for a minimum of two breeding seasons).
252 To determine the extent to which gene flow had occurred, we used our calculation of the proportion
253 of immigrant ancestry per individual to visualise the proportion of immigrant ancestry in the
254 breeding and fledged population from 2010-2019. We also compared the total number of fledged
255 offspring per dam, for dams where total lifetime reproduction was known and compared to the
256 number of fledged offspring descended from the most successful immigrant female over an eight-
257 year period post-translocation.

258 Do individuals with immigrant ancestry have a higher survival probability?

259 The effects of immigrant ancestry on early survival were tested at three discreet stages: from egg
260 to hatching (hatched 1/ did not hatch 0), from hatching to fledging (fledged 1 / did not fledge 0)
261 and from fledging to being recruited into the breeding population at any point during the
262 individual's lifetime (recruited 1/ did not recruit 0). These were analysed using generalised linear
263 mixed-models (GLMMs) with binomial errors using the lme4 package version 1.1.31 (Bates et al.
264 2018). Juvenile individuals that were taken from the Tiritiri Mātangi population (some with
265 immigrant ancestry) and used to establish and supplement the other hihi populations (in 2009-2010,
266 2010-2011, 2012-2013, 2016-2017 and 2017-2018), were excluded from analyses of survival to
267 recruitment. Immigrant ancestry, inbreeding coefficient and an interaction term between inbreeding
268 coefficient and immigrant ancestry were included in all models as predictors. This interaction was
269 used to test for differences in the effect of inbreeding between local and immigrant ancestry
270 individuals. Quadratic female age, in years, at the time of laying was also included as a fixed effect
271 to control for the strong age-structured reproduction found in hihi (Brekke et al. 2013; de
272 Villemereuil et al. 2019a; Low and Pärt 2009). All models included year and female identity as
273 random factors to account for individuals breeding in multiple breeding seasons and any
274 contribution of inter-year variation in environmental conditions. Every possible model was
275 generated using the dredge function in the MuMIn package version 1.47.1 (Barton and Barton
276 2015). Diagnostics of models were checked using DHARMA package (Figure S1; Hartig and Hartig
277 2017) and the final models were selected using AIC. Model averaging was performed across all
278 models within $AIC > 2$. We decided to use an information theoretic approach in this analysis as it
279 allows us to test competing hypotheses and interpret the more complex relationships in longitudinal
280 datasets by accounting for annual variation and repeated measures.

281

282 Does early survival differ between the first generation and subsequent generations of individuals
283 with immigrant ancestry?

284 The effects of immigrant ancestry on early survival in the first generation (F1 only, to test for
285 potential heterosis) and subsequent generations (F2+, to test for potential outbreeding) were tested
286 as above, with the added predictor of generation time (a binomial variable, F1 or F2+ generation)
287 and an interaction term of generation time and immigrant ancestry (as a binomial variable,
288 Immigrant/local ancestry). This interaction allowed us to test survival differences between
289 individuals with local or immigrant ancestry in the F1 compared to the F2+ generation. Individuals
290 with generation time equal or larger than F2 were assigned as F2+. Generation time was calculated
291 by averaging the parental generation and adding 1 (e.g., $(F0+F0)/2+1 = F1$; backcross is
292 $(F1+F0)/2+1 = F1.5$). All models included female ID as a random factor. Year was not included at
293 a random factor, as generation time and year are highly correlated.

294

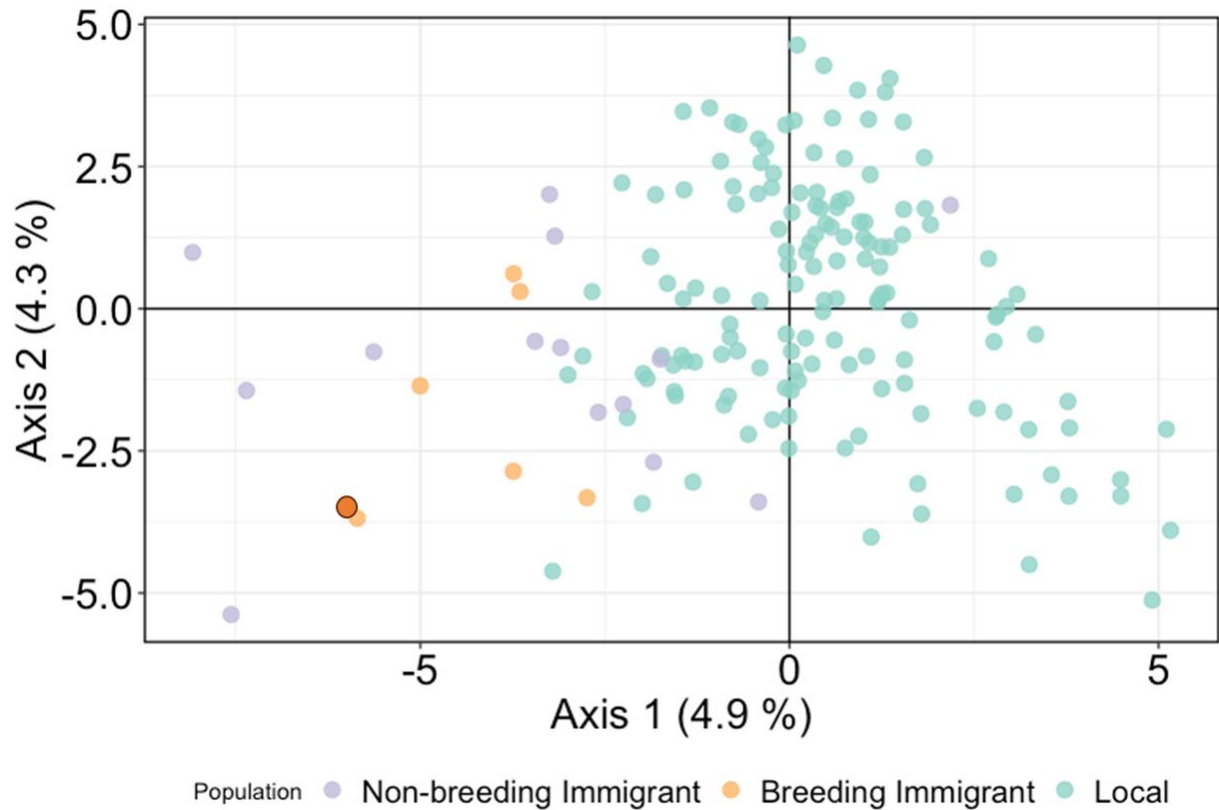
295 **Results**

296 Genetic differentiation between source and recipient population

297 Across 18 microsatellite loci, there was low genetic differentiation between the 20 immigrants from
298 Te Hauturu-o-Toi and the 144 breeding individuals in the Tiritiri Mātangi population in 2010 (F_{ST}
299 = 0.04, 95% CI = 0.02- 0.06), confirmed using a PCA (Figure 1). However, breeding immigrants
300 tended to cluster separately from locals, which suggests sufficient genic differentiation is present
301 to improve genetic diversity in the recipient population. Further, while expected heterozygosity was
302 marginally higher in the Te Hauturu-o-Toi individuals ($H_e = 0.70$) than in the Tiritiri Mātangi
303 population ($H_e = 0.65$) there was no significant difference between the groups (Monte Carlo
304 simulation, based on 1000 iterations; simulated p -value: 0.074). Lack of strong differentiation was
305 expected due to the short time frame since the Tiritiri Mātangi population establishment. Average
306 relatedness between individuals from Te Hauturu-o-Toi and Tiritiri Mātangi was lower ($r = 0.07$)

307 than within each population (Te Hauturu-o-Toi $r = 0.11$; $n = 20$; Tiritiri Mātangi $r = 0.08$; $n = 144$;
308 Table S2).

309



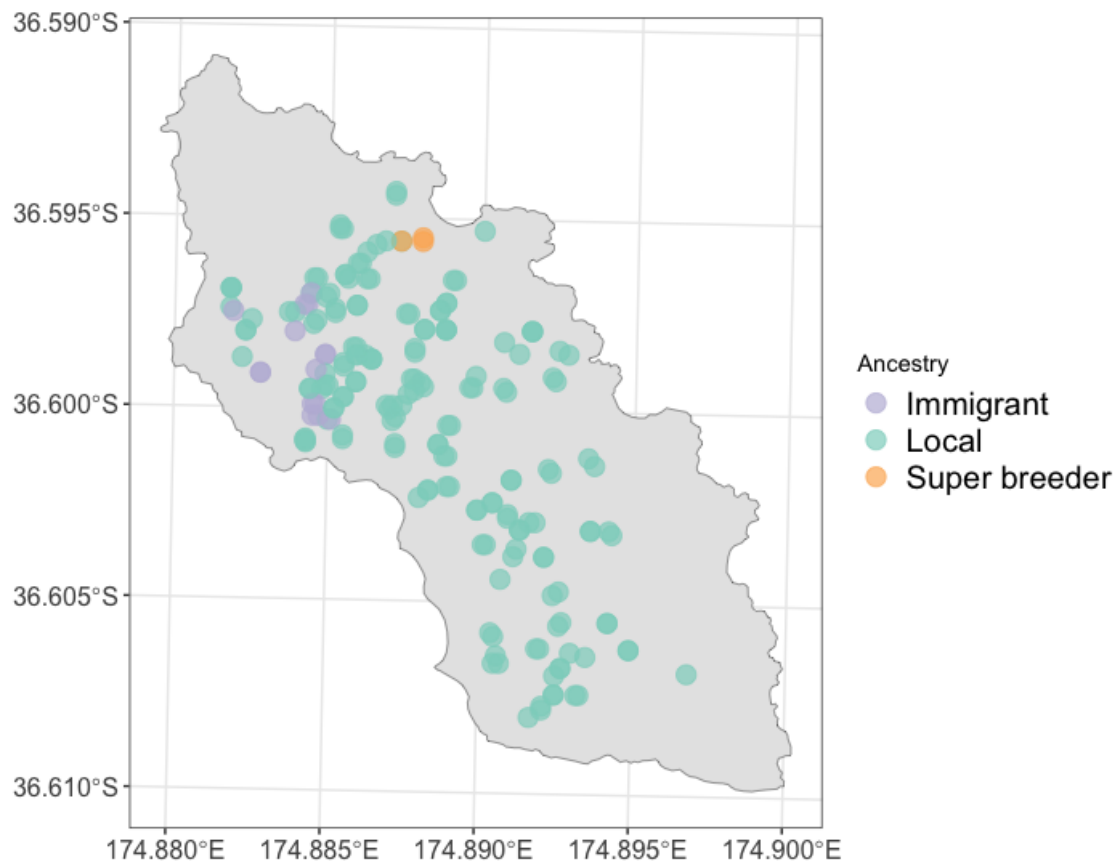
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311 **Figure 1** Principal Component Analysis based on microsatellite genotypes at 18 neutral loci for
312 breeding individuals with local inheritance on Tiritiri Mātangi during 2009 ($n = 144$, green dots),
313 non-breeding immigrants ($n = 14$, purple dots) and breeding immigrants from Te Hauturu-o-Toi
314 translocated during 2009 ($n = 6$, yellow dots), the female ‘super breeder’ (see text) is indicated by
315 a dark orange dot.

316

317 Immigrant establishment, survival, and reproduction

318 A total of 10 of the 20 immigrants survived the first-year post-release. The surviving individuals
319 included five males and five females. Of these individuals, four were first-year and six were older
320 (Table S3). All the surviving individuals attempted to breed by establishing a territory or through
321 extra-pair mating (Figure 2). Most individuals attempted to breed relatively close together in the
322 northern part of the island, in some of the more mature, high quality remnant forest patches (Figure
323 2).



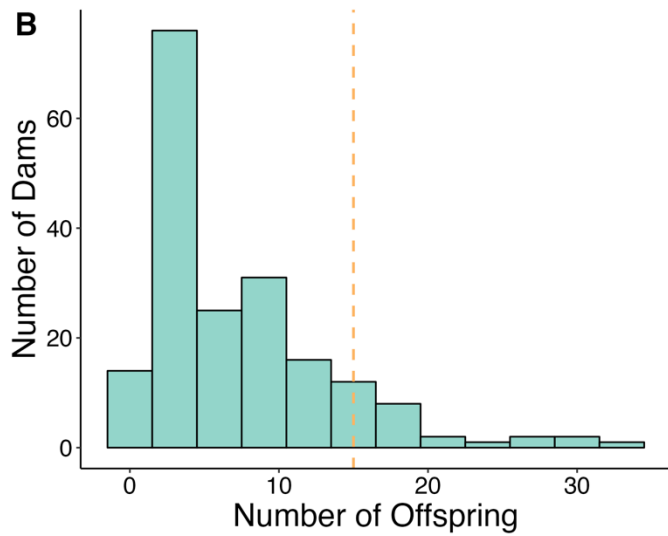
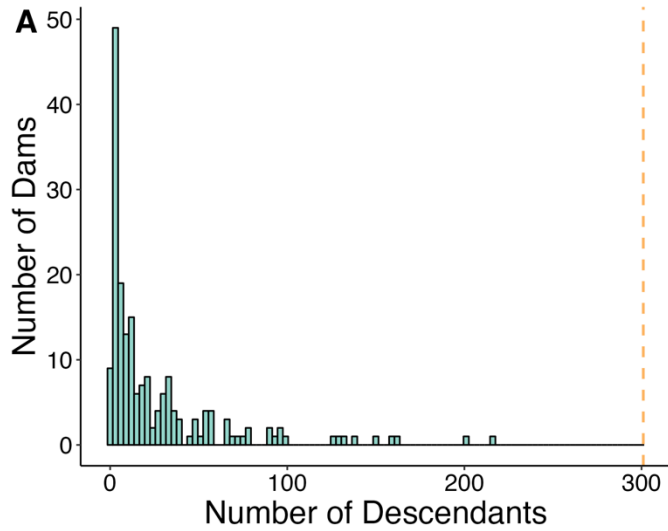
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325 **Figure 2** Map of Tiritiri Mātangi Island marking the use of nest boxes by the adults present in 2010
326 (after translocation) across their breeding lifetimes (2010-2019). Each territory contains two nest
327 boxes, one of which was occupied by the female. Colours indicate the ancestry of individuals
328 occupying the nest box. Please note that not all individuals that used nest boxes produced offspring.

329

330 Six (two males and four females) out of the 10 surviving immigrants successfully fledged offspring
331 (Table S3; 35%). Although all five surviving males attempted to fertilise eggs in the first breeding
332 season, only two successfully fledged offspring (total fledglings produced across their combined
333 lifetimes = 3). None of the immigrant males survived for longer than the first-year post-release.
334 While all surviving females produced eggs, only four out of the five successfully fledged offspring
335 in the first breeding season. Of the females that survived the first breeding season, all established
336 territories, and bred for a minimum of two and maximum of seven breeding seasons post-
337 establishment (total fledglings produced across their combined lifetimes = 28; Figure 3A-B). Gene
338 flow, defined as successful reproduction of immigrants, was achieved (Figure 4A-B). However, of
339 the six immigrants that fledged offspring following translocation, only the lineages of three (one
340 male and two females) were represented in the breeding population eight years after the
341 translocation and this represented only a small proportion of ancestry in the fledged population
342 (Figure 4B). The number of descendants per individual was highly variable (range = 1 – 301). The
343 most successful translocated individual was a juvenile female who fledged 11 offspring over three
344 breeding seasons, hereon referred to as the ‘super breeder’, this female left a total of 301
345 descendants from 2010-2019 (Figure 3A). The mean number of descendants for dams whose total
346 lifetime reproductive success is known, over the same time-period of 8 years, was 26.65 (CI 21.98-
347 33.01). Therefore, the super breeder produced more descendants than would be expected by chance
348 (Figure 3A). Despite the high number of descendants, the super breeder did not produce
349 substantially more offspring during her lifetime than other dams in the population (Figure 3B).

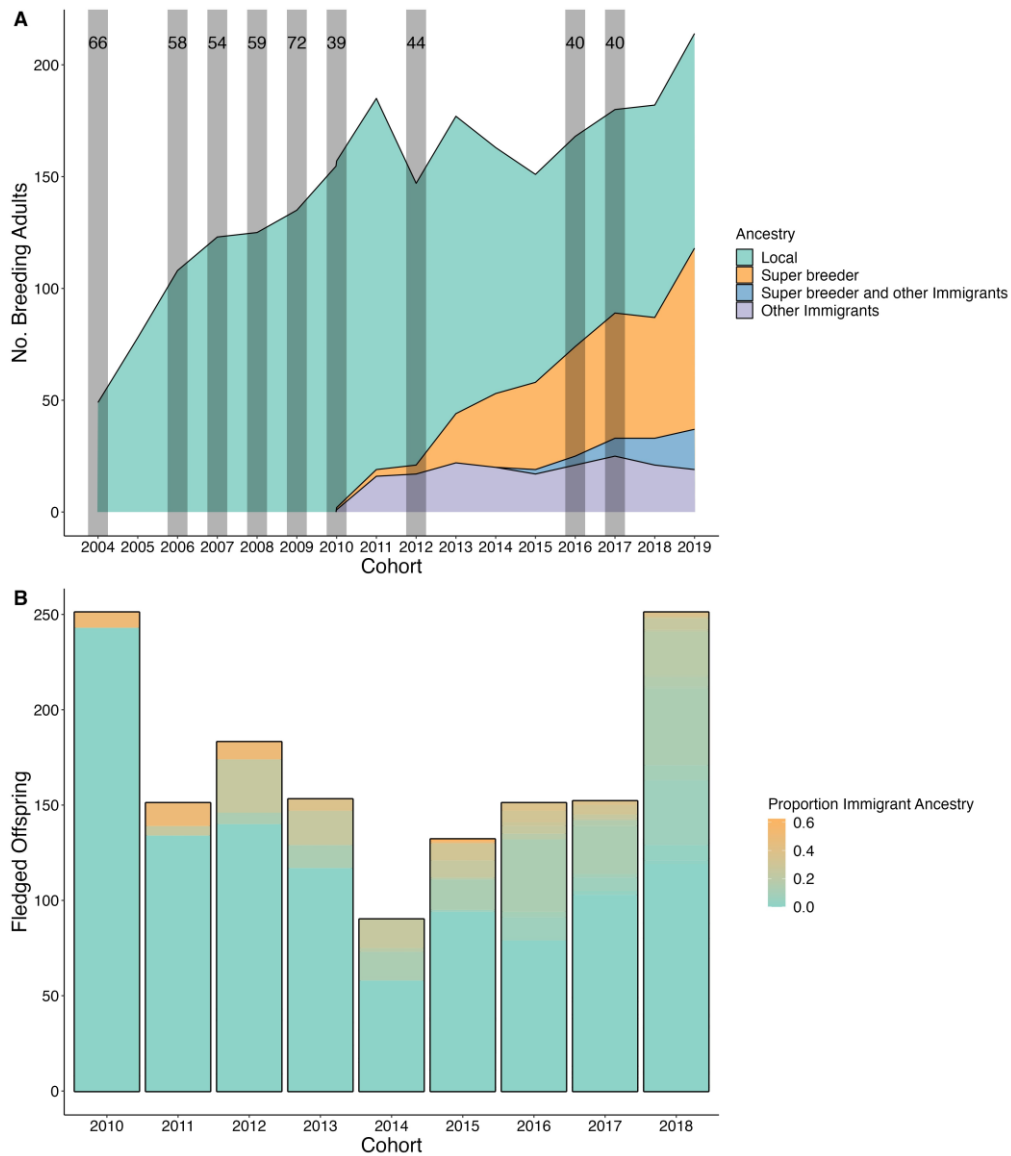
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351

352 Figure 3 A) Total number of fledged descendants per dam, for dams where total lifetime
 353 reproduction is known. Data was collected for 8 years from the first season they bred. The yellow
 354 dashed line indicates the number of fledged offspring descended from the super breeder over 8
 355 years. B) Total number of fledged offspring produced per dam, for dams where total lifetime
 356 reproduction is known, 8 years from the first breeding season for each dam. The yellow dashed line
 357 indicates the number of offspring fledged by the 'super breeder'.

358



359

360 **Figure 4** A) Total number of breeding individuals per cohort on Tiritiri Mātangi from 2004-2019.
 361 ‘Local’ category refers to breeding individuals descended from local Tiritiri Matangi pairings.
 362 ‘Super breeder’ category refers to breeding individuals descended from pairings between the super
 363 breeder and local individuals. ‘Super breeder and other Immigrants’ category refers to breeding
 364 individuals descended from pairings between the super breeder and other translocated immigrant
 365 individuals and finally ‘Other immigrants’ category refers to breeding individuals descended from
 366 immigrant pairings excluding the super breeder individual, following the 2010 translocation. Grey

367 shading indicates harvesting events in which individuals were removed from the population to
368 establish new ones (juveniles were harvested at random with respect to ancestry), the number of
369 individuals removed is annotated. B) Temporal patterns of total proportion of immigrant ancestry
370 in the population according to fledged offspring from 2010 to 2019. Pure local individuals from
371 Tiritiri Mātangi had an index of zero (green) and pure immigrants had an index of one (yellow).
372 Proportion of immigrant ancestry was calculated using the pedigree.

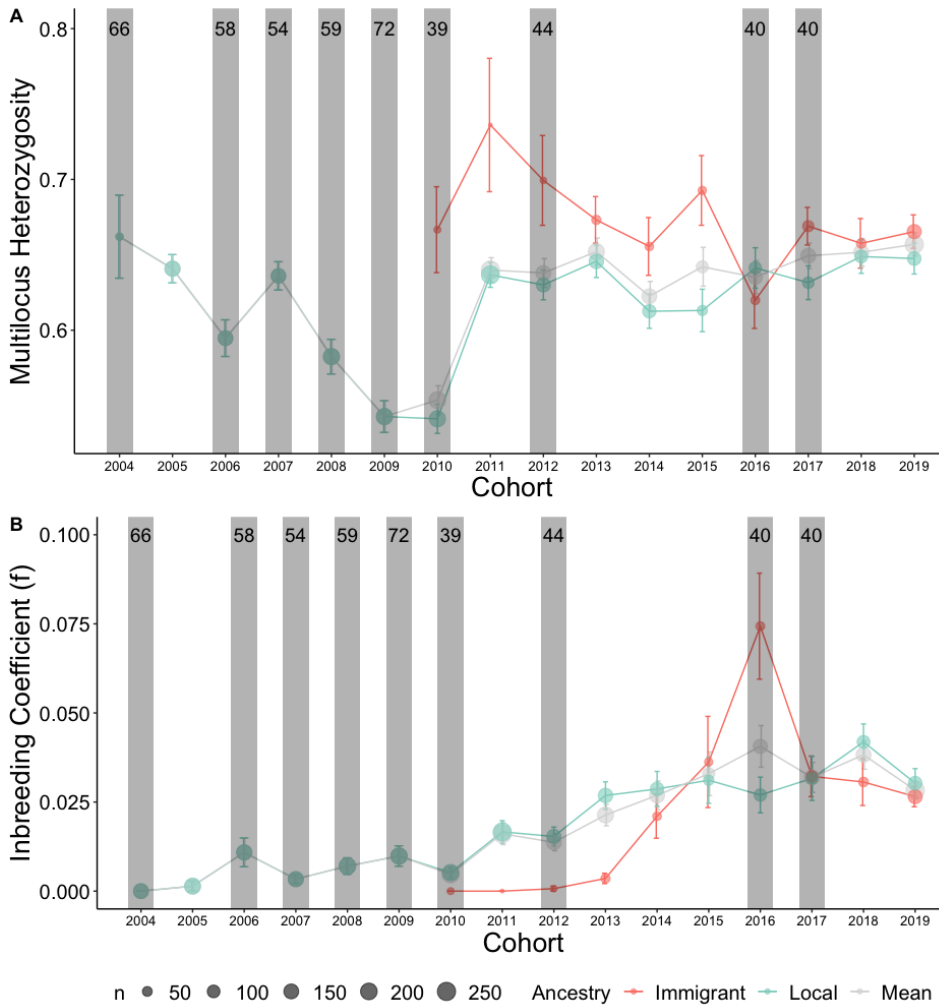
373

374 Changes in genetic diversity and inbreeding due to immigration

375 *Genetic diversity*

376 Gene flow led to changes in genetic diversity as well as inbreeding levels (Figure 4, 5, Table S4).
377 Neutral genetic diversity (MLH) increased in fledged individuals with immigrant ancestry in the
378 breeding season following the translocation (local ancestry: 0.64; immigrant ancestry 0.74; 95%
379 CI: 0.02 - 0.19)), and in the long-term there was a positive correlation between MLH and immigrant
380 ancestry (Microsatellites only: $R^2 = 0.10$; $P < 0.001$, Figure S2; Table S5; Figure S2). However,
381 on average across the population, MLH levels remained lower after supplementation than the
382 maximum previously observed in the recipient population (e.g., 2004/2005 breeding season; Figure
383 5; Table S4). Furthermore, no significant short-term change was observed in functional genetic
384 diversity (TLR-based MLH) of fledged individuals following the translocation (local: 0.35 CI: -
385 0.03- 0.07; post-translocation 0.41; 95% CI: -0.04- 0.15, Table S5; Figure S2), while there was a
386 marginally significant positive correlation between TLR MLH and immigrant ancestry ($R^2 = 0.13$;
387 $P = 0.044$; Table S5 and Figure S2).

388



390

391 **Figure 5** A) Mean multilocus heterozygosity (MLH) \pm SE based on microsatellite genotypes at 18
 392 neutral loci and B) mean pedigree-based inbreeding coefficient (f) \pm SE. Both A and B show fledged
 393 offspring in the population each year from 2004 to 2019 on Tiritiri Mātangi with individuals with
 394 local only (green), immigrant (orange) ancestry and the mean (grey) for the entire population. Grey
 395 shading indicates harvesting events in which individuals were removed from the population to
 396 establish new ones, the number of individuals removed is annotated.

397

398 *Inbreeding*

399 The level of inbreeding for local fledglings on Tiritiri Mātangi gradually increased from 2004 to
400 2019 as expected (Figure 5B). However, mean inbreeding level for fledglings with immigrant
401 ancestry also increased sharply from 2010, to overtake inbreeding levels in locals by 2016, before
402 declining again, so that no observable differences in inbreeding between immigrant and local
403 descendants remained by 2019 (Figure 5B). There was no correlation between proportion of
404 immigrant ancestry and inbreeding coefficient (Table S5 and Figure S2). However, inbreeding is
405 also likely to be underestimated as two of the six individuals that fledged offspring were closely
406 related ($r > 0.5$; Table S1) and two of the immigrants with the most successful lineages (including
407 the super breeder) were also related ($r = 0.22$; Table S1)

408

409 Do individuals with immigrant ancestry have a higher early survival probability?

410 Three models for survival to hatching were selected and averaged (Table S7). The strongest
411 predictor of survival to hatch was dam age, with survival to hatching decreasing with quadratic
412 female age. Inbreeding and immigrant ancestry were also included. However, these terms had very
413 low predictive power (Table 1). Two models for survival to fledging had AIC values below 2 and
414 model averaged (Table S7). Survival to fledging increased with dam age and with immigrant
415 ancestry. Inbreeding was also present in the averaged model for fledgling survival but had low
416 predictive power (Table 1). Two models for survival to recruitment were averaged and contained
417 inbreeding and immigrant ancestry, which again had low predictive power (Table 1).

418

419

420 **Table 1** The model averaged results (for models with $AIC < 2$; $S7$) of three separate generalised
 421 linear mixed models testing for the post-translocation effect of immigrant ancestry and inbreeding
 422 on survival at three stages of early life. Quadratic dam age was also included as a well-known
 423 predictor of early offspring survival. Year (cohort) and female ID were included as random effects
 424 to control for repeat measures from females across the lifespan and temporal variation across years.

	Fixed Effects		Random effects		# of Obs.
	Estimate	SE		Variance SD	
Hatching					2911
(Intercept)	2.546	0.169	Dam	1.324	1.151
Dam age	-0.148	0.042	Cohort	0.022	0.148
Inbreeding (<i>f</i>)	0.411	1.031			
Immigrant Ancestry	-0.159	0.464			
Fledging					2536
(Intercept)	-0.015	0.140	Dam	0.677	0.823
Dam age	0.126	0.030	Cohort	0.077	0.278
Immigrant Ancestry	1.399	0.574			
Inbreeding (<i>f</i>)	0.393	0.808			
Recruitment					1350
(Intercept)	-1.320	0.480	Dam	0.097	0.312
Inbreeding (<i>f</i>)	-0.603	1.178	Cohort	1.947	1.395
Immigrant Ancestry	0.021	0.263			

426

427 Does early survival differ between the first generation and subsequent generations of individuals
 428 with immigrant ancestry?

429 Survival to hatching was significantly lower in immigrant ancestry individuals compared to locals
 430 in the F1 generation. However, hatching success did not vary between immigrant and local ancestry
 431 individuals in the F2+. Hatching success increased from F1 to F2+, particularly in individuals with
 432 immigrant ancestry (Table 2, Figure 6). Fledging success did not significantly vary between
 433 immigrant and local ancestry individuals in the F1 or F2. However, fledging success decreased
 434 from F1 to F2+, particularly in individuals with immigrant ancestry, but this decline was again not

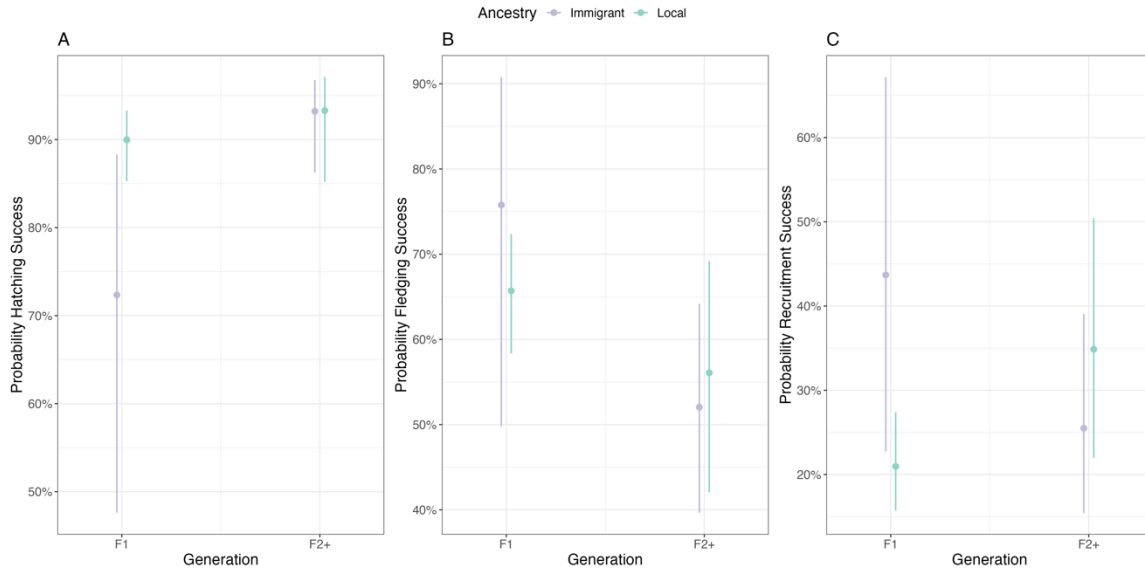
435 significant (Table 2, Figure 6). Survival to recruitment was significantly lower in local ancestry
 436 individuals compared to immigrant ancestry individuals in the F1 generation. However, in the F2+
 437 generation local ancestry individuals had a higher recruitment success. Recruitment success
 438 declined from F1 to F2+ in individuals with immigrant ancestry, while it increased from F1 to F2+
 439 in those with local ancestry (Table 2, Figure 6).

440

441 **Table 2** Model output for three separate generalised linear mixed models with a binomial
 442 distribution of errors testing for the effect of immigrant ancestry and generation (F1 or F2 and
 443 above) between three stages of early life survival. Female ID was included as random effects to
 444 control for repeat measures from females across the lifespan. Values in bold indicate statistically
 445 significant results ($p < 0.05$).

	Fixed effects			Random effects		Observations
	Estimate	CI	P	Variance	SD	
Hatching						699
(Intercept)	0.96	-0.10 – 2.02	0.075	Dam	0.76	0.87
Generation category	1.66	0.34 – 2.97	0.013			
Ancestry	1.23	0.09 – 2.37	0.034			
Interaction	-1.22	-2.76 – 0.32	0.12			
Fledging						616
(Intercept)	1.14	-0.01 – 2.29	0.051	Dam	0.60	0.77
Generation category	-1.06	-2.31 – 0.19	0.098			
Ancestry	-0.49	-1.67 – 0.69	0.416			
Interaction	0.65	-0.71 – 2.02	0.347			
Recruitment						374
(Intercept)	-0.25	-1.22 – 0.72	0.608	Dam	0.15	0.39
Generation category	-0.82	-1.96 – 0.32	0.158			
Ancestry	-1.07	-2.09 – -0.06	0.038			
Interaction	1.52	0.18 – 2.86	0.026			

446



447

448 **Figure 6** Interaction between immigrant ancestry and generation ('F1' refers to local-local and
 449 local-immigrant offspring in the first-generation following translocation while 'F2+' refers to
 450 offspring with entirely local or partially immigrant ancestry in the second generation onwards
 451 following translocation) at three stages of early life survival (note different y-axis scales) for three
 452 separate generalised linear mixed models with a binomial distribution of errors. Female ID was
 453 included as random effects to control for repeat measures from females across the lifespan.

454

455 Discussion

456 Despite mounting evidence that genetic rescue is a valuable tool for genetically imperilled species,
 457 attempting to establish gene flow in the wild remains difficult due to the uncertainty in selecting an
 458 appropriate population for supplementation and stochasticity in the establishment of translocated
 459 individuals. In this study, we show that in accordance with our first two predictions, some
 460 translocated individuals from the island of Te Hauturu-o-Toi, the only suitable source population
 461 for a genetic rescue attempt available for hihi, survived, and bred creating gene flow between the

462 populations. However, only three of the translocated individuals successfully left descendants in
463 the long-term on Tiritiri Mātangi Island. Two of these individuals were related according to
464 microsatellite data. Immigrant reproduction led to a marginal increase in genetic diversity in neutral
465 and functional loci in the years following the translocation.

466 Furthermore, our third prediction was not supported, as one immigrant female's lineage was
467 remarkably prolific. It is likely that her legacy and genetic over-representation in the population, as
468 well as relatedness with the other successfully breeding immigrant female, led to an increase in the
469 levels of inbreeding in the population, as a large proportion of the population shared her as a
470 common ancestor. Overall, no differences were observed in survival to hatch or recruit between
471 individuals with immigrant or local ancestry, and individuals with immigrant ancestry were more
472 likely to fledge in accordance with our fourth prediction and despite the increasing levels of
473 inbreeding in immigrant hybrids. Some support of our final prediction was found as heterosis was
474 evident in the hybrid F1 generation at the fledge and recruitment stage, but not survival at hatching.
475 However, this increase in survival in the F1 declined in subsequent generations, from the F2
476 onwards potentially due to outbreeding, except for survival at hatching which increased in the F2
477 hybrids to mirror hatching success levels of local ancestry individuals. Contrary to our final
478 prediction.

479

480 *Immigrant establishment phase*

481 The translocation to Tiritiri Mātangi lead to 50% mortality of released individuals, which is
482 consistent with mortality levels seen in previous hihi translocations (Armstrong et al. 2017; Brekke
483 et al. 2011a) and relatively low mortality considering the levels of mortality experienced through
484 translocation in other wild populations (Berger-Tal et al. 2020). All five remaining females and two

485 out of the five remaining males acquired territories. Lack of experience in the new site may have
486 relegated immigrants to floating (i.e. not holding a territory) or lower quality territories making
487 them less desirable partners (Brekke et al. 2015). However, those that established territories did so
488 relatively close to one another, in areas of more mature, high quality remnant forest (Morland, Pers.
489 Comm). Released individuals may have preferred habitat that more closely resembled the source
490 site (pristine native forest) or breeding near familiar/related individuals (but see Franks et al. 2020).
491 The three non-territorial males that survived, floated between territories, and were able to mate
492 through extra-pair copulations, although only two of these males had offspring successfully fledge
493 (Brekke et al. 2013).

494 Demographic stochasticity associated with the establishment of small numbers of individuals in
495 reintroduction is common (Armstrong and Seddon 2008). In hihi, survival of released individuals
496 into already established populations has not previously been observed to differ between the sexes
497 but tended to be lower when conspecifics are present (Castro et al. 1994; Richardson et al. 2017).
498 However, in this translocation event females had higher survival and were more successful (fledged
499 more offspring) than males. Demographic parameters of the recipient population are likely to play
500 a key role in the post-release survival, stress, and acclimatisation of the released individuals
501 (Armstrong and Seddon 2008). The adult sex-ratio on Tiritiri Mātangi was strongly male-biased in
502 the year of release, and during the establishment phase of the Te Hauturu-o-Toi individuals (in
503 2010 and 2011; Morland, Pers. Comm), which could explain the female-biased success in
504 establishment and long-term survival. In highly territorial species, with strong male-male
505 competition or male-biased adult sex-ratio, such as with hihi, potentially using females or
506 individuals at earlier stages of development, for example, eggs or juveniles may facilitate
507 establishment (Heber et al. 2013).

508

509 *Immigrant reproduction*

510 A large proportion of surviving immigrants failed to produce offspring in the first season, as
511 offspring died prior to fledgling, particularly those sired by males. Females tended to have longer
512 lifespans post-translocation, allowing them to produce more offspring and therefore increasing the
513 likelihood of leaving descendants. Males also tend to have a higher reproductive variance in hihi,
514 and reproductive success is particularly low in males that do not acquire a territory (Brekke et al.
515 2015). Establishing a territory in an unfamiliar environment, where local males have already
516 territories established, is likely to impact on immigrant male stress, survival, and ability to
517 successfully reproduce (Low 2005). Immigrants may have also had higher rates of early offspring
518 mortality if they carried a higher frequency of deleterious recessive alleles from the larger,
519 potentially more outbred population on Te Hauturu-o-Toi, despite both populations displaying low
520 levels of molecular diversity (de Villemereuil et al. 2019b). However, rates of early reproductive
521 failure were comparable between immigrant and local hihi females (Brekke et al. 2010) and it is
522 unlikely that males carry more recessive deleterious alleles than females, which suggests non-
523 genetic factors drove the higher early mortality in immigrant male-sired offspring. Differences in
524 reproductive variance between the sexes also support the use of females to increase the chances of
525 gene flow (as seen in Heber et al. 2013) between populations with caution taken not to impact the
526 source population sex ratio.

527

528 *Gene flow and immigrant representation*

529 Gene flow was achieved from this translocation as six individuals successfully fledged offspring
530 (30% of released individuals). Three out of the six immigrant lineages persisted long-term. The
531 ‘super breeder’ female lineage was significantly more successful than all but one individual in the

532 population during the same period. Hihi have been shown to avoid inbreeding through extra-pair
533 mating with less related individuals (Brekke et al. 2011b). Overall, there was low relatedness
534 between the immigrants and locals, potentially giving immigrants and their descendants a mating
535 advantage. Furthermore, the ‘super breeder’ was also closely related to one of the other immigrants
536 with a successful lineage. Similar levels of success by a single immigrant were observed through
537 natural dispersal into the highly inbred Isle Royale population of grey wolf (*Canis lupus*) (Adams
538 et al. 2011). The grey wolf population accumulated inbreeding which led to this small population
539 collapsing due to this highly successful migrant, lack of ongoing migration and continued small
540 population size (Hedrick et al. 2014).

541

542 *Changes in inbreeding and inbreeding depression due to immigration*

543 Levels of inbreeding in the hihi population also increased, with individuals with immigrant ancestry
544 experiencing a sharper increase and then decline to the same level observed in those with local
545 ancestry. Increased levels of inbreeding in the population are likely due to the overrepresentation
546 of one immigrant lineage, with 84% of breeding adults with immigrant ancestry in 2019 related to
547 the most successful female lineage. Inbreeding has been found to impact embryo and nestling
548 survival in a previous study using microsatellite-based methods (Brekke et al. 2010) and more
549 recently juvenile survival using a large panel of genome-wide single nucleotide polymorphism
550 markers (Duntsch et al. 2023). Despite the levels of inbreeding increasing in the population after
551 establishing gene flow, inbreeding was not a strong predictor of differences in early survival.
552 However, individuals with immigrant ancestry and to a lesser degree, lower inbreeding, were more
553 likely to fledge, which may reflect the higher success of the surviving immigrant lineages. This
554 suggests that gene flow did have some beneficial effects, as fledging success is one of the most
555 important drivers of population growth (Parlato et al. 2021).

556 However, population growth per se declined two years after the translocation and three harvesting
557 events that removed a total of 124 juveniles. Population size rebounded, but remains smaller than
558 it was at the time of the translocation (Parlato et al. 2021). Therefore, the benefits of heterosis lasted
559 in the F1 generation and declined in the following generations. The breakdown of epistatic
560 interactions may have been initially masked in the F1 but became more evident in F2 hybrids and
561 subsequent generations. From F2 onwards homozygosity increased, and co-adapted gene
562 complexes may have been broken down through recombination (Frankham et al. 2011; Lynch
563 1991). Furthermore, our ability to accurately estimate inbreeding for individuals with
564 immigrant/unknown ancestry is likely to be hampered by our assumption that immigrants are
565 unrelated to each other and the recipient population. In future, genome-based methods are more
566 likely to provide more precise global and region-specific inbreeding estimates (Alemu et al. 2021;
567 Duntsch et al. 2023).

568

569 *Changes in genetic diversity due to translocation*

570 Gene flow led to fledged individuals with immigrant ancestry displaying higher genetic diversity
571 across neutral and functional sites, particularly in the first generation. However, these changes were
572 relatively small and likely to be driven by the small number of individuals that successfully bred
573 and left descendants in the population. Despite locals and breeding immigrants displaying some
574 genetic structuring. But also, due to the low levels of immunogenetic (and genomic) diversity
575 present in source and recipient populations (de Villemereuil et al. 2019b; Grueber et al. 2015), and
576 small number of immune-genes and microsatellite markers used in the study, which provide relative
577 rather than absolute levels of genetic diversity. Low levels of immunogenetic diversity in hihi are
578 comparable to other threatened New Zealand avifauna with a similar history of recent population
579 decline and bottlenecks due to reintroduction management (Grueber et al. 2015). In hihi, low

580 immunogenetic diversity is likely to be linked to higher disease susceptibility. There was a severe
581 salmonella outbreak in the population before (loss of 26% of adults in February of 2006, Ewen et
582 al. 2007) and a less severe outbreak after (in September 2011; Alley et al. 2012) the genetic rescue
583 attempt took place. However, a detectable increase in neutral and to some extent functional
584 diversity provides support for developing management strategies to improve establishment and
585 integration of a higher number of immigrants that might lead a stronger/long-lasting effect of higher
586 genetic diversity.

587

588 **Conclusions**

589 There are very few studies where the outcomes of a genetic rescue attempt for the genetic
590 management of a threatened species are assessed over the adequate timeframes. In this study we
591 have shown that 10 years after recreating gene flow through artificial immigration, the genetic
592 benefits for the recipient population were small. Genetic diversity gains and heterosis were limited
593 to the first generation of hybrids and inbreeding accumulated in the subsequent generations as one
594 immigrant female was highly successful. This is due to a high degree of stochasticity in our ability
595 to establish, acclimatise, and integrate immigrants into already established populations. In highly
596 territorial species like the hihi, with a high degree of sexual selection, utilising females/eggs may
597 help reduce mortality and improve establishment success to bypass the impact of strong male-male
598 competition (Heber et al. 2013). As well as improving integration, it is also likely that more frequent
599 attempts to create gene flow between the hihi populations currently established will be required as
600 one event is unlikely to lead to the necessary improvements in viability.

601

602 **Data accessibility**

603 Hihi are of cultural significance to the indigenous people of New Zealand, the Māori, and are
604 considered a taonga (treasured) species whose whakapapa (genealogy) is intricately tied to that of
605 Māori. For this reason, genotypes for hihi will be made available by request on the recommendation
606 of Ngāti Manuhiri, the iwi (tribe) that affiliates as kaitiaki (guardians) for hihi. To obtain contact
607 details for the iwi, please contact the corresponding author.

608

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