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Scope for genetic rescue of an endangered subspecies though re-establishing natural gene flow with another subspecies

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Abstract

Genetic diversity is positively linked to the viability and evolutionary potential of species but is often compromised in threatened taxa. Genetic rescue by gene flow from a more diverse or differentiated source population of the same species can be an effective strategy for alleviating inbreeding depression and boosting evolutionary potential. The helmeted honeyeater *Lichenostomus melanops cassidix* is a critically endangered subspecies of the common yellow-tufted honeyeater. *Cassidix* has declined to a single wild population of ~130 birds, despite being subject to intensive population management over recent decades. We assessed changes in microsatellite diversity in *cassidix* over the last four decades and used population viability analysis to explore whether genetic rescue through hybridisation with the neighbouring *L. m. gippslandicus* subspecies constitutes a viable conservation strategy. The contemporary *cassidix* population is characterised by low genetic diversity and effective population size (N_e <50), suggesting it is vulnerable to inbreeding depression and will have limited capacity to evolve to changing environments. We find that gene flow from *gippslandicus* to *cassidix* has declined substantially relative to pre-1990 levels and argue that natural levels of gene flow between the two subspecies should be restored. Allowing gene flow (~4 migrants per generation) from *gippslandicus* into *cassidix*(i.e. genetic rescue), in combination with continued annual release of captive-bred *cassidix* (i.e. demographic rescue), should lead to positive demographic and genetic outcomes. Although we consider the risk of outbreeding depression to be low, we recommend that genetic rescue be managed within the context of the captive-breeding program, with monitoring of outcomes.

Introduction

Genetic diversity is an integral component of biodiversity and is positively linked to the viability and evolutionary potential of species (Frankham 1995; Reed & Frankham 2003; Harrisson *et al.* 2014). Populations are expected to lose genetic diversity at a rate proportional to the inverse of their effective population size (N_e), thus small populations rapidly lose genetic diversity through drift (random loss of alleles across generations) (Wright 1931; Frankham 1996). Since most taxa of conservation concern have experienced strong population contractions, avoiding loss of fitness and evolutionary potential as a result of inbreeding and genetic drift is a key challenge that must be addressed in management plans (Lacy 1987; Pierson *et al.* 2015).

Genetic rescue and genetic restoration can be effective management strategies when a species, subspecies or population is reduced to a small number of individuals (<1000) and is experiencing inbreeding depression and loss of genetic variation (Frankham 2015; Whiteley *et al.* 2015). Genetic rescue is typically defined as the short-term introduction of novel genes into a small population to counteract genetic load (expression of deleterious genes) and primarily describes the rapid increase in fitness (survival and fertility) associated with the injection of novel diversity (Weeks *et al.* 2011). Genetic restoration is defined as ongoing gene flow that both counteracts genetic load in the short-term and aims to increase genetic diversity and evolutionary potential in the longer-term (Hedrick & Fredrickson 2010; Weeks *et al.* 2011). Novel genetic diversity is typically sourced from other more diverse populations/individuals of the same species. A decision on the amount of gene flow to introduce into a recipient population must balance the costs of genetic swamping and potential outbreeding depression (the decrease in individual fitness that may arise when distinct lineages are crossed), with the ongoing risks of inbreeding depression and genetic load (Frankham *et al.* 2011; Weeks *et al.* 2011). For genetic rescue it is not recommended that translocations/introductions from a source population exceed levels equivalent to 20% of the recipient population in the first instance, to prevent 'genetic swamping' (Hedrick & Fredrickson 2010). For genetic restoration, ongoing gene flow equivalent to one genetically effective migrant per generation has traditionally been considered sufficient to alleviate inbreeding depression and genetic load, without swamping locally adapted alleles (Mills & Allendorf 1996; Pimm *et al.* 2006; Weeks *et al.* 2011). However, the classical one-migrant-per-generation rule is based on theoretical assumptions that are likely to be violated in natural systems (e.g. island model of migration, genetic equilibria) and in many conservation scenarios it is likely that higher levels of gene flow (up to ten genetically effective migrants

per generation) would be required to alleviate inbreeding depression (Lacy 1987; Mills & Allendorf 1996; Fernández *et al.* 2008; Sánchez-Molano *et al.* 2013).

Genetic rescue/restoration strategies have been successfully applied in a number of management cases around the world across an array of taxa, including the Florida panther *Puma concolor coryi* and greater prairie chicken *Tympanuchus cupido* in the United States (Westemeier *et al.* 1998; Pimm *et al.* 2006; Hedrick & Fredrickson 2010), the Mexican wolf *Canis lupus baileyi* (Hedrick & Fredrickson 2010), the mountain pygmy possum *Burramys parvus* in Australia (Weeks *et al.* 2015), the Swedish adder *Vipera berus* (Madsen *et al.* 2004), and the South Island robin *Petroica australis* in New Zealand (Heber *et al.* 2013). Despite successful precedents, genetic rescue/restoration strategies remain under-utilised in management of endangered taxa because of concerns about outbreeding depression, and loss of locally adapted alleles though swamping (Weeks *et al.* 2011; Frankham 2015; Hamilton & Miller 2015; Whiteley *et al.* 2015).

The helmeted honeyeater *Lichenostomus melanops cassidix* (hereafter *cassidix*), named for its distinctive erect crown feathers or 'helmet', is a subspecies of the common south-east Australian yellow-tufted honeyeater. *Cassidix* is sedentary, and forms monogamous breeding pairs that occupy and defend territories. Pairs typically produce up to 4 broods each year, with an average of 2 offspring per brood (Smales *et al*. 2009). Individuals begin breeding at approximately 2 years of age and can live as long as 14 years (Smales *et al*. 2009). The average generation time is approximately 3 years (Smales *et al*. 2009). Additional details on the reproductive system of *cassidix* are provided in Appendix A.

Cassidix is critically endangered under national legislation (Environment Protection and Biodiversity Conservation Act 1999). In recent decades *cassidix* has been restricted to low-lying areas of dense riparian vegetation dominated by mountain swamp gum *Eucalyptus camphora*, although historically *cassidix* also occurred in taller, open riparian forest (Smales *et al.* 1990; Blackney & Menkhorst 1993; McMahon & Franklin 1993; Menkhorst 2008). *Cassidix* has suffered population decline throughout the 1900s as a result of increasing degradation and loss of habitat, bush fires and competition from bell miners *Manorina melanophrys* (Smales *et al.* 1990; Menkhorst 2008). Since the 1960s, *cassidix* has been the subject of conservation efforts, including the creation of the Yellingbo Nature Conservation Reserve in 1965 and on-going habitat restoration (Menkhorst 2008). Large forest fires in 1983 wiped out former colonies at Cockatoo and Upper Beaconsfield and reduced the natural

range of *cassidix* to a single wild population located in a small part (<5 km^2) of the Yellingbo reserve (Figure 1) (Menkhorst 2008). By 1989 the Yellingbo population had declined to only 50 individuals (15 breeding pairs) (Menkhorst 2008). Intensive population management commenced in 1989 with the establishment of a captive breeding program at Healesville Sanctuary (Victoria, Australia). Subsequent management actions have included supplementation of the Yellingbo population with captive-reared birds, attempted establishment of new wild populations with captive-bred birds, nest protection, removal of competitors (bell miners) and monitoring (Menkhorst & Middleton 1991; Menkhorst 2008). Since releases commenced from the captive breeding program in 1995, captive-reared offspring have been released at Yellingbo Nature Conservation Reserve in most years (mean = 5.2 ± 6.1 SD per year; range 0-18) (Helmeted Honeyeater Recovery Team (HHRT) records; Appendix Y). Population size has fluctuated over the last two decades, but after the 2015 breeding season, the wild Yellingbo population of *cassidix* was estimated to consist of 130 individuals (23 breeding pairs) with an additional 17 potential breeding pairs in captivity (Appendix B). Six additional release sites were established in the former range of *cassidix* in Bunyip State Park near Tonimbuk and Labertouche North, ~30 km south-east of Yellingbo (Figure 1), where small numbers of captive-bred birds were released most years between 2001 and 2012. However, birds re-introduced into these areas have failed to establish colonies and currently fewer than five birds, including a breeding pair, remain in Bunyip State Park (HHRT records).

Analyses support *cassidix* as a population that is distinct from the other three *L. melanops* subspecies (hereafter *melanops*, *meltoni* and *gippslandicus)* morphologically and genetically in allele frequencies, but not phylogenetically (Pavlova *et al.* 2014). The former range of *cassidix* did not overlap with the distribution of *melanops* (coastal New South Wales) or that typically recognised for *meltoni* (widespread across eastern Australia inland of the Great Dividing Range). However, *meltoni* has been recorded in parts of the historical range of *cassidix*, including Yellingbo, especially as seasonal visitors during drought years (Blackney & Menkhorst 1993); there have been no records of *meltoni* breeding with *cassidix* and in most instances *meltoni* individuals have left Yellingbo by the beginning of the breeding season (HHRT records). The former range of *cassidix* abutted and probably overlapped with the distribution of *gippslandicus* (distributed through Gippsland in eastern Victoria; Figure 1), the subspecies which is the most phenotypically similar to *cassidix* (Wakefield 1958). Although *cassidix* and *gippslandicus* currently occupy different habitats (*gippslandicus*, is

more associated with taller, riparian open-forest habitat, and cassidix is restricted to lowland swamp forest), it is unclear whether this has been the case historically (Blackney & Menkhorst 1993). Multi-locus coalescent analysis suggests that *cassidix* and *gippslandicus* diverged from a common ancestor 4 - 281 thousand years ago, but may have continued to experience some natural gene flow post-divergence (Pavlova *et al.* 2014). Although *gippslandicus* has not been recorded in Yellingbo, it occurs in Bunyip State Park where captive-bred *cassidix* have been released (Figure 1). Several natural breeding attempts between *cassidix* and *gippslandicus* in lowland swamp habitat regenerating after being burnt by wildfire in 2009 at Bunyip State Park (from both combinations of sexes-by-subspecies) were observed to have resulted in fledged offspring, suggesting genetic compatibility between the two subspecies (HHRT unpublished data).

Despite an intensive recovery program, the *cassidix* population remains very small and critically endangered (Garnett *et al.* 2011). Substantial resources have been allocated to the management of *cassidix* through the implementation of a National Recovery Plan to promote the future persistence of the subspecies (Menkhorst 2008). A key objective of the recovery plan is to maintain genetic diversity and evolutionary potential of *cassidix.* Explicit performance criteria related to genetic management are that there be no decrease in heterozygosity (relative to levels at the onset of the plan), that there be no evidence of inbreeding depression in captive or wild populations and that, at any point in time, 95% of the heterozygosity in the wild population be maintained in the captive population (Menkhorst 2008). Data on inbreeding depression in *cassidix* are very limited, although some evidence of lower egg fertility in captive, relative to wild, *cassidix* has previously been reported (6/19 captive eggs were infertile, whereas none of a small sample of 4 wild eggs were infertile; Hemmings *et al.* 2012).

Here, we assess genetic variation at twelve microsatellite loci in samples of *cassidix* spanning the last four decades and explore the potential for genetic rescue and restoration of extant cassidix through the introduction of low levels of hybridisation with the neighbouring *gippslandicus* subspecies. Our study addressed three key questions. First, is there evidence that genetic drift and/or inbreeding has affected the genotypic composition and eroded genetic diversity in the wild Yellingbo *cassidix* population from pre-1990 to 2013? Second, is there evidence of past natural gene flow from *gippslandicus* into the *cassidix* gene pool, and if so, has the extent of gene flow between the subspecies declined relative to pre-1990 levels? Third, would introducing gene flow between *gippslandicus* and *cassidix* improve the

likelihood of population persistence of *cassidix*? Incorporating data from intensively monitored wild and captive populations and genetic information into population viability models, we assess the likely effects of a range of supplementation-based management scenarios on key population parameters related to viability. We discuss whether the relevant goals of the National Recovery Plan for the Helmeted Honeyeater are being met and make recommendations concerning the ongoing management of *cassidix*.

Methods

History of the cassidix *captive breeding program*

The captive breeding program was established in 1989 primarily to produce birds for release to the wild. Initially, nestlings were removed from wild nests and hand reared. Later, clutches of eggs were removed from wild *cassidix* nests and placed into *gippslandicus* nests in captivity (although pairs of *gippslandicus* have been used as foster parents in the captive breeding program they have never been bred with *cassidix* in captivity). Based on advice from the studbook keeper, the captive population has been periodically supplemented with additional targeted birds taken from the wild. In October 1992 a total of 21 *L. m. cassidix* (out of a captive population of 23) died following an accidental toxic dose of vitamin D3 delivered as a dietary supplement. The task of rebuilding the captive population began in the following breeding season (1993/94) using the same cross-fostering technique and targeting nests of pairs that had already contributed to the wild population (i.e. had produced independent young at some point). Under this strategy during the 1993/94 breeding season four two-egg clutches were cross-fostered, resulting in six independent captive young. In subsequent years, management policy changed to rebuild the captive population as quickly as possible, so the restriction on target pairs was removed. This policy switch was influenced by the low breeding success at Yellingbo the previous year combined with the lack of success of wild-wild translocations undertaken over the previous two years, which heightened the importance of the captive colony.

Current captive management

Since around 2005, the size of the captive population has been relatively stable, between 28 and 32 adult birds (14 – 16 potential breeding pairs). Breeding during this period has been managed using a studbook and pedigree-derived values of mean kinship (MK) generated for all individuals (MK is the average co-efficient of kinship of a bird to each living, non-founder

other bird in the pedigree; Lacy 1995). The aim of the program is to minimise genetic change by maximising breeding contribution across all adult birds. This has been done using a number of strategies that have been progressively adjusted over time. To maximise breeding opportunity and output, all birds are paired in a breeding environment. Pairs are decided based on individual MK values, and birds with the lowest mean kinship to the overall managed population are prioritised for the best breeding opportunities, as their alleles are most at risk of being lost to the next generation (Ballou & Lacy 1995). As well as MK, individual breeding history and temperament are considered when pairing to try to ensure contribution. At the beginning of each breeding season, MK is recalculated and birds are re-paired. Because pairs can realistically produce as many as four clutches within a breeding season, re-pairing also takes place within a breeding season to reduce the likelihood that a small number of successful pairs will dominate the gene pool. As needed, eggs or chicks are exchanged between the wild and captive population, and releases are managed to avoid the release of full siblings to the same wild site within Yellingbo.

Sampling

DNA was extracted from blood or tissue samples of: 98 captive (born in captivity) and 147 wild (born at Yellingbo) *cassidix* collected between 1990 and 2013; 80 wild *gippslandicus* collected between 1989 and 2000; and two *cassidix/gippslandicus* hybrids sampled from a *cassidix* reintroduction site in Bunyip State Park in 2011 (Figure 1) (details in Appendix C). To explore trends in genetic diversity metrics in the Yellingbo *cassidix* population, we partitioned the wild *cassidix* samples into five different time periods according to each bird's hatch year (or estimated hatch year) (Table 1). The number and width of time intervals were chosen to ensure that each time period was represented by adequate numbers of both captive and wild individuals. Given a generation time of approximately 3 years (Smales *et al.* 2010), each time period (with the exception of 1993-2003, where samples were limiting) represented approximately 1-2 generations. The same time periods were used to partition captive *cassidix* samples, except the earliest time period for captive samples was 'pre-1993 captive', which included wild-caught nestling *cassidix* originally used to found the captive breeding program in 1989. Almost all of the original founding birds from the 'pre-1993 captive' sample died from an accidental excess of vitamin D dietary supplement in October 1992 and were replaced with new individuals. Thus, the genotypic composition of the captive breeding population is expected to differ between the 'captive pre-1993' and 'captive 1993-2000' samples. Microsatellite genotypes for 12 loci were used in analyses described below (details

in Appendix D). The locus Mcyμ7 was monomorphic in the *cassidix* sample and so was only included in analyses that also included *gippslandicus*. Because the two *cassidix/gippslandicus* hybrids were sampled from a re-introduction site and not from Yellingbo, they were only included in the STRUCTURE analysis to test whether they appeared as genetic hybrids.

Is there evidence that genetic drift has affected the genotypic composition and eroded genetic diversity in the wild cassidix *Yellingbo population?*

We used a range of metrics to examine changes in genetic diversity in the wild Yellingbo *cassidix* population from pre-1990 (before the establishment of the captive breeding program) through to 2013. Mean allelic richness across loci (*AR*; a measure of allelic diversity standardised to the minimum sample size) was calculated in FSTAT 2.9.3 (Goudet 2001). Mean unbiased expected heterozygosity across loci (H_e) was calculated in GENALEX 6.5 (Peakall & Smouse 2012). Since a key objective of the National Recovery Plan to maintain wild genetic diversity in the captive population (specifically that 95% of wild heterozygosity be maintained in the captive population), we also calculated AR and H_e for the captive population sample in each time period. To identify specific alleles that may have been lost from the population through drift, we calculated allele frequencies for captive and wild population samples for each time period using GENALEX. As small populations lose genetic diversity through drift, individuals are expected to become more genetically similar. To assess whether average relatedness among individuals has increased over time, mean pairwise relatedness (*R*, Queller and Goodnight 1989) among wild *cassidix* individuals for each time interval was calculated in COANCESTRY 1.0.1.5 (Wang 2011). We chose to present estimates based on Queller and Goodnight's (1989) *R*, but it was highly correlated with the other estimators calculated by the software (r = 0.7-0.9). To test whether genetic relatedness in recent time periods was greater than Pre-1990 baseline levels of relatedness, we compared observed differences in relatedness values between Pre-1990 and each of the other time periods, with expected distributions of differences in relatedness values generated using COANCESTRY. To generated expected distributions, individuals were randomly shuffled between the Pre-1990 time period and each of the other time periods, with 1000 randomization steps.

Is there evidence for a decline in effective population size of the wild cassidix *population?*

For the wild Yellingbo *cassidix* samples we estimated two measures of effective population size: the effective number of breeders (N_{eD}) (related to inbreeding and reflecting the parental

generation) and the variance genetic effective size (N_{eV}) (related to allele frequency changes through drift and reflecting the progeny generation) (Waples 2005). Estimates of N_{eD} and N_{eV} were obtained using a single sample LD method (Waples 1989; Waples & Do 2008) and a temporal method, respectively, as implemented in NeEstimator 2.01(Do *et al.* 2014). Because the methods implemented in NeEstimator are sensitive to small sample sizes, samples from 1990-1992 were combined with samples from 1993-2003 and samples from 2004-2009 were combined with samples from 2010-2013 (Table 2). For the single sample method, N_{eD} was estimated using the monogamous mating model. For the temporal method, N_{eV} was estimated between all pairs of sampling periods using the method of Jorde and Ryman (2007), assuming generation time of 3 years (Smales *et al.* 2010), with individuals sampled under Plan I, assuming a census size of 100 (Appendix B). For both methods, alleles with a frequency below 0.02 were removed from the analysis to reduce bias (P_{crit} = 0.02) (Waples & Do 2008). Because our samples will include several overlapping generations, our estimates of N_{eD} and N_{eV} will be downward biased due to Wahlund effect (Waples *et al.* 2014). However, because all samples are similarly affected by overlapping age structures, we expect estimates of N_{eD} and N_{eV} to be biased in the same direction and thus comparable across samples. To calculate the adjusted effective number of breeders (N_b) and effective population size per generation (N_e), we applied a correction for biases of age structure using the method outlined in Waples *et al.* (2014; assuming: age of reproductive maturity = 2 years, reproductive lifespan = 10 years, and variation in age-specific fecundity = 0.13; Appendix A). In addition to the methods implemented in NeEstimator, changes in mutation-scaled effective population size between a subset of samples from pre-1990 and post-1995 were also estimated using MIGRATE-N (see below).

Bayesian clustering (STRUCTURE) was used to assess changes in the genotypic structure of *cassidix* over time (Pritchard *et al.* 2000; Falush *et al.* 2003). STRUCTURE was run on *cassidix* only genotypes for *K* values 1-10 using the admixture model with correlated allele frequencies. Twenty replicate runs of 3 x 10^6 Markov Chain Monte Carlo (MCMC), after an initial burn-in period of 10^6 repetitions, were performed for each value of *K*. Results were summarised using STRUCTURE HARVESTER v6.93 (Earl & Vonholdt 2012). The most likely number of clusters (*K*) was selected using the Evanno et al. (2005) Δ*K* method, which finds the point of greatest change in the distribution of LnP(D). Cluster probabilities were averaged over the twenty runs for the most likely value of *K* using the greedy algorithm and

1000 random input orders implemented in CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007). Results were visualised in DISTRUCT (Rosenberg 2004).

Is there evidence of past natural gippslandicus *contribution to the* cassidix *gene pool and has the extent of gene flow between the subspecies declined relative to pre-1990 levels?*

We hypothesise that the range reduction of *cassidix* to a single primary population where *gippslandicus* does not occur (Yellingbo) may have reduced the genetic contribution of *gippslandicus* to the *cassidix* gene pool relative to pre-1990 levels. We used two complementary approaches, STRUCTURE and MIGRATE-N v 3.6.11 (Beerli & Felsenstein 1999, 2001; Beerli & Palczewski 2010), to explore, indirectly and directly, changes in gene flow from *gippslandicus* to *cassidix* through time.

STRUCTURE was run using genotype data from both subspecies to assess the genetic distinctiveness of, and levels of admixture between, *cassidix* and *gippslandicus* through time. Although STRUCTURE results are presented with individuals grouped according to whether they were captive- or wild- born and their estimated hatch year, this information was not used by the STRUCTURE clustering algorithm. Allele frequency changes and loss of genetic diversity through bottlenecking and genetic drift are expected to lead to genetic differentiation among small, isolated population units (Frankham *et al.* 2010). We explored whether the genetic distinctiveness of contemporary *cassidix* has increased over time, as would be expected from a reduction in gene flow between *gippslandicus* and *cassidix*. STRUCTURE was run using genotypes of all *cassidix* and *gippslandicus* individuals, including the two hybrid individuals, using the same settings as described above.

Bayesian coalescent modelling in MIGRATE-N was used to quantify directly the direction and magnitude of gene flow from *cassidix* to *gippslandicus* and vice-versa for two time periods: (1) pre-1990; and (2) post-1995. We compared results between these two periods to explore whether gene flow between the two subspecies has been reduced relative to pre-1990 levels. For each time period, MIGRATE-N model estimated four parameters: mutation-rate (μ) -scaled effective population sizes ($\Theta = 4N_e\mu$) for *cassidix* (Θ_c) and *gippslandicus* (Θ_g) and mutation-rate (μ) -scaled migration rates ($M = m/\mu$) from *cassidix* to *gippslandicus* ($M_{c\rightarrow g}$) and from *gippslandicus* to *cassidix* ($M_{g\rightarrow c}$). Each time period was represented by a subset of twenty randomly-chosen wild individuals of each subspecies owing to the computational intensity of the procedure. The *cassidix* pre-1990 sample consisted of wild

birds born prior to the establishment of the captive breeding program (pre-1990) and the post-1995 sample consisted of wild birds born after 1995 (see Appendix E). For *gippslandicus* most individuals were selected from the locations closest to Yellingbo (Reefton and Starvation Creek; Figure 1) with additional samples from Wellington River and Nowa Nowa (Figure 1); spatial overlap of *gippslandicus* sampling distributions between time periods was good (Appendix E). MIGRATE-N was run using the Brownian approximation to the step-wise mutation model. We set Uniform priors on both Θ and M (U[0-50] and U[0-100], respectively). Four heated chains were run using default (or recommended) temperatures, auto-tune option was implemented (details in Appendix F). We ran 50 replicate chains for each locus, recording samples every 250 steps for a total of 5000 samples per replicate chain. The first 2.5×10^6 steps of each replicate chain were discarded as burn-in steps. Convergence was assessed by examining the posterior distribution of parameters across loci, and ESS values (Appendix F). Posterior probability distributions for the parameters from MCMC simulations were used to estimate the posterior probabilities that Θ_c, $M_{c \to g}$ and $M_{g \to c}$ had declined between the two time periods (R scripts are provided in Appendix F).

Simulating re-introduction of gene flow with gippslandicus *on* cassidix *key population parameters related to population viability*

To explore the predicted effects of re-introducing gene flow from *gippslandicus* on key *cassidix* population parameters related to population viability, we simulated a range of potential management scenarios using the population viability analysis software VORTEX 10.1 (Lacy & Pollak 2015). VORTEX simulates the effects of deterministic forces, as well as demographic, environmental and genetic stochasticity on key population parameters, including population size, time to extinction, allelic diversity, heterozygosity and inbreeding. Six different management scenarios (Table 4) were run using the parameters outlined in Appendix A. Scenario 1 represented the 'do-nothing' management scenario, with no supplementation of the *cassidix* population (Table 4). For management scenarios 2-6, supplementation of the current wild *cassidix* population was simulated with the annual addition of captive *cassidix* (i.e. to represent the current management strategy of demographic rescue from a genetically similar source via releases from the captive breeding program) and/or two different levels of assisted periodic migration from *gippslandicus* (i.e. to represent genetic rescue/restoration from a genetically divergent source) (Table 4). Supplementation with *gippslandicus* was performed every three years to represent gene flow on a per-

generation time-scale, allowing comparison with MIGRATE-N estimates (assuming a generation time of approximately three years; Smales *et al.* 2010). To assess the potential for genetic rescue to have rapid positive impacts on the *cassidix* population, each scenario was run for a period of 50 years, and model outputs for each year were averaged across 500 replicate runs. Eleven microsatellite loci were modelled and were subject to mutation with a rate of 10^{-4}. The wild *cassidix* population was simulated using allele frequencies from the most recent wild *cassidix* population sample (2010-2013). The *gippslandicus* source population for translocations was simulated using allele frequencies from the most recent *gippslandicus* population sample (1994-2000). Because in VORTEX supplementation of additional *cassidix* (i.e. from the captive-breeding program) would be by default via new, unrelated individuals with novel genetic diversity (which would unrealistically overestimate the amount of genetic diversity being added to the population), we simulated a second *cassidix* population with identical population parameters (except for a relaxed mortality rate; see Appendix A) and allele frequencies to the first to act as a more realistic source population for supplementation of the wild *cassidix* population. Inbreeding depression is modelled in VORTEX as the number of lethal equivalents per diploid individual (6.29 in our case; see Appendix A. The percentage of inbreeding depression that is due to recessive lethal alleles (50% in our case; see Appendix A) is modelled by assigning each founder individual unique recessive lethal alleles at loci that are internal to the VORTEX model (i.e. independent of the loci described above). Inbred descendants with two copies of the same recessive allele are killed before they can reproduce.

Results

Is there evidence that genetic drift has affected the genotypic composition and eroded genetic diversity in the wild cassidix *Yellingbo population?*

Genetic data suggest there has been a loss of genetic diversity (number of alleles, allelic richness, heterozygosity) in the wild *cassidix* population since pre-1990 (Table 1; Figure 2). Allelic richness and expected heterozygosity in the 2010-2013 wild *cassidix* population sample declined to around 84% and 90% of pre-1990 levels, respectively. Heterozygosity also declined in the captive population (Figure 2). Eleven alleles (typically minor frequency alleles: frequency <0.05) appear to have been lost from the population, as they were absent from the 2004-2009 and 2010-2013 captive and wild *cassidix* samples (Table 1; details in Appendix G). Another allele (BMC2_189) was sampled up to 2004-2009, but not in 2010-

2013 (Table 1; details in Appendix G). Mean pairwise relatedness values (Queller and Goodnight's *R*) among wild *cassidix* in each time period increased from -0.05 in the wild pre-1990 population sample to 0.06 in the 2010-2013 population sample (Figure 2). Levels of relatedness significantly ($P< 0.05$) increased in the last two time periods (2004-2009; 2010-2013), relative to Pre-1990 baseline levels.

Is there evidence for a decline in effective population size of the wild cassidix *population?*

There was a clear trend of declining contemporary effective population size over time, with estimates of N_{eD}, $N_{b,}$ N_e and N_{eV} very low (<50 for the most recent time period) (Tables 2 and 3). Large 95% confidence intervals around the single sample N_{eD} estimate for the Pre-1990 sample may have reflected smaller sample sizes for this time period (N=25), thus we urge caution in the interpretation of the estimate for this time period (Table 3) (Tallmon *et al.* 2010). Because the temporal method is based on changes in allele frequencies over time, it relies on there being sufficient time between the two samples for drift to change allele frequencies. Thus, weaker effects of drift in earlier time periods (due to larger effective population sizes) may explain the unrealistically high estimates of N_{eV} between Pre-1990 and 1990-2003 (Table 3). MIGRATE-N analysis estimated ~56% decline in wild *cassidix* mutation-scaled effective population size from pre-1990 to post-1995 (mode Θ_{c_pre} = 1.88, 95% HPD 0.63–3.13; mode Θ_{c_post} = 0.82, 95% HPD 0.00–1.63; with a posterior probability that $\Theta_{c\_pre} > \Theta_{c\_post}$ = 90%; details in Appendix F).

STRUCTURE analysis on *cassidix* genotypes revealed changes in the genotypic structure of the *cassidix* population over time, consistent with strong shifts in allele and genotype frequencies (including loss of alleles) over time due to genetic drift (Figure 3). According to the delta *K* method, the most likely number of clusters was three (Figures H2 and H3 in Appendix H). However, we present the *K*=2 plot here since it more clearly represents the main patterns of substructure (Figure 3). Most of the substructure appeared to be driven by differences in the genotypic composition (in the sense of random differences in allele/genotype frequencies due to strong founder effects/drift) between the captive and wild populations (especially pre-2004) (Figure 3). Overall, most wild *cassidix* individuals were assigned to the 'white' genetic cluster (mean assignment to white cluster = 0.60; Figure 3) and most captive *cassidix* individuals were assigned to the 'grey' genetic cluster (mean assignment to grey cluster = 0.57; Figure 3). Observed changes in the genotypic composition of the captive population from pre-1993 to1993-2003 (i.e. shift from higher representation of

the white cluster to higher representation of the grey cluster) were consistent with strong founder effect/drift following the effectively complete replacement of the captive population in 1992 (Figure 3). Increasing representation of the grey genetic cluster in the wild post-2004 is consistent with increasing genetic similarity between the wild and captive due to both loss of genetic diversity through drift and releases of captive-bred individuals at Yellingbo (Figure 3; Appendix B).

Evidence that gene flow between gippslandicus *and* cassidix *has declined relative to pre-1990 levels*

STRUCTURE analysis on the dataset containing both subspecies partitioned *cassidix* and *gippslandicus* individuals into two genetic clusters (Figure 4; Appendix I). One genetic cluster included only *cassidix* (grey; Figure 4), while the other had *cassidix* as well as *gippslandicus* individuals (black; Figure 4). The proportion of *cassidix* assigned to the *cassidix*-exclusive (grey) cluster increased over time (Figure 4). Increasing distinctiveness of the more contemporary captive and wild *cassidix* population samples relative to the *gippslandicus* and pre-1990 wild *cassidix* samples is consistent with loss of genetic diversity over time and stochastic changes in allele frequencies through drift. Assignment of *cassidix* individuals to the 'black' cluster could in some instances reflect genetic contribution from *gippslandicus* in previous generations, as was observed for the two known hybrid birds (Figure 4).

MIGRATE-N supported a decrease in gene flow from *gippslandicus* to *cassidix* between the pre-1990 and post-1995 time periods: 4.06 (95% HPD 0.70-11.17) migrants per generation vs 1.03 (0.000-4.16), respectively, with a posterior probability of 95% that gene flow has decreased between the time steps (Appendix F). There was also support for a decrease in the amount of gene flow in the other direction (from *cassidix* to *gippslandicus*) between the pre-1990 and post-1995 time periods: 5.98 (1.60-14.92) migrants per generation vs 3.90 (0.72-9.80), respectively, with a posterior probability of 86% that gene flow has decreased between the time steps. When we compared gene flow within time steps, the posterior probability that gene flow from *cassidix* to *gippslandicus* was larger than the converse increased from 75% to 94% between the two time steps.

Simulating effects of re-introducing gene flow with gippslandicus *on* cassidix *population viability*

Of the six management scenarios simulated, the best demographic and genetic outcomes were achieved using strategies that combined annual supplementation of the wild population with captive-bred *cassidix* (i.e. demographic rescue) and periodic supplementation with *gippslandicus* individuals (i.e. genetic rescue) (scenarios 5 and 6; Figure 5). Under the 'do nothing' management scenario (scenario 1), the mean number of individuals and genetic diversity (number of alleles and heterozygosity) decreased most strongly and inbreeding increased substantially over the 50 year simulation period (Figure 5). Under scenarios 1 and 2, 87% and 7% of simulated populations went extinct after 50 years, respectively. Population size decreased for the scenarios where only 4 *gippslandicus* individuals per generation (and no *cassidix*) were added to the wild population (scenario 3), although the decline was less pronounced than for scenario 1 (Figure 5). The greatest population growth was achieved through supplementation with both *cassidix* and *gippslandicus* (scenarios 5 and 6), but positive population growth was also achieved through supplementation with 8 *gippslandicus* per generation (and no *cassidix;* scenario 4) and *cassidix*-only supplementation (scenario 2). Although higher population growth was achieved under the *cassidix*-only supplementation scenario (scenario 2) than under the two *gippslandicus*-only scenarios (scenarios 3 and 4), genetic outcomes were better under the *gippslandicus*-only scenarios (Figure 5). All six scenarios resulted in loss of alleles, but for all four scenarios where gene flow from *gippslandicus* was introduced (scenarios 3-6), heterozygosity increased, inbreeding decreased and none of the simulation populations went extinct over the 50 year period.

Discussion

Have relevant goals of the Helmeted Honeyeater National Recovery Plan been met?

Despite an intensive recovery program, the helmeted honeyeater population remains very small, low in microsatellite marker genetic diversity, and critically endangered. One of the key objectives of the recovery plan was to maintain genetic diversity and evolutionary potential of *cassidix* (Menkhorst 2008). Meeting this objective requires that there be no decrease in heterozygosity (relative to levels at the onset of the plan) and that there be no evidence of inbreeding depression in captive or wild populations (Menkhorst 2008). Here we show that genetic diversity (allelic richness, expected heterozygosity) has continued to decline over the last two decades despite the implementation of intensive management strategies. There has also been a steady increase in mean levels of relatedness among individuals in the wild population over time (albeit non-significant). Levels of heterozygosity

in the most recent captive population sample represent 93% of the heterozygosity in the corresponding wild population sample, only slightly below the target of 95% set in the National Recovery Plan. Allelic richness was slightly higher in the captive 2010-2013 sample relative to the corresponding wild sample, suggesting that the allelic diversity present in the wild population is well-represented in the captive population.

All effective population size estimates (including our corrected estimate of per generation effective population size) for the most recent wild *cassidix* population sample were consistently below 50. There was evidence of a decline in effective population size over time, which was corroborated by MIGRATE-N estimates of a ~56% decline in mutation-scaled effective population size in wild *cassidix* post-1995. Frankham et al. (2014) recently advocated that the minimum acceptable effective population size to avoid inbreeding depression in wild populations on a short time-scale, and to limit loss of fitness to ≤10% over five generations, is 100. In the longer-term, evolutionary potential (i.e. ability to respond to environmental changes through evolutionary adaptations) is best promoted by maintaining much larger global effective population sizes above 1000 (Frankham *et al.* 2014). Although increasing the *cassidix* local effective population size above 1000 in the short-to-medium term may not be realistic, populations with a local $N_e < 1000$ can still achieve global $N_e > 1000$ if they receive gene flow from other populations, as we are proposing here with *cassidix* and *gippslandicus* (Jamieson & Allendorf 2012).

Loss of genetic diversity in cassidix *as a result of drift may be exacerbated by reduced gene flow from neighbouring* gippslandicus

Estimates of recent gene flow (MIGRATE-N analysis) between *cassidix* and *gippslandicus* are consistent with recent divergence (Pavlova *et al.* 2014). Comparison of MIGRATE-N estimates of gene flow between the pre-1990 and post-1995 time periods suggests that the genetic contribution from *gippslandicus* into *cassidix* has declined by approximately 3.03 migrants per generation. Because no gene flow from *gippslandicus* into *cassidix* is expected from the time *cassidix* was restricted to Yellingbo (where *gippslandicus* has not been recorded), MIGRATE-N estimates of gene flow greater than zero are likely to be reflecting past gene flow. MIGRATE-N estimates of positive gene flow will have arisen because many of the alleles in the *cassidix* population still have an ancestor in the *gippslandicus* population, which likely came into the *cassidix* gene pool before *cassidix* was restricted to Yellingbo. Although MIGRATE-N will partially reflect historical signatures of gene flow (and

so overestimate current gene flow between *cassidix* and *gippslandicus* for the pre-1990 and post-1995 time periods), there was strong support for a reduction in gene flow between the subspecies over time. A decrease in gene flow from *gippslandicus* to *cassidix* is further corroborated by increasing genetic distinctiveness of *cassidix* relative to *gippslandicus* and to the pre-1990 wild *cassidix* population sample over time (Figure 4).

Could re-introducing low levels of gene flow from gippslandicus *save the helmeted honeyeater from extinction?*

The current *cassidix* population is characterised by very low levels of genetic diversity and an effective population size that is well below the threshold that is considered necessary to avoid inbreeding depression on a short term (Frankham *et al.* 2014). The population has responded only modestly to intensive management, and the very limited available data indicate possible inbreeding depression (Hemmings *et al.* 2012). Given this bleak outlook for the future persistence of *cassidix*, an alternative management strategy is required.

VORTEX simulations indicated that without ongoing management actions, the wild *cassidix* population will continue to decline, experience loss of genetic diversity and increased levels of inbreeding and be at high risk of extinction. The best demographic and genetic outcomes were achieved using strategies that combined the current management strategy of annual supplementation of captive-bred *cassidix* (i.e. demographic rescue) with periodic supplementation with *gippslandicus* individuals (i.e. genetic rescue/restoration). Because supplementation with only *gippslandicus* individuals did not lead to strong population growth, our models support the continued implementation of annual releases of captive-bred *cassidix* from the captive breeding program. Continual addition of captive-bred birds would buffer the wild population against stochastic fluctuations, with positive outcomes for growth and persistence (Hufbaur *et al.* 2015). Although supplementation with captive-bred *cassidix* resulted in positive population growth, supplementation of the *cassidix* population with *gippslandicus* (with or without captive-bred *cassidix*) had a stronger positive effect on genetic outcomes than supplementation with captive-bred *cassidix* alone. Our results are consistent with experimental evidence that supplementation with genetically distinct individuals ('genetic rescue') has much stronger positive effects on the size, fitness and evolutionary potential of small populations than supplementation with additional genetically similar individuals ('demographic rescue') (Hufbauer *et al.* 2015). Relatively high levels of exchange occur between the captive and wild populations under the current management strategy

(reflected in very similar genotypic composition since 2004) and, to-date, persistence of *cassidix* in the wild has relied of demographic rescue. However, because VORTEX simulations cannot test for outbreeding depression between the captive and wild populations (e.g. loss of fitness of captive-bred birds in the wild due to adaptation to captive conditions), additional information about the fitness of captive-bred birds relative to wild birds would be useful for assessing the value of demographic releases from the captive breeding program longer-term.

Our study adds additional weight to the recommendation by Pavlova *et al*. (2014) that, within an adaptive management framework (sensu Weeks *et al.* 2011), gene flow between *cassidix* and *gippslandicus* should be introduced at controlled levels, with close monitoring of outcomes (e.g., phenotype, survival and breeding success of hybrids). Although our VORTEX simulations highlight the likely positive effects of genetic rescue/restoration (e.g. reducing inbreeding depression), our simulations cannot test for outbreeding depression. Thus the possibility of outbreeding depression must still be assessed within a risk assessment framework that considers the potential risks of outbreeding and genetic swamping, and the potential risks of inbreeding depression and loss of evolutionary potential if action is not taken (Frankham *et al.* 2011). Risk of outbreeding depression is greatest when one of the four following criteria apply to the populations that are proposed to be mixed: 1) the populations are distinct species; 2) there are fixed chromosomal differences between the populations; 3) there has been no gene flow between the populations within the last ~500 years; or 4) the populations are strongly adapted to different environments (Frankham *et al.* 2011). We argue that none of these criteria are likely to apply in the case of proposed hybridisation between *cassidix* and *gippslandicus. Cassidix* and *gippslandicus* are recently diverged on evolutionary timescales (Pavlova *et al*. 2014) and MIGRATE-N analysis supports recent gene flow between the two subspecies. Genetic compatibility is further supported by the observation of natural hybridization events between *gippslandicus* and *cassidix* individuals at the Bunyip State Park re-introduction sites, where hybrid offspring from both combinations of sexes-by-subspecies have been successfully raised to fledging. These hybridization events occurred in lowland swamp habitat (HHRT unpublished data) despite there being evidence to suggest that *cassidix* and *gippslandicus* may have historically been associated with different habitat types (i.e. lowland swamp habitat compared to taller open-forest habitat, respectively). Given recent hybridization events, likely historic range overlap and records of *cassidix* colonies in open-forest habitat prior to the 1980s, we argue that there is a low risk of outbreeding

depression as a result of adaptation to habitats, especially relative to the risk of extinction if gene flow from *gippslandicus* is not introduced (Blackney & Menkhorst 1993). Nonetheless, we stress that any managed hybridisation be strictly controlled, with ongoing monitoring of potential fitness outcomes and loss of phenotypic distinctiveness. We recommend that introduction of gene flow from *gippslandicus* be introduced, at least initially, as part of the captive-breeding program, to allow greater control of the amount of gene flow, close monitoring of fitness outcomes and detection of any evidence of outbreeding depression. Controlled hybridisation in the captive-breeding program will mean outbreeding depression can be properly tested for before any gene flow from *gippslandicus* is introduced into the wild *cassidix* population.

How much gene flow is enough?

The value of genetic rescue/restoration as a management strategy for improving fitness and evolutionary potential has been demonstrated across a wide range of taxa, but identifying the suitable amount of gene flow to introduce between genetically distinct units in a conservation context is challenging (Frankham 2015; Hufbauer *et al.* 2015). Too much gene flow could create genetically indistinguishable populations and result in loss of unique diversity from the recipient population (Gonçalves da Silva *et al.* 2015). Although guidelines are available for the cases where there has been substantial isolation between source and recipient populations (Hedrick 1995), we are unaware of any study that has examined a case where there is very recent/ongoing natural gene flow. Our recommendation is that gene flow into the captive population should be augmented back to its past levels. Our pre-1990 estimate of *gippslandicus* to *cassidix* gene flow equivalent to 4.06 (0.70-11.17) migrants per generation, combined with results of population viability analysis, suggest that the introduction of ~4 migrants per generation (in conjunction with continued annual releases of *cassidix* from the captive breeding program) should be sufficient to avoid harmful effects of inbreeding depression without exceeding the level under which distinctiveness arose, and more than necessary for the spread of beneficial alleles (Hedrick & Fredrickson 2010; Lowe & Allendorf 2010; Frankham *et al.* 2011; Weeks *et al.* 2011; Pickup *et al.* 2013).

Concluding remarks

We assessed changes in genetic diversity over recent decades in the last remaining wild population of *cassidix*, a critically endangered subspecies of yellow-tufted honeyeater.

Despite intensive management efforts, genetic diversity has continued to decline suggesting that the contemporary *cassidix* population is vulnerable to inbreeding depression and has low ability to evolve in response to changing environments. We found evidence that recent gene flow between *gippslandicus* and *cassidix* in the wild, likely a natural feature of the interaction between these two subspecies, has been disrupted due to the range reduction of *cassidix* to a single location geographically isolated from *gippslandicus*. In light of our findings, we suggest the captive management strategy be adjusted to allow low levels of gene flow from *gippslandicus* into *cassidix* (~4 migrants per generation), in combination with continued annual releases of captive-bred *cassidix*. Although we consider the risk of outbreeding depression as a result of hybridisation between *cassidix* and *gippslandicus* to be low, we stress that any management action should be conducted within the context of the captive breeding program, with appropriate monitoring of outcomes. Adjusting conservation strategies to focus on maintaining and restoring gene flow processes is likely to have strong benefits for promoting long-term viability and evolutionary potential in many threatened taxa, including *cassidix* (Crandall *et al.* 2000; Frankham 2015).

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812 **Data Accessibility**

813 Sampling information and microsatellite genotypes have been submitted to Dryad:
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815 **Author Contributions**

816 All authors contributed to the development of concepts presented here. NM, BQ, MM, ML
817 and PM provided field data and samples. AP, RR, JB and KH conducted genetic screening.
818 KH and AGS conducted analyses. KH wrote the original manuscript. All authors contributed
819 to revisions of the manuscript.

820 Table 1. Sample sizes (N), number of alleles (#Alleles), the proportion of alleles lost in each
821 time interval (i.e. proportion of alleles that were missing from subsequent captive and wild
822 samples) (see Appendix G for additional details), mean allelic richness (AR) and mean
823 heterozygosity (He) for wild Yellingbo *cassidix* population samples in each time interval.

Time interval	N	#Alleles	Prop. alleles lost	AR	He
Pre-1990	25	47	0.02	3.6	0.51
1990-1992	47	46	0.17	3.5	0.51
1993-2003	22	39	0.05	3.2	0.51
2004-2009	17	37	0.02	3.2	0.47
2010-2013	36	37	-	3.1	0.46

Table 2. The effective number of breeders (N_{eD}) was estimated using a single sample method (LD method, P_{crit} = 0.02, CI = JackKnife on loci) implemented in NeEstimator for wild Yellingbo *cassidix* population samples in each time interval. Because the method is sensitive to small sample sizes, samples from 1990-1992 were combined with samples from 1993-2003 and samples from 2004-2009 were combined with samples from 2010-2013. To correct for biases of age structure we used the method outlined in Waples *et al.* (2014) to calculate the adjusted effective number of breeders (N_b) and effective population size per generation (N_e).

Time interval	N	N_{eD}	Lower 95% CI	Upper 95% CI	N_b	N_e
Pre-1990	25	88.0	42.9	543.5	100.0	85.0
1990-2003	69	89.6	60.1	146.9	101.8	86.6
2004-2013	53	42.1	29.3	63.0	47.8	40.6

Table 3. Estimates of variance genetic effective size (N_{eV}) and upper and lower 95% confidence intervals estimated for wild Yellingbo *cassidix* population samples using a temporal method (Jorde and Ryman, P_{crit} = 0.02, CI = Parametric) implemented in NeEstimator. The analysis was run for all comparisons of three time intervals. Because the method is sensitive to small sample sizes, samples from 1990-1992 were combined with samples from 1993-2003 and samples from 2004-2009 were combined with samples from 2010-2013.

Comparison	N_{eV}	Lower 95% CI	Upper 95% CI

Pre-1990 to 1990-2003	485.1	280.4	745.1
Pre-1990 to 2004-2013	62.4	35.1	96.5
1990-2003 to 2004-2013	32.7	18.7	50.6

841 Table 4. Descriptions of VORTEX simulation scenarios. The number and sex of *cassidix* (i.e. from the captive breeding program) and
842 *gippslandicus* individuals supplemented and the intervals between supplementation of the wild *cassidix* population are specified.

Scenario	Description	Number of *cassidix* to supplement	Interval between *cassidix* supplements (years)	Number of *gippslandicus* to supplement	Interval between *gippslandicus* supplements (years)
1.	No supplementation	-	-	-	-
2.	Supplementation with *cassidix* from captive breeding program	3 males + 3 females	1	-	-
3.	Supplementation with 4 *gippslandicus* per generation	-	-	2 males + 2 females	3
4.	Supplementation with 8 *gippslandicus* per generation	-	-	4males + 4 females	3
5.	Supplementation with *cassidix* from captive breeding program and 4 *gippslandicus* per generation	3 males + 3 females	1	2 males + 2 females	3
6.	Supplementation with *cassidix* from captive breeding program and 8 *gippslandicus* per generation	3 males + 3 females	1	4males + 4 females	3

Figure captions

Figure 1. Locations in south-eastern Australia of *cassidix* (black square) and *gippslandicus* (black circles) samples used for genetic analysis. Hollow squares indicate former *cassidix* colonies wiped out during severe bushfires in 1983. Two *cassidix/ gippslandicus* hybrids were sampled at a reintroduction site in Bunyip State Park (black star). Altitude is indicated by grey shading.

Figure 2. Changes in mean allelic richness across loci (A), mean unbiased expected heterozygosity across loci (B), single-sample corrected effective population estimates (C) and Queller and Goodnight *R* relatedness (D) over time in the wild Yellingbo *cassidix* population samples (black). In (A) and (B) allelic richness and heterozygosity, respectively, of captive *cassidix* population samples for each time period are shown in grey. The 95% credible limits around *Ne* estimates are shown in (C). Shaded area in (D) bounds the 95% confidence interval about the null hypothesis of no difference between the baseline Pre-1990 levels of relatedness and each time interval, as determined by permutation.

Figure 3. Summary of results of STRUCTURE analysis (*K*=2) run for captive and wild *cassidix* individuals: plots indicate proportional assignment of individuals (bars) to the 'white' or 'grey' genetic cluster. The dotted vertical line divides captive and wild *cassidix* samples and sampling time periods are indicated on the x-axis.

Figure 4. Summary of results for STRUCTURE analysis run on both subspecies (*K*=2): plots indicate proportional assignment of individuals (bars) to either the 'grey' or 'black' genetic cluster. Thick white lines separate time periods along the x-axis. Two known wild *cassidix/gippslandicus* hybrids are indicated (last two columns in wild *cassidix* plot). Plots represent a single analysis but are split into captive *cassidix*, wild *cassidix* and wild *gippslandicus* for clarity.

Figure 5. Results of six VORTEX simulation scenarios are shown for mean values of: A) number of individuals; B) number of alleles; C) heterozygosity (H_e); and D) inbreeding coefficient (F), calculated each year for a 50 year period across 500 iterations. Simulation scenarios are 1) no supplementation (dark blue); 2) Annual supplementation with captive-bred *cassidix* (red); 3) supplementation with 4 *gippslandicus* individuals per generation (green); 4) supplementation with 8 *gippslandicus* individuals per generation (purple); 5) annual supplementation with captive-bred *cassidix* and addition of 4 *gippslandicus*

875 individuals per generation (black); and 6) annual supplementation with captive-bred *cassidix*
876 and addition of 8 *gippslandicus* individuals per generation (light blue) (additional details in
877 Table 4). A generation was equivalent to three calendar years.