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Genetic drift outweighs natural selection at toll-like receptor (*TLR*) immunity loci in a re-introduced population of a threatened species

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Abstract

During population establishment, genetic drift can be the key driver of changes in genetic diversity, particularly while the population is small. However, natural selection can also play a role in shaping diversity at functionally important loci. We used a well-studied, re-introduced population of the threatened Stewart Island robin (N = 722 pedigreed individuals) to determine whether selection shaped genetic diversity at innate immunity toll-like receptor (*TLR*) genes, over a 9-year period of population growth following establishment with 12 genetic founders. We found no evidence for selection operating with respect to *TLR* diversity on first-year overwinter survival for the majority of loci, genotypes and alleles studied. However, survival of individuals with $TLR4_{BE}$ genotype was significantly improved: these birds were less than half as likely to die prior to maturity compared with all other *TLR4* genotypes. Furthermore, the population frequency of this genotype, at a two-fold excess over Hardy–Weinberg expectation, was increased by nonrandom mating. Near-complete sampling and full pedigree and reproductive data enabled us to eliminate other potential causes of these patterns including inbreeding, year effects, density dependence, selection on animals at earlier life history stages or genome-level association of the $TLR4_{E}$ allele with 'good genes'. However, comparison of observed levels of gene diversity to predictions under simulated genetic drift revealed results consistent with neutral expectations for all loci, including *TLR4*. Although selection favoured $TLR4_{BE}$ heterozygotes in this population, these effects were insufficient to outweigh genetic drift. This is the first empirical study to show that genetic drift can overwhelm natural selection in a wild population immediately following establishment.

Keywords: bottleneck, colonization, conservation, heterozygosity, pedigree, *Petroica*, survival

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Introduction

When unoccupied habitat (e.g. an offshore island or forest fragment) becomes colonized by a few individuals, genetic change relative to the source population occurs at the initial founding event and subsequently as a result of genetic drift while the population remains small (Nei *et al*. 1975). These forces tend to reduce genetic diversity of the population, although the genetic makeup of the descendent population can also be shaped by natural selection if the environment differs from that of the source population (Schluter 2000). If selection pressures are strong, their action on founder diversity can act in opposition to, or in combination with, genetic drift (Haldane 1927; Clegg *et al*. 2002). For example, mechanisms of diversifying selection may maintain genetic diversity in the face of drift if animals with versatile (e.g. heterozygous, *sensu* Lerner 1954) genotypes proliferate (e.g. Bensch *et al*. 2006), whereas in cases where strong directional selection occurs, genetic diversity is expected to be rapidly eroded (Ejsmond & Radwan 2011).

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Much attention has been given to the relative influences of these forces in newly established populations, as they have bearing on several issues not only in evolution but also in conservation biology, including the ability to introduce and recover small numbers of individuals of threatened species in new habitat. The relative effects of genetic drift and natural selection in small founder populations have been the subject of a number of simulation and laboratory-based studies (e.g. Ejsmond & Radwan 2011; Ellison *et al.* 2013). Recent studies have also compared pre- and postbottleneck change in putatively neutral genetic diversity (e.g. microsatellites) to changes in functional diversity (e.g. genes of the major histocompatibility complex, *MHC*), indicating that a combination of genetic drift and selective forces is at play during population bottlenecks z(Piertney & Oliver 2006; van Oosterhout 2009; Radwan *et al.* 2010; Sutton *et al.* 2011). These empirical findings are similar to those obtained from simulation studies (Ejsmond & Radwan 2011), although conflicting results continue to be reported (e.g. smaller loss of functional diversity than neutral: Oliver & Piertney 2012; Strand *et al.* 2012; greater loss of functional diversity: Eimes *et al.* 2011). Certainly, the effects of genetic drift and natural selection in small populations are difficult to disentangle using molecular markers alone (Alcaide 2010; Bollmer *et al.* 2011; Sutton *et al.* 2011; van Oosterhout 2013).

In most studies of selection and genetic drift in natural populations, the influence of drift is quantified using a sample of presumed neutral markers, such as microsatellites (e.g. Agudo *et al.* 2011; Eimes *et al.* 2011; Oliver & Piertney 2012; Taylor *et al.* 2012). In the current study, we take advantage of a complete and reliable pedigree that has been constructed for our study population based on breeding observations and offspring banded in the nest (Townsend & Jamieson 2013a), as well as detailed data of the reproductive success of all breeders and genotypes of their offspring. This detailed knowledge of the contributions of each animal to annual changes in the genetic composition of the population allows us to use pedigree data to generate expectations under chance, thus avoiding tacit assumptions associated with using microsatellites (e.g. selective neutrality, unbiased genome representation, independent assortment among loci).

Recently, Gratten *et al.* (2012) employed extensive pedigree information in a well-studied island population of Soay sheep (*Ovis aries*) to show that changes in the frequency of a particular coat-colour allele were driven by selection, not drift. Our study differs from this previous work in two ways. First, whereas the Soay sheep population was established several decades prior to the study (Overall *et al.* 2005), our island population has been sampled over a period of natural population growth immediately following human-assisted establishment, including genotyping of the 12 genetic founders. This allows us to address the roles of selection and drift immediately following colonization of a new site. Second, unlike the Soay sheep study population, our population is isolated, preventing low rates of immigration or emigration that might mask any effects of drift or selection.

We scored variation at seven innate immunity toll-like receptor (*TLR*) loci, whose genes encode binding proteins that initiate innate and acquired aspects of the immune response (Brownlie & Allan 2011). Although the majority of *TLR* sequence variation is subject to purifying selection (Barreiro *et al.* 2009; Mukherjee *et al.* 2009), several *TLR* genes are thought to evolve under balancing selection (Ferrer-Admetlla *et al.* 2008) and show high levels of diversity in wild populations (Alcaide & Edwards 2011; Bergman *et al.* 2012; Grueber *et al.* 2012). The main technical advantage of using Toll-like receptors (*TLRs*) in studies of nonmodel species is the rarity of gene duplication in derived lineages (Alcaide & Edwards 2011). The more commonly used major histocompatibility complex (*MHC*) is typified by a high degree of copy number variation, especially in passerine birds (Babik 2010; Bollmer *et al.* 2010). High levels of gene duplication in *MHC* can lead to co-amplification of multiple loci during PCR, complicating genotyping and increasing the probability of PCR artefacts (reviewed by Babik *et al.* 2009).

Toll-like receptors are a particularly interesting family of genes to study the immunogenetics of wild populations, as together they play a crucial role in host front-line defence against a wide diversity of pathogens (i.e. bacteria, viruses, fungi; Uematsu & Akira 2008). Variation in *TLR* sequences has also been associated with variation in resilience to infections (Villaseñor-Cardoso & Ortega 2011). In the current study, we do not have cause-of-death information for animals that died, nor specific information about the disease environment of the habitat; therefore, we cannot make specific a priori predictions about the relationship between any *TLR* locus and survival. Therefore, rather than establishing the genetic mechanisms underpinning variation in individual fitness traits (e.g. Acevedo-Whitehouse *et al.* 2005; Gratten *et al.* 2012), our goal is to test predictions centred on population-level processes.

Previous authors have highlighted the important role played by chance in influencing changes in population genetic diversity after colonization (e.g. Kaeuffer *et al.* 2007; Bouzat 2010). Because only strong selection is expected to overcome drift in small populations (Bouzat 2010; Hedrick 2012), we asked whether selection operating on *TLR* diversity would overcome the genetic drift

that occurs during population establishment. The unique advantages of our study system, as described above, allow us to address this question explicitly by examining population growth as it unfolded, correlating survival rates of descendants with their genotypes. Our ability to trace founder representation, mating patterns and genotype transmission as the population grew from 12 genetic founders to 346 adults enables us to execute the first empirical study to distinguish several selective and demographic forces from chance, during the earliest stages of colonization.

Materials and methods

Our analysis proceeded in two major steps: first, we determined whether there was evidence for selection on *TLR* genotypes in the population. Second, we tested whether the magnitude of selection detected was sufficient to outweigh the influence of genetic drift on temporal changes in genetic diversity of the population.

Study population, sampling and genotyping

Our study animal is a nonmigratory forest passerine, the Stewart Island robin *Petroica australis rakiura*. This subspecies is threatened on the mainland of Stewart Island, and therefore a number of individuals were re-introduced to Ulva Island where introduced predators were eradicated (Laws & Jamieson 2010; Townsend & Jamieson 2013a). The study took place over a 9-year period of natural population growth: from the time the robins were translocated and released over two breeding seasons (2000/2001 and 2001/2002) to the end of the 2009/2010 breeding season. All 722 known (banded) birds were included in the pedigree (excluding founders), of which we genotyped 656 (91%).

Natural selection was defined here by differential survival of *TLR* genotypes. Adult robin annual survival is generally high on Ulva Island (approximately 90%; IGJ, unpublished data), so our fitness trait of choice focussed on the birds' most vulnerable period: first-year over-winter survival. Robins are able to breed in the season after they hatched (i.e. aged approximately 1 year), so this measure can also be interpreted as survival to maturity. Systematic monitoring during each breeding season indicates that any birds not sighted each year are likely to have died since the previous season (see Laws & Jamieson 2010 for detailed field methods). The genotyped birds included 96% of all known birds that survived to maturity ($N = 517$, of 538 survivors) and 76% of known birds that died over their first winter ($N = 139$, of 184 birds that died). Of the 9% ($N = 66$) of known birds (survived or died) that were not included, the majority ($N = 58$) were unsampled, and most of these ($N = 34$) were animals that died in the last year of the study (during the winter of 2010). Six sampled birds were excluded from the final molecular data set as a result of poor-quality DNA and/or genotyping. Our final data set included 99% of all known breeders [as defined as pairs that produced at least one nestling, regardless of whether it survived ($N = 246$)].

Blood samples were collected as part of routine monitoring, and DNA was extracted using a standard Chelex protocol (Walsh *et al.* 1991; Casquet *et al.* 2012) for previous studies (Taylor *et al.* 2007; Townsend *et al.* 2012). DNA sample concentrations were standardized to 15 ng/μl and samples genotyped at seven *TLR* loci using a total of 13 SNPs diagnostic for alleles identified in the study by Grueber *et al.* (2012) (Table S1, Supporting information). These SNPs inform the allelic state of approximately 6.4 kb of coding sequence in total (range, 522–1279 bp per *TLR* locus, Table S1, Supporting information). All birds were genotyped for all SNPs in single multiplexes on two Sequenom iPLEX MassARRAY chips (service provided by GenomNZ). SNP genotypes were distilled into allele haplotypes following Grueber *et al.* (2012). *TLR21* exhibited synonymous variants for the SNPs studied here, so two *TLR21* DNA haplotypes were pooled into a single amino acid variant, giving three 'alleles' for this locus (Grueber *et al.* 2012). Due to a high degree of sequence similarity between robin *TLR2A* and *TLR2B* sequences, *TLR2A* could not be genotyped, and only one SNP from *TLR2B* could be genotyped. *TLR2B* exhibited two synonymous variants, expected to be selectively neutral. For all remaining loci, each DNA haplotype translated as a unique amino acid variant.

Tests of selection

For our survival analyses, we defined *TLR* diversity in a number of ways, each of which represents a subtly different potential target of natural selection. First, we used generalized linear mixed effects modelling (GLMM) to estimate the effect of *TLR* heterozygosity on survival, both as a composite metric, and for each locus individually. Composite heterozygosity was quantified using standardized observed multilocus heterozygosity (referred to here as *H*), calculated with the package Rhh (Alho *et al.* 2010) for R (R Core Development Team 2011). We chose this metric as it controls for differing levels of heterozygosity among loci (see Results), as well as any missing genotypes (only 0.3% of the data set). The effect of heterozygosity at individual loci on survival was estimated by fitting separate models for each locus, where heterozygosity was coded as a 0/1 (for homozygote or heterozygote, respectively)

predictor. Aside from heterozygosity predictors, these survival models also included inbreeding coefficient F (calculated from pedigrees, see Townsend & Jamieson 2013a) and 'cohort' (breeding year) as the random factor. Our composite heterozygosity model also included the $H \times F$ interaction, following previous observations in this population by Townsend & Jamieson (2013b) that the effect of microsatellite-based multilocus heterozygosity on survival varied with F. See Methods S1 (Supporting information) section 'Generalized linear mixed modelling' for more details.

Second, it is possible that certain alleles or genotypes affect survival independently of heterozygosity per se. We therefore tested whether allele frequencies of each locus differed between birds that survived vs. died using per-locus F_{ST} (Weir & Cockerham 1984) calculated with FSTAT (Goudet 1995). We tested the influence of genotypes by focussing on those loci with >2 alleles (*TLR4*, *TLR5*, *TLR21*). In this part of the analysis, we followed the approach of Sepil *et al.* (2012) by modelling the effect of each genotype on survival separately, where each individual is coded with a 1/0 predictor indicating presence/absence of the genotype in question. These models all included F and the random factor cohort, as mentioned above. AIC_C values of all genotype models were ranked (following Burnham & Anderson 2002), along with a 'base' model that included only F and cohort. Where a genotype model ranked highly (i.e. was ≥2 AIC_C from the next best model and superior to the base model), we interpreted this as evidence that the genotype in question influenced survival (Sepil *et al.* 2012).

We found evidence that one particular genotype ($TLR4_{BE}$) conferred a survival advantage and was present in excess of Hardy–Weinberg equilibrium (HWE) expectation (see Results). We therefore examined processes other than selection that could lead to deviations from HWE. Birds entered our data set at banding age (approximately 2 weeks old), so possible selection pressures occurring prior to this (e.g. during fertilization, egg development, early nestling stages) were tested by examining whether observed genotype frequencies in offspring deviated from predictions under Mendelian inheritance, given observed parental genotype combinations. We compared annual production of $TLR4_{BE}$ offspring to predictions generated using Monte Carlo simulations (see Methods S1 section 'Prediction of Mendelian frequencies', Supporting information). We also tested whether the excess of $TLR4_{BE}$ individuals could be attributed to nonrandom mating by comparing annual frequencies of $TLR4_{BE}$ to those expected under random mating (see Methods S1 section 'Simulation of random mating', Supporting information).

Predictions under genetic drift

To forecast changes in annual levels of gene diversity expected under genetic drift, we wrote a genedropping simulation (MacCluer *et al.* 1986; Gratten *et al.* 2012) in R (R Core Development Team 2011). As genedropping simulates transmission of alleles through the known pedigree following Mendelian inheritance only, it can be used to predict levels of genetic diversity in the absence of selection while accounting for known pedigree relationships, inbreeding [which has been gradually increasing (Jamieson 2011)], disproportionate reproductive success among individuals (Jamieson 2011) and increasing population size. We used allele frequencies among the living descendants (adults only) to calculate annual gene diversity (D) for each locus using $1 - \sum_{i=1}^{n} p_i^2$ (Hedrick 2000), where p_i is the frequency of the i th allele at a locus. The means and 95% quantiles of $N = 5000$ simulations were taken as our predicted values and compared with equivalent values calculated using the observed *TLR* allele frequency data. The pedigree used for genedropping was 98.1% complete; see Methods S1 section (Supporting information) 'Genedropping' for specific details regarding handling of the small proportion of missing pedigree data. Unless stated otherwise, all analyses were conducted using functions available in the base package of R (R Core Development Team 2011).

Results

Of the 656 genotyped animals, >99% were successfully genotyped at all SNPS for all 7 *TLR* loci, and all were genotyped at ≥3 *TLR* loci. For two unsampled founders and two other unsampled birds, full genotypes could be inferred because of extensive first-degree pedigree relationships with genotyped animals (following Grueber *et al.* 2012), permitting their inclusion in the analysis.

Effects of TLR diversity on survival

There was little effect of composite heterozygosity at seven *TLR* loci on juvenile robin survival [GLMM model-averaged standardized coefficient (± adjusted SE): $\beta_H = -0.300$ (±0.196), relative importance = 0.54, model set provided in Table S2, Supporting information; Fig. 1]. The negative coefficient for H suggests that survivors showed slightly decreased overall *TLR* heterozygosity (Fig. 1), but this effect was estimated with poor precision, as the standard error was large. The final model also included individual inbreeding [where $\beta_F = -0.136$ (±0.198), relative importance = 0.32, Table S2, Supporting information], but no evidence of an

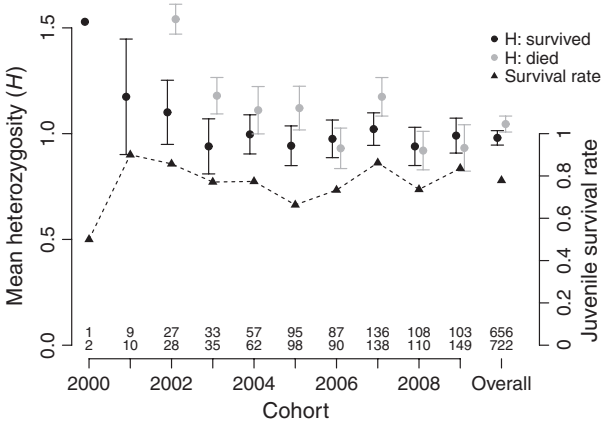

Fig. 1 Mean toll-like receptor (*TLR*) *H* of juvenile robins that survived (black circles) or died (grey circles) over their first winter; error bars indicate 95% confidence intervals (1.96 × standard error of the mean). Also shown is the total proportion of juveniles that survived (triangles). Sample sizes (along the *x*-axis) show the total number of juveniles genotyped at ≥3 loci (numerator) as a proportion of the total number produced (denominator; the large disparity at 2009 represents a number of unsampled dead birds).

$H \times F$ interaction. When examining the effect of heterozygosity at each gene separately, none of the seven *TLR* loci showed a strong relationship between heterozygosity and probability of survival, although most effects tended negative (heterozygotes showed lower survival than homozygotes), consistent with the multilocus result (except *TLR15* where a nonsignificant improvement in survival with heterozygosity was observed, Fig. S1, Supporting information). All single-locus final models also included *F* in their top model sets.

Allele frequencies were very similar among individuals that survived and died: mean F_{ST} across loci = −0.001 (ranging from −0.002 for *TLR1LA*, *TLR1LB* and *TLR2B* to 0.001 for *TLR5* and *TLR15*; Fig. S2, Supporting information). The confidence interval (bootstrapping over loci) for the overall value was −0.002 to 0, so we concluded that overall allele frequencies of surviving and dead juveniles did not differ.

When comparing the influence of particular genotypes, for the three loci with greater than two alleles (*TLR4*, *TLR5* and *TLR21*), a convincing survival effect was observed for *TLR4* only (Table 1A). All genotypes for both *TLR5* and *TLR21* were poor predictors of overwinter survival (note the ΔAIC_C column in Table 1B and Table 1C, absence of a distinct 'best model' ≥2 AIC_C from the next best model). Within *TLR4*, only one genotype was a useful predictor of survival: relative to all other genotypes, and accounting for any effects of *F*, $TLR4_{BE}$ genotype provided a large survival advantage to those birds that contained it (indicated by the positive value for β, Table 1A). Models containing all other *TLR4* genotypes were poor predictors of survival (Table 1A).

Relationship between $TLR4_{BE}$ genotype and survival

Although the effect of $TLR4_{BE}$ genotype would be considered statistically significant at $\alpha = 0.05$ ($\beta < 1.96 \times SE_\beta$, Table 1A), we evaluated its biological significance using the model-averaged parameter estimates. The fitted survival probability for $TLR4_{BE}$ birds (given a mean *F* and averaged across cohorts) was calculated as 91.3%, while the fitted mean survival of all other *TLR4* genotypes was 79.3%. Of the 645 birds genotyped for *TLR4*, 60 (9.3%) were $TLR4_{BE}$ [6 of these died, 12.9 expected based on contingency table (Table S3, Supporting information)]. These values equate to a selection coefficient against non-$TLR4_{BE}$ genotype animals of 0.142 and a relative risk of over-winter death of $TLR4_{BE}$ individuals of 0.440 (95% CI = 0.203, 0.954; standardized against a risk of death of 1 for non-$TLR4_{BE}$ animals).

As an independent test of the selective benefit of $TLR4_{BE}$ genotype, we compared the observed proportion of $TLR4_{BE}$ animals produced each year with the number expected under Mendelian inheritance, given parental genotypic combinations, where an excess is expected at 1 year of age. At age 2 weeks, the observed proportion of $TLR4_{BE}$ juveniles produced was generally similar to Mendelian predictions: values for all years were consistent with simulated data, and aside from slightly elevated observed values in the first 3 years, all observations were very close to simulated means (Fig. 2A). At age 1 year (i.e. among surviving birds only), observed annual proportions of $TLR4_{BE}$ individuals were also very close to simulated mean predictions, but there was a consistent pattern of slight excess in every year of the study except for 2006 (Fig. 2B; probability of observing 8 or more excesses across 9 years = 0.020). The very small magnitude of these excesses (Fig. 2B) is consistent with the relatively small number of 'additional' birds that are thought to have survived as a result of having $TLR4_{BE}$ genotype ($N = 6.9$; Table S3, Supporting information).

Although the Ulva Island population had 12 genetic founders, only one of these (band combination 'blue metal') carried the $TLR4_E$ allele (genotype $TLR4_{AE}$). Thus, all $TLR4_{BE}$ animals in the population are descendants of this founder, and it is possible that the observed survival benefit of the genotype may be confounded by other heritable factors. This is particularly plausible when considering that blue metal and his first partner 'white metal' are the highest contributors to the descendent Ulva population, together contributing nearly 40% of the descendent gene pool (I. G. Jamieson, unpub-

Table 1 Effect of toll-like receptor (TLR) genotypes on first-year survival probability of Ulva Island robins, for three loci with more than two alleles in the population [A: *TLR4* (5 alleles); B: *TLR5* (3 alleles); C: *TLR21* (3 alleles)]. Only years with mixed survival are included (i.e. data from 2001 is excluded)

Model*	β_{ij} ($\pm$ SE_β)†	Deviance	AIC_C	ΔAIC_C	w_i	N_{ij}‡	$Survival_{ij}$ (%)§
(A): *TLR4*							
Base + *BE*	**1.007 (0.448)**	**659.5**	**667.6**	**—**	**0.511**	**60**	**90**
Base + *AA*	0.463 (0.303)	663.4	671.4	3.82	0.076	95	84
Base	—	666.3	672.4	4.79	0.047	645¶	78¶
Base + *BD*	−0.443 (0.394)	664.7	672.7	5.14	0.039	34	71
Base + *BC*	0.456 (0.456)	664.8	672.8	5.24	0.037	42	86
Base + *DD*	−1.491 (1.437)	664.9	672.9	5.34	0.035	2	50
Base + *CE*	−0.942 (0.932)	664.9	673.0	5.40	0.034	5	60
Base + *AC*	−0.272 (0.278)	665.0	673.0	5.40	0.034	81	74
Base + *CC*	−0.847 (0.889)	665.0	673.1	5.53	0.032	6	67
Base + *BB*	−0.230 (0.283)	665.2	673.3	5.69	0.030	79	75
Base + *EE*	−1.025 (1.449)	665.4	673.4	5.85	0.027	2	50
Base + *AD*	−0.236 (0.352)	665.4	673.5	5.90	0.027	48	75
Base + *AE*	−0.239 (0.428)	665.6	673.6	6.03	0.025	31	74
Base + *AB*	−0.078 (0.234)	665.8	673.8	6.22	0.023	137	77
Base + *CD*	−0.079 (0.589)	665.8	673.9	6.31	0.022	17	76
Base + *DE*	—	—	—	—	—	6	100
(B): *TLR5*							
Base + *AC*	−1.203 (0.697)	663.6	671.7	—	0.313	9	56
Base	—	666.3	672.4	0.69	0.222	646¶	78¶
Base + *BB*	−0.293 (0.260)	665.1	673.2	1.49	0.149	94	73
Base + *AA*	0.190 (0.202)	665.5	673.5	1.82	0.126	247	80
Base + *BC*	−0.612 (0.883)	665.9	674.0	2.27	0.101	6	67
Base + *AB*	0.086 (0.196)	666.2	674.2	2.52	0.089	290	80
Base + *CC*	—	—	—	—	—	0	—
(C): *TLR21*							
Base + *AB*	−0.310 (0.239)	664.2	672.3	—	0.275	122	75
Base	—	666.3	672.4	0.08	0.264	645¶	78¶
Base + *AA*	0.245 (0.233)	664.8	672.8	0.54	0.210	511	79
Base + *BB*	0.301 (1.129)	665.8	673.9	1.55	0.127	5	80
Base + *AC*	0.121 (1.137)	665.9	673.9	1.61	0.123	5	80
Base + *CC*	—	—	—	—	—	2	100
Base + *BC*	—	—	—	—	—	0	—

Model shown in bold is the most parsimonious model supported by the data for each locus (where the 'best' model is ≥2 AIC_C units from the next best model); models could only be fitted for genotypes with mixed survival.

*Generalized linear mixed effects model with a binomial response (survived or not), where the base model contains individual inbreeding coefficient *F* and cohort as a random factor. Genotype models include base parameters plus a 1/0 binary predictor for presence/absence of the specified genotype.

†Parameter estimates for the specified genotype (ij, as indicated in the 'Model' column) namely effect size coefficient (β) and its standard error (SE_β).

‡Number of animals containing the specified genotype (ij) — note that these vary widely, as a result of uneven allele frequencies in the population (see Fig. S2, Supporting information).

§Survival rate of animals containing the specified genotype.

Overall sample size and survival rate for this locus (all genotypes combined).

lished data). If the apparent survival benefit of $TLR4_{BE}$ genotype is due to co-inheritance of 'good genes' from blue metal, this may also explain why blue metal's lineage dominated. Our 'good genes' hypothesis predicts a positive correlation between degree of relatedness to the blue metal lineage (calculated using the pedigree and denoted $R_{BM,}$ see Methods S1 section 'Relatedness to blue metal', Supporting information) and probability of survival, but only a weak relationship was observed, with a very large standard error (standardized effect size $\beta_{R_{BM}} = 0.038 \pm 0.202$ SE; RI = 0.19, Table S4, Supporting information). After accounting for the effect of R_{BM} in the model, there remained a strong positive influence of $TLR4_{BE}$ genotype on probability of survival (standardized effect size $\beta_{BE} = 1.003 \pm 0.448$ SE; RI = 1.00, Table S4, Supporting information). There was no interaction

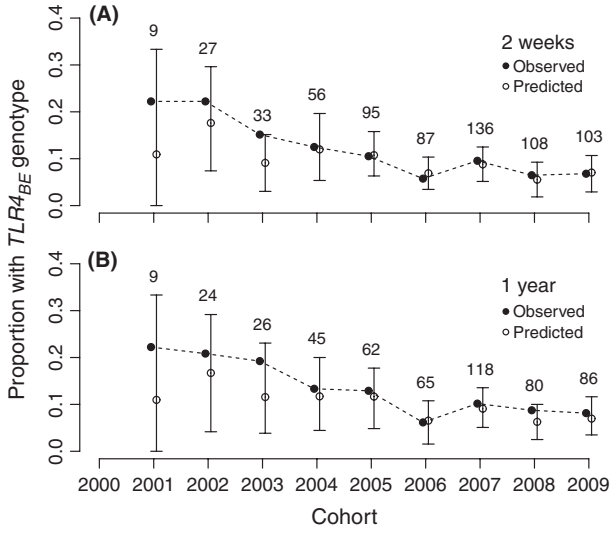

Fig. 2 Proportion of locally produced juveniles [A: at banding age (2 weeks); B: at maturity (1 year)] in each cohort with the $TLR4_{BE}$ genotype (filled circles), compared with simulated predictions under Mendelian inheritance (open circles; error bars indicate the 95% quantiles of 5000 randomization simulations). Values above points indicate the number of genotyped offspring at that stage for which both parents were also genotyped.

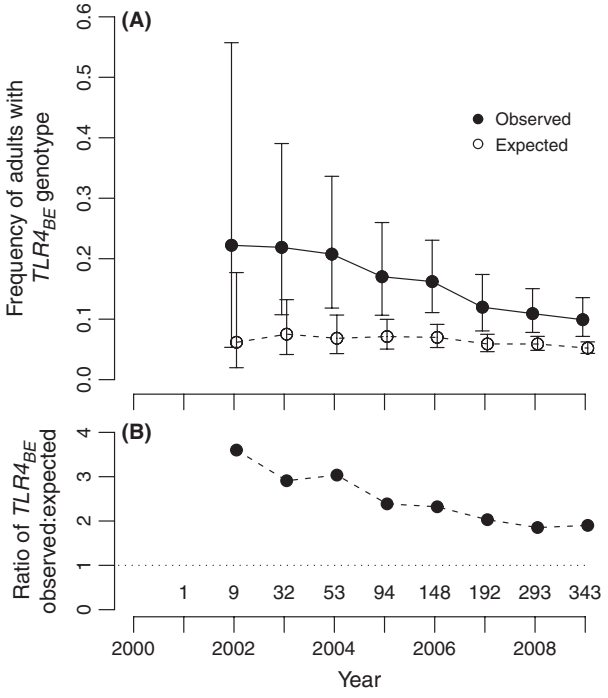

Fig. 3 Frequency of the $TLR4_{BE}$ genotype over time in the non-founder adult population. In (A) filled circles indicate observed proportion of adults with this genotype [error bars are Agresti–Coull 95% confidence interval for proportions (Agresti & Coull 1998), evaluated using the R package binom (Sundar 2009)]; open circles indicated expected frequency of $TLR4_{BE}$ genotypes under Hardy–Weinberg (error bars are 95% confidence intervals derived using logit transformation for frequencies, Sutton *et al*. 2011). Panel (B) shows the ratio of observed/expected frequencies in each year, where a value of 1 (horizontal dotted line) is expected under Hardy–Weinberg equilibrium. The size of the genotyped adult population in each year is indicated along the *x*-axis of B.

between R_{BM} and presence/absence of $TLR4_{BE}$ genotype in the final model, indicating that the selective advantage of $TLR4_{BE}$ genotype is consistent across animals that are both closely related and distantly related to blue metal. This latter result suggests that the correlation between $TLR4_{BE}$ genotype and survival is unlikely to result from a genome-wide influence of 'good genes' generally in blue metal. In addition, the lack of a strong relationship between R_{BM} and survival suggests that blue metal's lineage did not necessarily dominate because of intrinsic 'good genes'.

Temporal patterns in $TLR4_{BE}$ frequency

There was a decline in the frequency of $TLR4_{BE}$ genotype in the adult population, which was not accounted for by increasing the precision of genotype frequency estimates as population size increased [GLM with binomial error, standardized slope = −0.913 (95% CI: −1.43, −0.39), Fig. 3A: filled circles]. Although the observed frequency of $TLR4_{BE}$ animals always exceeded the expected frequency under Hardy–Weinberg equilibrium (HWE; Fig. 3A: open circles), the magnitude of this deviation decreased [ratio observed/expected frequencies declined: GLM with quasi-Poisson error, standardized slope = −0.533 (95% CI: −0.65, −0.42), Fig. 3B]. Nevertheless, there remained nearly a two-fold excess of $TLR4_{BE}$ adults by the end of the study period (observed number in 2009 = 34; expected under HWE = 17.9; Fig. 3A). The slightly higher than expected production of $TLR4_{BE}$ offspring by observed pairs in the first 3 years of the study (Fig. 2) was insufficient to account for the ultimate excess of $TLR4_{BE}$ adults in 2009 [4.6 excess $TLR4_{BE}$ birds over mean Mendelian predictions in 2001–2003 (Fig. 2) <16.1 excess $TLR4_{BE}$ adults in 2009 (Fig. 3A)].

The temporal trend in the magnitude of deviation from HWE frequency of $TLR4_{BE}$ is unlikely to result from unidentified selection pressure occurring on either the $TLR4_B$ or$TLR4_E$ alleles, as their frequencies have remained relatively constant over the study period (Fig. S3, Supporting information). Similarly, it is unlikely that there is undetected selection on other *TLR4* genotypes as none of these showed deviations from expected frequencies at any point during the study (Fig. S4, Supporting information). Furthermore, our observation that the proportion of 2-week-old juveniles produced (2004

onward) was generally similar to the number expected under Mendelian inheritance (Fig. 2A) indicates that temporal patterns in $TLR4_{BE}$ frequency are unlikely to result from selection at early life history stages. Taken together, the declining number of $TLR4_{BE}$ individuals is unlikely to result from undetected viability selection on this locus and probably signifies decrease in the magnitude of deviation from HWE.

To test whether early deviation from HWE was caused by nonrandom mating, we compared the number of $TLR4_{BE}$ 2-week-olds (i.e. prior to overwinter viability selection) produced by observed pairings to simulated data where breeding animals were randomized within each year (see Methods S1 section 'Simulation of random mating', Supporting information). In the first 5 years of the study, observed pairings produced an excess of $TLR4_{BE}$ offspring (2001–2005, notably 2002, where the excess would be considered statistically significant, Fig. 4), while production was consistent with random mating from 2006 onward. The combination of high $TLR4_{BE}$ survival and early excess productivity apparently explains the ultimate excess of $TLR4_{BE}$ individuals in 2009: over the entire study period, a total of 17.1 additional $TLR4_{BE}$ 2-week-olds were produced over mean random mating expectations (62 observed, 44.9 expected; Fig. 4).

In the latter part of the study, when $TLR4_{BE}$ production was consistent with random mating (i.e. the period

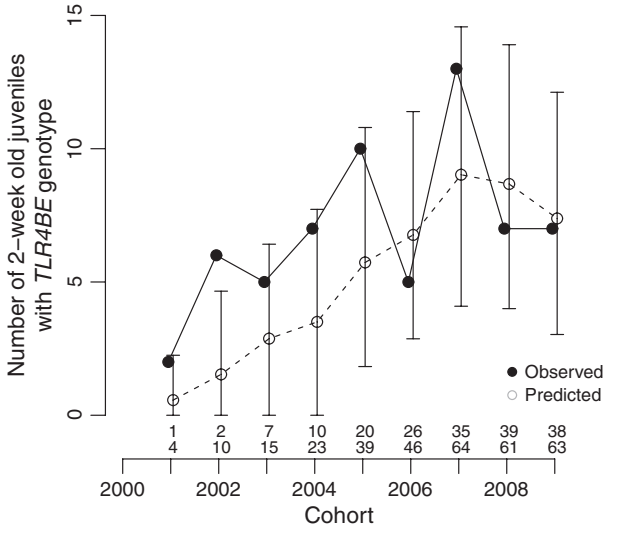

Fig. 4 Production of 2-week-old juveniles with $TLR4_{BE}$ genotype (filled circles), compared with predictions under random mating (open circles; error bars indicate 95% quantiles of 5000 simulations). Values along the *x*-axis indicate the number of pairs: the numerator is the number of established pairs (those that had bred together in previous years and therefore held fixed during the randomization – see Methods S1, Supporting information); the denominator is the total number of pairs.

2006–2009, Fig. 4), $TLR4_{BE}$ genotype remained the most useful predictor of survival (statistically significant effect at $\alpha = 0.05$; Table S5, Supporting information). Thus, the observed survival benefit of $TLR4_{BE}$ genotype overall was not limited to the early period of the study when population density was low and juvenile survival was relatively high (Fig. S5, Supporting information).

Role of genetic drift

We next examined whether the $TLR4_{BE}$ survival advantage detected above, or indeed possible undetected survival advantages associated with diversity at any loci, was sufficient to outweigh genetic drift and drive temporal patterns of overall genetic diversity at each locus (gene diversity, *D*) in the Ulva Island robin population. Our genedropping simulations revealed that for every year of the study, observed values of *D* for every locus were consistent with predictions under genetic drift (Fig. 5). These results implicate drift as the predominant force influencing gene diversity.

Gene diversity for *TLR2B* was often slightly above mean predictions (Fig. 5), possibly indicating a form of weak balancing selection that was not detected by our main analysis (e.g. selection operating on traits other than first-year over-winter survival). Conversely, observed levels of *TLR21* gene diversity were slightly lower than neutral predictions, which may indicate undetected positive selection. In both of these cases, however, observed values were generally consistent with simulated predictions (i.e. within simulated confidence intervals, Fig. 5).

Discussion

The Ulva Island robin population presents an ideal study system for investigating the evolutionary and population genetic processes that occur in a small population following colonization of a new habitat. This unique data set results from intensive monitoring efforts that have provided full pedigree and reproductive data for founders and all descendants for several generations, as well as near-complete genetic sampling. Over the 9-year period of natural population growth, there was little evidence that *TLR* diversity was under natural selection, as defined by the probability of juvenile robins surviving their most vulnerable stage, their first winter. There were no associations between survival and particular alleles for any loci, and none of the loci tested showed a relationship between overall heterozygosity and survival.

We detected viability selection for one genotype, $TLR4_{BE}$, which conferred a 0.44 relative risk of overwinter death as compared to birds with all other *TLR4*

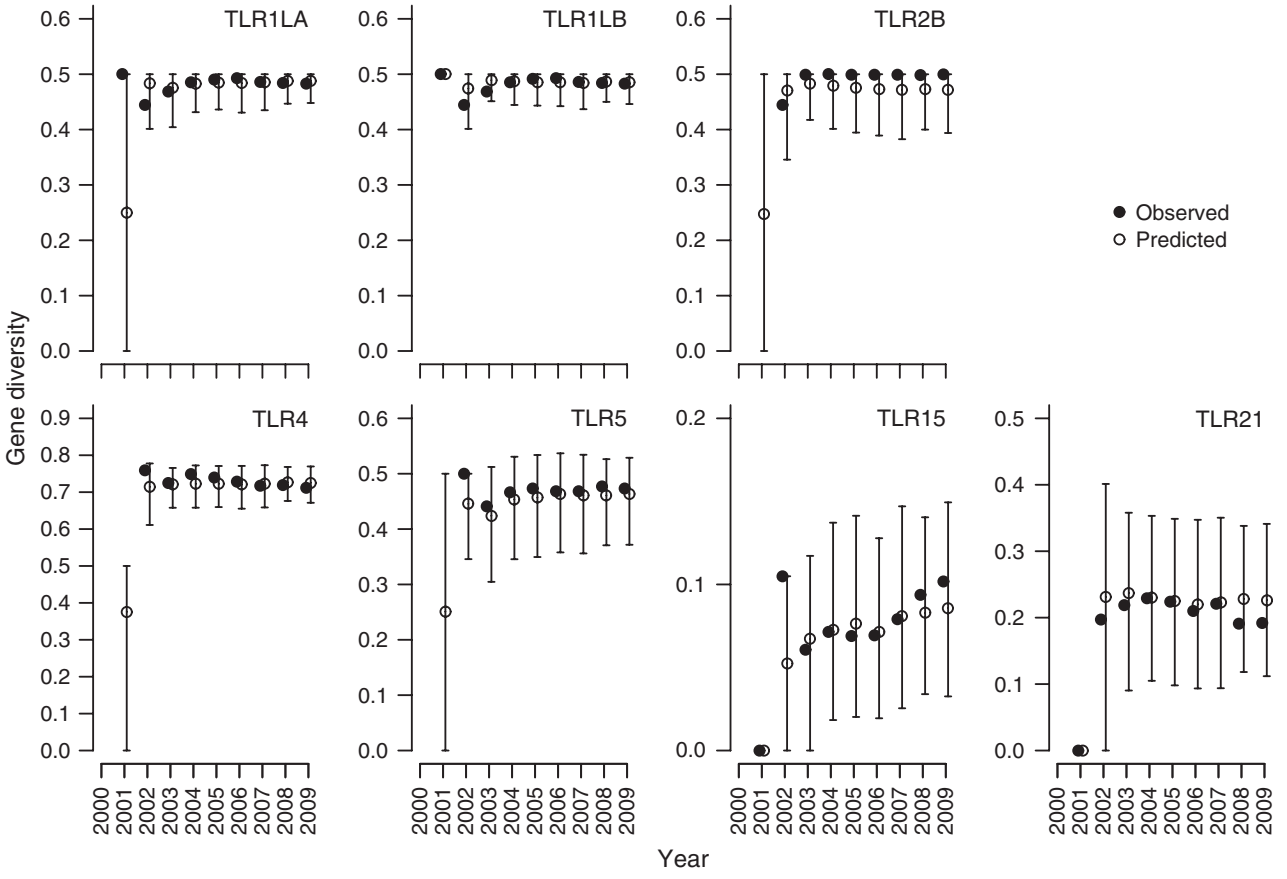

Fig. 5 Changes in annual gene diversity (*D*) among the adult population at each toll-like receptor (*TLR*) locus (filled circles are observed values, open circles are the mean of 5000 genedropping simulations assuming neutrality, where the error bars are the 95% quantiles of simulated distributions).

genotypes. This effect remained after accounting for inbreeding and year effects, and an ultimate two-fold excess of this genotype over Hardy–Weinberg expectations did not result from selection on the $TLR4_B$ or $TLR4_E$ alleles, other *TLR4* genotypes, nor selection at earlier life history stages. Neither could this pattern be attributed to association between $TLR4_{BE}$ genotype and 'good genes', nor density dependence. Therefore, the excess of $TLR4_{BE}$ in 2009 is probably due to improved viability of animals with this genotype in combination with nonrandom mating in the first half of the study. Multiple mechanisms could give rise to a deviation from random mating: for example, inbreeding avoidance, mating to minimize allele sharing or mating of compatible genotypes; distinguishing among these with our data set was beyond the scope of this study.

Despite detecting statistically significant selection operating on the $TLR4_{BE}$ genotype, the combined effect of this and other processes was insufficient to drive (deterministically) overall gene diversity (*D*) at the *TLR4* locus beyond predictions under genetic drift. While the selection coefficient we report (0.142) could be considered relatively large [recent sequence-based estimates of selection for human *HLA* genes were all ≤0.044 (Kawashima *et al.* 2012; Yasukochi & Satta 2013)], it is worth noting that the genotype under selection, $TLR4_{BE}$, was at a relatively low frequency (see Fig. 3A). Its selective benefit may therefore be diluted when considering patterns at the locus level, as in the gene diversity metric. We also observed minor (nonsignificant) deviations from predictions under genetic drift at two other loci (Fig. 5), neither of which were found to be important predictors of over-winter survival. It is possible that these deviations result from a combination of subtle effects on multiple traits, including or in addition to overwinter survival. Nevertheless, the combined strength of these effects was insufficient to result in statistically significant deviations from neutral predictions.

With currently available data, we are unable to determine the specific mechanism by which $TLR4_{BE}$ genotype confers a survival advantage to juvenile robins, although heterozygote advantage has been previously reported for several genes (reviewed in Hedrick 2012), and adaptive significance of particular immunity

genotypes has also been observed (as opposed to heterozygosity per se; e.g. Sepil *et al.* 2012). Neither the $TLR4_B$ nor $TLR4_E$ alleles have a unique amino acid residue at any position (Table S6, Supporting information). The *TLR4* gene is well studied in mammals, and evidence suggests that the avian ortholog has similar functions (e.g. Saponaro *et al.* 2011). The protein has been associated with resistance to a number of common environmental pathogens that are probably present in the forest environment of Ulva Island, including gram-negative bacteria, fungi, viruses and protozoa (Akira *et al.* 2006; Vinkler *et al.* 2009; Villaseñor-Cardoso & Ortega 2011). Importantly, variation at *TLR4* has been associated with variation in susceptibility to several infectious diseases (Leveque *et al.* 2003; Villaseñor-Cardoso & Ortega 2011). Our results are therefore not at odds with theoretical predictions of *TLR* immune function, although targeted investigation of disease pressure in this population (e.g. following Ewen *et al.* 2007; Middleton *et al.* 2010) would be challenging, given the diversity of potential candidate pathogens.

Despite apparent selection for a heterozygote genotype, it is possible that in the long term, the Ulva robin population may lose genetic diversity if population growth is limited. This can occur, for example, if selection favours the $TLR4_B$ and $TLR4_E$ alleles, to the detriment of the $TLR4_A$, $TLR4_C$ and $TLR4_D$ alleles. Selection coefficients for all other *TLR4* genotypes were unknown because they were not useful predictors of survival (Table 1A). As a result, it is difficult to forecast the time frame over which such losses of genetic diversity may occur. A simulation analysis that incorporates uncertainty of selection coefficients of all genotypes may be useful for estimating the long-term equilibrium state of the five *TLR4* alleles once this relatively small population reaches its demographic equilibrium (estimated carrying capacity approximately 250 pairs, IGJ unpublished data). Further investigation into the nature and role of nonrandom mating in this population may also provide valuable information for predicting long-term changes in genetic diversity.

This is the first empirical study to examine the roles of selection and drift immediately following a population bottleneck of a wild population. Our results support the general assertion that drift plays a significant role in determining the genetic makeup of small populations (Bouzat 2010). Our findings demonstrate that this can be true even where natural selection is operating. We provide empirical support for the theoretical prediction that neutral processes can overwhelm selection when a population is small and assert that genetic drift is therefore a significant concern during the establishment phase of colonization. In a conservation context, translocations to establish new populations in protected or restored areas, or to re-introduce extirpated species to their former range, are key strategies for the management of threatened species (Seddon 2010; Thomas 2011). Where the aim is to establish a self-sustaining population, conservation-motivated translocations may be conducted without provision for ongoing, supplementary translocations. Isolated populations lacking opportunity for genetic exchange are likely to experience significant genetic drift, particularly while they remain small (Weiser *et al.* 2012, 2013), and the resulting losses of genetic diversity may impact on the population's ability to respond to future environmental changes, such emerging infectious diseases (O'Brien & Evermann 1988). Our results show that genetic drift can be the major determinant of the genetic makeup of a population, even when natural selection confers a survival advantage to a heterozygote genotype, such as those associated with the innate immune system.

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C.E.G., I.G.J. and G.P.W. designed the study; C.E.G. and I.G.J. collected the data; C.E.G. analysed the data; and C.E.G., I.G.J. and G.P.W. wrote the article.

Data accessibility

Raw data from this manuscript (individual data including genotypes and survival, pairwise juvenile production, pedigree data, population-level juvenile production) have been deposited in the DRYAD data repository (doi:10.5061/dryad.k3311).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 SNPs genotyped in this analysis.

Table S2 Top model set (top $2AIC_C$) of models for the effect of inbreeding (*F*) and composite heterozygosity at seven TLR loci (*H*) on juvenile robin survival.

Table S3 Observed (and expected) number of robins that survived to 1 year or not, based on *TLR4* genotype (birds from 2001 excluded, for consistency with the main survival analysis).

Table S4 Model-averaged standardised predictors of first year overwinter survival.

Table S5 Effect of *TLR4* genotypes on first year survival probability of Ulva Island robins, data restricted to the period 2006 onwards.

Table S6 Characteristics of the variable amino acids of each *TLR4* haplotype.

Fig. S1 Forest plot of standardised coefficients for effects of *TLR* heterozygosity on over-winter survival of Ulva robins; positive values indicate that heterozygotes are more likely to survive than homozygotes; also shown is the multilocus effect of *H* for comparison.

Fig. S2 Allele frequencies for the seven genotyped loci in birds that survived ("Alive", $N = 507$) or not ("Died", $N = 139$) their first winter (for consistency with the main analysis, these data exclude years where all offspring survived, and birds with fewer than 3 loci genotyped).

Fig. S3 Change in observed frequencies of each of *TLR4* allele in the adult population over time.

Fig. S4 Frequencies of each *TLR4* genotype over time among the non-founder adult population.

Fig. S5 Number of surviving juveniles produced by each pair in each breeding season, including all known Ulva robin breeding pairs (i.e. including ungenotyped animals); $N = 475$ pair-years.

Fig. S6 Correlation between relatedness of genotyped animals. to blue-metal and to his first partner white-metal ($N = 624$ individuals with non-zero relatedness to either); points shown may represent overlay of multiple individuals with equal relatedness.

Data S1 Methods.