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The genetic rescue of two bottlenecked South Island robin populations using translocations of inbred donors

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Populations forced through bottlenecks typically lose genetic variation and exhibit inbreeding depression. 'Genetic rescue' techniques that introduce individuals from outbred populations can be highly effective in reversing the deleterious effects of inbreeding, but have limited application for the majority of endangered species, which survive only in a few bottlenecked populations. We tested the effectiveness of using highly inbred populations as donors to rescue two isolated and bottlenecked populations of the South Island robin (*Petroica australis*). Reciprocal translocations significantly increased heterozygosity and allelic diversity. Increased genetic diversity was accompanied by increased juvenile survival and recruitment, sperm quality, and immunocompetence of hybrid individuals (crosses between the two populations) compared with inbred control individuals (crosses within each population). Our results confirm that the implementation of 'genetic rescue' using bottlenecked populations as donors provides a way of preserving endangered species and restoring their viability when outbred donor populations no longer exist.

1. Introduction

The rapid and widespread decline of many plant and animal populations owing to anthropogenic influences has created an urgent need to address the effects of reduced genetic diversity and inbreeding on population viability. The loss in genetic variation that typically accompanies severe bottlenecks and population fragmentation can decrease fitness, and limit the ability of a population to respond to changing environmental challenges, such as climatic extremes, pollutants and novel pathogens [1–3]. Similarly, inbreeding depression, the reduction in fitness of individuals born to closely related parents, can reduce population viability and increase the risk of extinction of endangered species [4].

Previous attempts to restore genetic variability and mitigate the negative effects of inbreeding in bottlenecked populations have focused on the introduction of outbred individuals or augmentation using captive-reared individuals [5–7]. For example, the introduction of outbred individuals led to a rapid increase in the fitness of wild inbred populations of both greater prairie chickens (*Tympanuchus cupido*, [8]) and European adders (*Vipera berus*, [9]). These approaches rely on the availability of suitable outbred or captive donor populations. However, most endangered species survive only as a series of small, fragmented populations, with each likely subject to loss of genetic variation and increased levels of inbreeding [2].

Theoretical models suggest that crosses between two or more inbred populations should decrease the severity of inbreeding depression in the hybrid offspring, if recessive deleterious alleles in one population become masked by alleles in the second population, and vice versa [10–12]. Experiments with laboratory populations of fruitflies (*Drosophila melanogaster*) and houseflies

(*Musca domestica*) have confirmed that immigration of individuals into inbred lines can lead to rapid improvements in fitness traits such as viability, productivity and survival [13–15]. In one of the few studies to use inbred donors in the genetic rescue of a wild animal, Fredrickson et al. [16] translocated inbred Mexican wolves (*Canis lupus baileyi*) to both captive and reintroduced populations of this species. No outbred individuals were available as donors, as only three captive lineages of Mexican wolves survived from a founding population of seven animals. Despite low genetic variation and fixed deleterious alleles within each lineage, crosses experienced increases in a number of fitness measures [16].

Despite the apparent success of the genetic rescue technique using inbred donors in the laboratory and with semi-captive populations, the general effectiveness of using inbred individuals as donors for the management of wild populations of endangered species is not clear. Captive populations are typically provided with ad libitum food, a benign environment, and limited exposure to parasites and pathogens. Under such relaxed selection, hybrid individuals arising from crosses of inbred lines may survive and reproduce that would otherwise not do so in the wild. This could lead to an overestimate of the benefits of donors to an inbred population, especially if the benefit is small.

The objective of this study was, therefore, to test whether the exchange of individuals between inbred populations reduces levels of inbreeding depression using two severely bottlenecked and isolated South Island robin (*Petroica australis*) populations in New Zealand. A total of 31 females were moved between islands just prior to the breeding season. We then assessed the effects of the translocation by comparing genetic diversity and key fitness traits such as juvenile survival, recruitment, sperm quality, and immunocompetence between hybrid birds (crosses between the two populations) and inbred control birds (crosses within each population).

2. Material and methods

(a) Study populations

In 2008 and 2009, we conducted experimental translocations between two isolated and inbred populations of the South Island robin on Allports and Motuara Islands, Marlborough Sounds, New Zealand. Both robin populations were founded with only five individuals each in 1973, and the founders of each population originated from different parts of New Zealand [17]. We refer to the descendants of the five founders on each island as 'inbred' individuals, even though they might not be the product of recent within-family matings. At the time of the translocation, the two populations had been isolated for nearly four decades (approx. 10 generations, [18]) and were showing signs of inbreeding depression, such as reduced hatching success and problems with immune system function [19,20]. Although the islands differ in size (Allports Island: 16 ha, Motuara Island: 59 ha), the density of robins is similar on both islands (Allports: approx. 60 adult individuals, approx. 3.9 birds per ha; Motuara: approx. 300 adult individuals, appox. 5.1 birds per ha; S.H., personal observation).

A total of 31 female robins were exchanged between Allports and Motuara Islands. In 2008, a total of 15 randomly chosen females were translocated from Allports to Motuara, and 10 females were translocated from Motuara to Allports. Owing to the mortality of some translocated females (see below), an additional translocation of three females from each island was carried out in 2009 to ensure that more than 5 females were recruited into each recipient population. For reference purposes, we refer to the year after the 2008 translocation as '1 year post-translocation', and the second year after the 2008 translocation as '2 years post-translocation', despite the follow-up translocation of six individuals in 2009. The number of individuals chosen for the experimental translocations was selected on the basis of leaving enough non-manipulated individuals on each island to act as controls as well as ensuring that enough females survived the translocation to breed. Only females were translocated in order to minimize disturbance to territorial boundaries that would occur if males were moved, and to ensure that new 'mixed' pairs would form upon release (i.e. by not translocating pairs we forced translocated birds to re-pair with an individual from the other island). Post-release survival was similar on both islands (Allports: $n = 7$, 53.8%; Motuara: $n = 10$, 55.6%). This is lower than is normally found with translocation of this species [18]; however, all surviving females exhibited normal breeding behaviour. The offspring of the mixed pairs (translocated female with male native to the island) are hereafter referred to as 'hybrid'.

To obtain estimates of genetic variability in each island population prior to the translocation (2008), and 1 and 2 years after the initial translocation (2009 and 2010, respectively), we collected blood samples from 658 individuals (greater than 90% of adult individuals and their offspring in each island population) in three consecutive years (before the translocation in 2008, and in 2009 and 2010). Blood samples were collected via brachial venipuncture, and approx. 10–30 µl of blood was stored in 1 ml of Queen's Lysis Buffer (0.01 M Tris-HCl, 0.01 M NaCl, 0.01 M Na-EDTA (pH 7.5), 1% (v/v) *n*-lauroylsarcosine; pH 7.5; [21]) at room temperature. In order to calculate levels of genetic variation, individuals were genotyped at 28 microsatellite loci. A detailed description of the genotyping procedure and the calculation of genetic variability is given in the sections 'microsatellite markers and genotyping' and 'population genetic characteristics' in the electronic supplementary material.

(b) Survival and recruitment

Throughout the breeding seasons of 2009 and 2010, a thorough search was repeated on both islands to determine the survival and recruitment rate of inbred and hybrid individuals. Survival and recruitment on Allports could be determined accurately, as the smaller size and terrain of the island allowed it to be surveyed entirely. The survival and recruitment rates on Motuara, however, represent minimum estimates owing to the larger size of the island, presence of inaccessible areas and possibility of missing individuals even after repeatedly combing the island. We were, however, able to infer the survival and recruitment of some individuals on Motuara through allocating parentage to captured individuals using paternity analysis. In total, we were able to successfully assign the paternity of 358 individuals (see section 'paternity assignment' in the electronic supplementary material).

To determine the accuracy of our survival estimates, we estimated detectability separately for each island using a Bayesian approach within the software WinBUGS [22]. We modelled the probability (p_i) of each individual being identified in year i as a product of both its survival rate and its probability of being observed, given that it was alive that year (re-sighting probability). Recapture histories for the three study years of $n = 90$ individuals on Allports Island and $n = 173$ individuals on Motuara Island were used to estimate values of p_i and 95% credible intervals. A bird that was observed in a given year was recorded as 1 in the recapture history for year i, and as 0 if it

was not observed or dead. These gaps in each bird's individual recapture history were used to derive annual values of p [23]. A total of 100 000 samples after discarding the first 10 000 were used to produce an estimate of the detectability of individuals on each island. Detection rates of robins were very high on both islands with $p_i = 98$ per cent (95% CI = 92–100%) on Allports and $p_i = 92$ per cent (95% CI = 86–96%) on Motuara Island. Thus, we can be confident that our estimates of survival are reasonably accurate.

Robins are sexually monomorphic until they reach sexual maturity [24]. We, therefore, assigned the sex to 267 robins banded as nestlings and fledglings, using molecular techniques in order to test whether there was a difference in survival to adulthood between the sexes (see section 'molecular sex determination' in the electronic supplementary material).

(c) Sperm quality

One of the most important determinants of male fertility is the number of morphologically normal sperm [25–28]. This is because morphologically abnormal sperm are likely to suffer from reduced velocity or compromised direction of movement (midpiece and tail normalities; [29]), or are unable to penetrate the ovum (acrosomal dysfunction; [30,31]). In passerine birds, the sperm midpiece is thought to consist of a single mitochondrial helix that is coiled around the flagellum ([32,33]; but see [34]) and provides the energy for movement [35]. It is, therefore, possible that gross abnormalities in the size of the midpiece (e.g. partial or complete aplasia) restrict energy production, swimming speed or the lifespan of spermatozoa [36,37]. Reduced midpiece length could also negatively affect fertilization success or competitiveness, as it results in a decrease in the quantity or size of mitochondria contained in each spermatozoon [38], and hence in reduced power output and swimming velocity [35,39]. A positive correlation between midpiece length and fertilization success has indeed been found both *in vitro* [40] and *in vivo* [41]. Midpiece length and abnormalities in midpiece structure have been found to be highly heritable in both mammals and birds [38,42].

To test whether there is a difference in sperm abnormalities and morphometry between inbred and hybrid birds, we collected sperm samples from males in reproductive condition in 2009 and 2010 using the gentle massage technique [43]. Both inbred and hybrid individuals were sampled in both years. Samples were stored in 10 per cent formalin until analysis in the laboratory. To estimate the proportion of abnormal sperm, 10 fields of view were randomly chosen per sample and sperm were counted and scored for abnormalities (mean number of sperm scored $\pm$ s.d. = 104 $\pm$ 77, range = 2–240, $n_{\text{samples}} = 33$). Sperm morphology was categorized either as normal, or with structural abnormalities in the various sperm components (head, midpiece and tail). In samples with very low sperm counts (less than 50 sperm, $n_{\text{individuals}} = 10$), all detected sperm were scored for abnormality.

Owing to its importance in fertilization success, we used sperm midpiece length (measured to the nearest 0.1 µm) as the morphometric trait of interest in this study. To obtain morphometric data, sperm were photographed using a Leitz Laborlux S microscope with a Spot Insight QE video camera at 250× magnification. Total length, head length, midpiece length and tail length were measured using LEICA IM50 v. 4.0 software (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). We calculated the number of sperm that would have to be measured in order for the sample to reflect within-male variation with 95% accuracy following Calhim et al. [44]. Based on the results of the accuracy estimates, which revealed high intra-male variation in sperm morphometry, a minimum of 15 sperm per male was measured whenever possible (see the electronic supplementary material, figure S1). All sperm measurements and abnormality screens were conducted by S.H. and blind as to the identity of the bird.

(d) Phytohaemagglutinin assay

In March 2010, 19 1-year-old South Island robins including 10 inbred and nine hybrid birds were caught and their immune systems challenged using the phytohaemagglutinin (PHA) immune test [45]. This assay consists of subcutaneously injecting an immunostimulant (the kidney bean lectin PHA) into an individual's wing web (patagium), and measuring the inflammatory response in form of the extent of the swelling at the injection site after a standardized interval, usually 24 h ([45,46]; but see [47,48]). In response to PHA, T lymphocytes proliferate and stimulate the activation of macrophages, heterophils, B lymphocytes and basophils [46,49]. The dense infiltration of leucocytes in the postcapillary venules causes inflammation at the injection site [49] that can then be used as a general index of one aspect of cell-mediated immunity [46].

Prior to capture, the birds were fed several mealworms (*Tenebrio molitor*) to ensure that they would not dehydrate during the holding period. A fresh PHA suspension was made each day by mixing 5 mg of PHA-P (Sigma, USA; Lot no. L-8754) with 1 ml of pyrogen-free phosphate-buffered saline in a sterile 1.5 ml Eppendorf vial to ensure that the solution was not contaminated (5 mg is the amount needed for 20 birds). Patagium thickness was measured three times immediately prior to injection using a digital micrometer (Mitutoyo, 395–371 Tokyo, Japan). The micrometer was set back to zero and the wing closed in between measurements to ensure independence of measurements. The patagium was then sterilized with a cotton swab dipped in ethanol. A 50 µl aliquot of the 5 mg ml^{-1} PHA suspension was drawn into a sterile $27\frac{1}{2}$ gauge syringe. After ensuring there were no air bubbles in the suspension, it was injected subcutaneously into the left patagium of each bird. The birds were then kept overnight for a period of 14 h in individual holding cages fitted with a perch, and with ad libitum access to mealworms and water. Owing to the required confinement of individuals over the experimental period, we conducted the immune challenge in the post-breeding season, as adults could be retained in captivity without affecting breeding activities. We chose the 14 h overnight holding period in order to minimize the risk of interfering with territory loss caused by the prolonged absence of the territory holder, and taking into account the daylight hours at that time of the year. Navarro et al. [47] and Møller & Cassey [48] found no significant increase in the response to PHA after a period of 6 h, hence, justifying the modification of Smits et al.'s [45] original protocol by reducing the holding time and thereby the stress imposed on the birds ([50]; but see [51]). After 14 h, the measurements of patagium thickness at the injection site were repeated as performed prior to injection, and the birds were subsequently released into their respective territories. The cell-mediated immune response was calculated as the difference between mean pre- and post-injection measurements of patagium thickness. All injections and measurements were conducted by S.H. to ensure consistency of the sampling method. To minimize measurement bias, the experiment was conducted in a semi-blind fashion in that the birds were handled by an assistant and passed to S.H. with a piece of cloth covering the colour identification bands and hence concealing the status of the bird (inbred or hybrid).

3. Results

On both islands, hybrid birds had significantly lower levels of homozygosity by locus (HL), compared with inbred

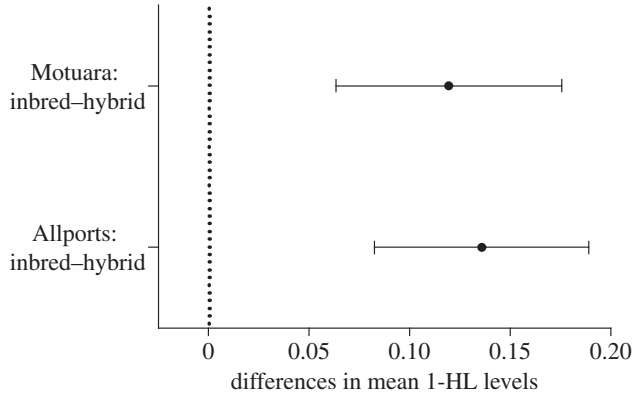

Figure 1. Effect size estimations of pair-wise comparisons of mean level of HL between inbred and hybrid individuals and their 95% confidence intervals (CIs). Differences are significant if the 95% CIs do not include 0.

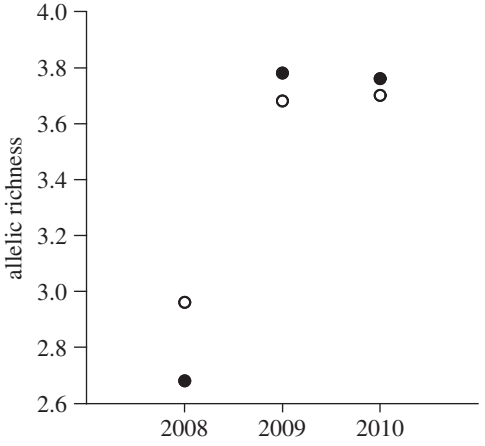

Figure 2. Changes in allelic richness over time in two island populations of South Island robin before 2008 and after 2009–2010 the reciprocal translocations (filled circles, Allports Island; open circles, Motuara Island)

birds (i.e. significantly higher levels of heterozygosity). For ease of interpretation, we present levels of heterozygosity as 1-HL, so that higher values of 1-HL correspond to higher levels of heterozygosity. On Allports Island, 1-HL in inbred birds had a mean $\pm$ s.e. of 0.582 $\pm$ 0.01 ($n = 128$), whereas hybrids averaged 0.718 $\pm$ 0.02 ($n = 41$; one-way ANOVA: effect size = 0.136, 95% CI = 0.082–0.189, $p < 0.0001$; figure 1). Similarly, mean 1-HL of robins on Motuara Island was lower in inbred (0.543 $\pm$ 0.005, $n = 459$) than in hybrid birds (0.663 $\pm$ 0.02, $n = 30$; one-way ANOVA: effect size = 0.12, 95% CI = 0.064–0.176, $p < 0.0001$).

On Allports Island, mean allelic richness was significantly higher 1 year after the translocation (3.78 $\pm$ 0.30, $n = 43$) compared with prior to the translocation (2.68 $\pm$ 0.20, $n = 45$; Wilcoxon signed-rank test: $z = 3.94$, $p < 0.001$), but did not change significantly from 1 year post- to 2 years post-translocation (3.76 $\pm$ 0.30, $n = 50$; Wilcoxon signed-rank test: $z = -2.032$, $p = 0.063$; figure 2). Similarly, mean allelic richness on Motuara Island increased significantly from 2.96 $\pm$ 0.22 ($n = 82$) to 3.68 $\pm$ 0.33 ($n = 70$) following the translocation (Wilcoxon signed-rank test: $z = 3.372$, $p < 0.001$). There was no significant change in allelic richness two years following the translocation (3.7 $\pm$ 0.31, $n = 122$; Wilcoxon signed-rank test: $z = -0.706$, $p = 0.52$; figure 2). Table 1 summarizes the genetic properties of the study populations pre- and 1 and 2 years post-translocation.

Table 1. Genetic properties of the study populations pre- and 1 and 2 years post-translocation. *N* alleles/locus, mean number of alleles sampled per locus; Eff *n* alleles/locus, effective number of alleles per locus; H_{exp}, expected heterozygosity; H_{obs}, observed heterozygosity; F_{IS} and F_{ST} values, $^{**}p < 0.01$, $^{***}p \leq 0.001$. *F*-statistics and effective number of alleles per locus were calculated using GENODIVE v. 2.0 software; all other genetic information was calculated using FSTAT v. 2.9.3

island	status	*n*	*n* alleles/locus	Eff *n* alleles/locus	allelic richness	H_{exp}	H_{obs}	homozygosity by locus (HL)	F_{IS}	F_{ST}		
										pre-translocation	1 year post	2 years post
Allports	pre-translocation	45	2.68	2.15	2.68	0.462	0.511	0.397	−0.092***	—	0.005	0.017***
	1 year post	43	3.79	2.44	3.78	0.518	0.535	0.366	−0.034		—	−0.006
	2 years post	50	3.79	2.55	3.76	0.527	0.554	0.346	−0.052**			—
Motuara	pre-translocation	82	2.96	2.17	2.96	0.489	0.492	0.446	−0.019	—	0.001	0.006***
	1 year post	70	3.68	2.34	3.68	0.521	0.493	0.453	0.054***		—	−0.0001
	2 years post	122	3.75	2.41	3.70	0.527	0.507	0.439	0.036**			—

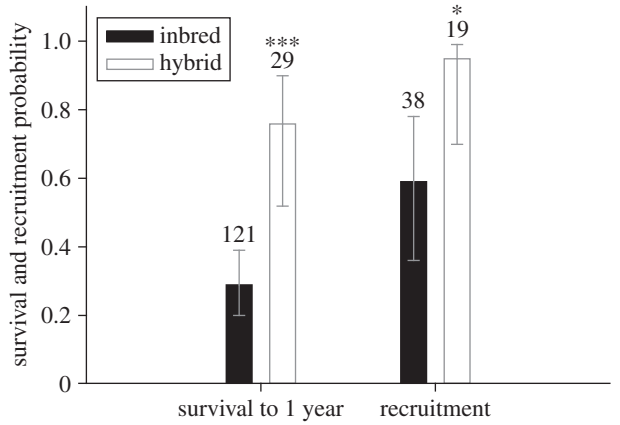

Figure 3. Comparison of survival to 1 year and recruitment between inbred and hybrid individuals; error bars represent 95% CIs; sample sizes (number of individuals) and significance are indicated above each bar (*$p < 0.05$, ***$p < 0.001$).

The observed increases in genetic diversity were accompanied by improvement of several fitness measures. Juvenile survival was significantly higher in hybrid individuals compared with inbred ones: 76 per cent of hybrids survived to age one (95% CI = 52–90%, $n = 29$) compared with only 29 per cent of inbred birds (95% CI = 20–39%, $n = 121$; generalized linear mixed model: $p = 0.0001$). Furthermore, 95 per cent of hybrid individuals alive at age one or two were recruited into the breeding population (95% CI = 70–99%, $n = 19$), compared with only 59 per cent of inbred individuals (95% CI = 36–78%, $n = 38$; generalized linear mixed model: $p = 0.016$; figure 3). As robins reach sexual maturity at age one, greater than 40 per cent of inbred individuals alive at age one or two were either unable to establish a territory, or unable to find a mate. This was only the case for 5 per cent of hybrid individuals.

The proportion of abnormal sperm was significantly lower in hybrid individuals (estimate = 13, 95% CI = 3–44%, $n_{males} = 16$) compared with inbred individuals (estimate = 46%, 95% CI = 22–72%, $n_{males} = 17$; generalized linear mixed model: $p = 0.039$; figure 4). Hybrid males also had significantly longer sperm midpieces—a measure of sperm swimming ability and longevity [36]—(estimate = 94.8 µm, 95% CI = 92.9–96.8 µm, $n_{males} = 16$) compared with inbred males (estimate = 92.5 µm, 95% CI = 90.8–94.2 µm, $n_{males} = 16$; generalized linear mixed model: $p = 0.029$; figure 4).

Finally, hybrid individuals had a stronger immune response, as measured by the swelling of the patagium following challenge with PHA (mean ± s.e.: 0.099 ± 0.02 mm, $n = 9$) than inbred birds (mean ± s.e.: 0.012 ± 0.03 mm, $n = 10$; one-way ANOVA: $p = 0.006$; effect size = −0.09, 95% CI = −0.15 to −0.03; figure 5).

After controlling for factors such as time of capture and sex, there were no significant differences in body mass between inbred and hybrid nestlings, fledglings or adults (all $p > 0.13$). Time to recruitment was not significantly shorter for hybrids (1.11 years, $n = 18$), than for inbred individuals (1.23 years, $n = 22$; $p = 0.736$).

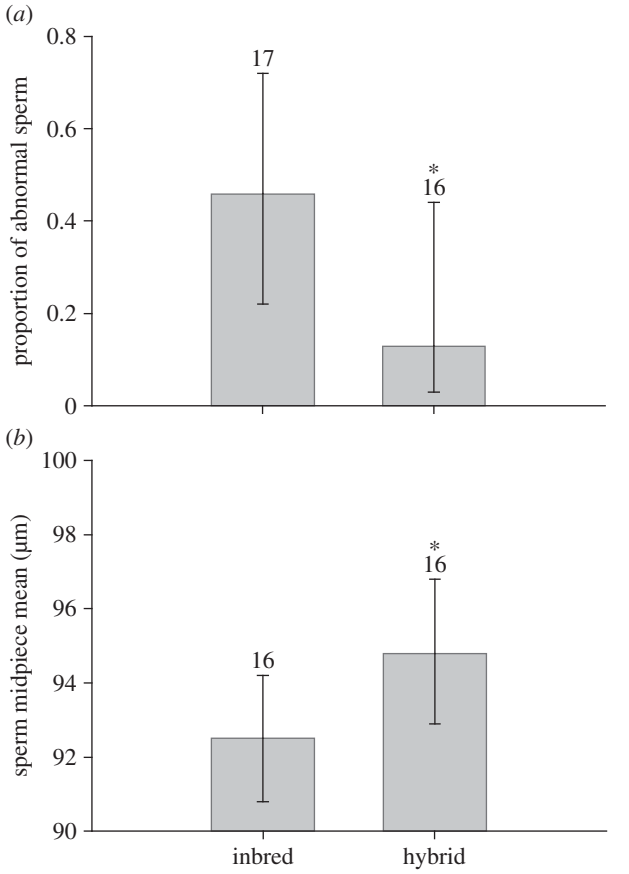

Figure 4. Sperm characteristics of 1- and 2-year-old inbred and hybrid males; error bars represent 95% CIs; sample sizes (number of individuals) and significance are indicated above each bar (*$p < 0.05$). (*a*) Proportion of total abnormal sperm and (*b*) mean sperm midpiece length (µm).

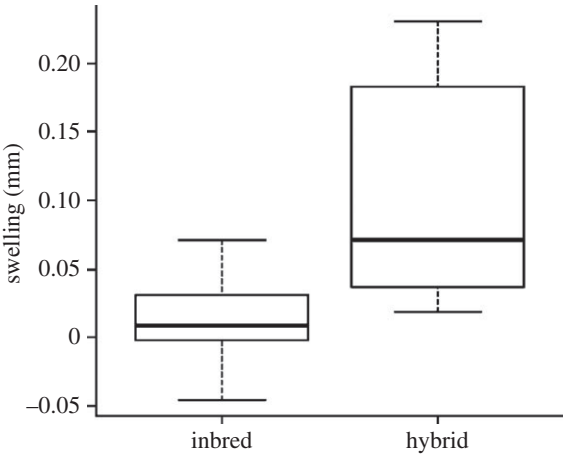

Figure 5. Comparison of swelling of the wing web (mm) between 1-year-old inbred control and hybrid South Island robins following immune challenge with phytohaemagglutinin (PHA); the lower and upper bars represent minimum and maximum values, respectively; the bottom and top of each box show the 25th and 75th percentiles, respectively; the thick black line indicates the median value; sample sizes (number of individuals) and significance are indicated above/within the box plots (**$p < 0.01$).

4. Discussion

Several studies of natural populations have shown that gene flow from outbred populations into inbred populations can reverse the detrimental effects of inbreeding and increase fitness measures over the short-term [8,16,52–55]. Our results demonstrate that—in the absence of outbred donor populations—we were likewise able to significantly increase levels

of genetic diversity using severely bottlenecked populations as donors.

In concert with increased genetic diversity, key fitness measures such as survival and recruitment showed a significant improvement in hybrid individuals compared with inbred ones. Increased survival provides individuals with more reproductive opportunities, and can potentially increase lifetime reproductive success. Furthermore, the proportion of abnormal sperm in hybrid males was on average only a third of that found in inbred males. This is consistent with other studies that have found inbreeding or heterozygosity effects on ejaculate quality [36,56–58]. The significantly longer sperm midpieces of hybrid individuals indicate they may also be more competitive than sperm of inbred males. Inbreeding has been linked with high levels of hatching failure in birds [59], and sperm abnormalities may be one of the causes of this problem.

The significant increase in one aspect of cell-mediated immunity in hybrid robins is consistent with the reversal of an expected decline in host immunity with inbreeding [60,61]. The relationship between the degree of inbreeding and immune system strength is thought to be mediated through reduced genetic diversity at major histocompatibility (MHC) loci in inbred individuals [62,63]. The significantly increased levels of both heterozygosity and allelic richness found in hybrid robins potentially reflect increased genetic diversity at MHC loci. This in turn could explain the stronger response to a novel antigen in the genetically more diverse hybrids (see also [64–66]). Several studies have also found evidence for a positive correlation between immunocompetence and survival (e.g. [67,68]; but see [69]). Our findings of reduced survival and a weaker ability to mount an immune defence in inbred individuals potentially represent one mechanism of how inbreeding depression might affect viability.

Our findings suggest that reciprocal translocations between inbred populations could be a valuable short-term measure to prevent a species from becoming extinct (see also [5–7]). However, such improvements will only be temporary if the focal populations remain small and isolated, as genetic drift and inbreeding will again decrease genetic diversity over the long-term [18,70]. If these conditions cannot be alleviated, then the periodical introduction of new genetic stock may be needed as part of a wider management scheme and complemented with other management strategies aimed at the restoration or conservation of suitable habitat.

The long-term viability of a population on the verge of extinction cannot be guaranteed unless the initial circumstances that led to the decline, such as habitat loss, are reversed. Considering the current rate and scale at which populations of a wide range of species are becoming fragmented and bottlenecked, the implementation of 'genetic rescue' using highly inbred populations as donors nonetheless provides a way of preserving endangered species and restoring their viability when outbred donor populations no longer exist.

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