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Evidence of the phenotypic expression of a lethal recessive allele under inbreeding in a wild population of conservation concern

Amanda E. Trask\(^1\)*, Eric M. Bignal\(^2\), Davy I. McCracken\(^3\), Pat Monaghan\(^4\), Stuart B. Piertney\(^1\) and Jane M. Reid\(^1\)

\(^1\)Institute of Biological & Environmental Sciences, School of Biological Sciences, University of Aberdeen, Zoology Building, Tillydrone Avenue, Aberdeen AB24 2TZ, UK; \(^2\)Scottish Chough Study Group, Kindrochaid, Bridgend, Isle of Islay, Argyll PA44 7PT, UK; \(^3\)Future Farming Systems, Scotland’s Rural College, Auchincruive, Ayr KA6 5HW, UK; and \(^4\)College of Medical, Veterinary & Life Sciences, University of Glasgow, Graham Kerr Building, Glasgow G12 8QQ, UK

Summary

1. Deleterious recessive alleles that are masked in outbred populations are predicted to be expressed in small, inbred populations, reducing both individual fitness and population viability. However, there are few definitive examples of phenotypic expression of lethal recessive alleles under inbreeding conditions in wild populations. Studies that demonstrate the action of such alleles, and infer their distribution and dynamics, are required to understand their potential impact on population viability and inform management responses.

2. The Scottish population of red-billed choughs (*Pyrrhocorax pyrrhocorax*), which currently totals < 60 breeding pairs and is of major conservation concern, has recently been affected by lethal blindness in nestlings. We used family data to show that the pattern of occurrence of blindness within and across affected families that produced blind nestlings was exactly 0\(^/\)C1\(^2\), matching that expected given a single-locus autosomal lethal recessive allele. Furthermore, the observed distribution of blind nestlings within affected families did not differ from that expected given Mendelian inheritance of such an allele.

3. Relatedness estimates showed that individuals from affected families were not more closely related to each other than they were to individuals from unaffected families that did not produce blind nestlings. Blind individuals tended to be less heterozygous than non-blind individuals, as expected if blindness was caused by the expression of a recessive allele under inbreeding. However, there was no difference in the variance in heterozygosity estimates, suggesting that some blind individuals were relatively outbred. These results suggest carriers of the blindness allele may be widely distributed across contemporary families rather than restricted to a single family lineage, implying that the allele has persisted across multiple generations.

4. Blindness occurred at low frequency (affecting 1\(^-\)6% of observed nestlings since 1981). However, affected families had larger initial brood sizes than unaffected families. Such high fecundity of carriers of a lethal recessive allele might reflect overdominance, potentially reducing purging and increasing allele persistence probability.

5. We thereby demonstrate the phenotypic expression of a lethal recessive allele in a wild population of conservation concern, and provide a general framework for inferring allele distribution and persistence and informing management responses.

**Key-words:** conservation genetics, disease aetiology, extinction, genetic disorder, heterozygote advantage, inbreeding depression, inheritance, mutation, Peters’ anomaly

*Correspondence author. E-mail: a.trask@abdn.ac.uk

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Introduction

Small and isolated populations, which are often of conservation concern, are expected to experience some degree of inbreeding given random or non-random mating. Deleterious recessive alleles are consequently more likely to be expressed, causing inbreeding depression in viability and fecundity (Charlesworth & Charlesworth 1999; Charlesworth & Willis 2009). Indeed, inbreeding depression has been observed in numerous wild populations (e.g. Grueber et al. 2010; Agudo et al. 2012; Mattila et al. 2012; Reid et al. 2014), potentially increasing extinction risk and necessitating management intervention (Crnokrak & Roff 1999; Hogg et al. 2006; O’Grady et al. 2006).

Inbreeding depression is thought to be primarily caused by the cumulative effects of partially or fully recessive deleterious alleles of small effect expressed across numerous loci (Charlesworth & Charlesworth 1999). However, large-effect deleterious recessive alleles can also contribute to inbreeding depression (Laikre 1999; Remington & O’Malley 2000; Charlesworth & Willis 2009). Small-effect and large-effect alleles can have different implications for the management of inbred wild populations. Inbreeding depression stemming from numerous small-effect alleles may be difficult to alleviate as appropriate management practices such as minimizing breeding between relatives (Hagen et al. 2011) or translocating unrelated individuals into a population (Vilà et al. 2003; Hogg et al. 2006) may not be feasible. Conversely, large-effect alleles might potentially be more amenable to management intervention if carriers can be identified and removed from the breeding population (Laikre, Ryman & Thompson 1993; Laikre 1999; Ralls et al. 2000). However, if carriers are numerous or widely distributed within a population then removing them may substantially reduce effective population size (e.g. Ralls et al. 2000; Hammerly, Morrow & Johnson 2013), thereby increasing subsequent inbreeding, genetic drift, demographic stochasticity and extinction risk. Key steps towards informing management strategies for small, wild populations are therefore to identify large-effect alleles contributing to inbreeding depression, and to establish the distribution and identity of carriers.

However, direct evidence of phenotypic expression of large-effect deleterious recessive alleles under inbreeding conditions in small, wild populations remains scarce, meaning that the possible impacts of such alleles are rarely incorporated into management strategies. Experimental breeding of wild-sampled individuals has shown that lethal recessive alleles do exist in wild populations (e.g. in monkey-flower, Mimulus guttatus, Willis 1992; fruit fly, Drosophila melanogaster, Kusakabe et al. 2000; bluefin killifish, Lucania goodei and zebrasfish, Danio rerio, McCune et al. 2002). Lethal recessive haplotypes, comprising major genomic inversions and multiple genes inherited as a single unit, are also known to exist in wild populations of house mice (Mus domesticus, r haplotype, Manser et al. 2011) and ruff (Philomachus pugnax, Küpper et al. 2016). Additionally, in pedigreed captive populations of threatened species, large-effect recessive alleles that would likely be lethal if expressed in the wild have been inferred (Laikre 1999). Examples include chondrodystrophy (dwarfism) in California condor (Gymnogyps californianus, Ralls et al. 2000), diaphragmatic hernias in golden lion tamarins (Leontopithecus rosalia, Bush et al. 1980) and blindness in grey wolves (Canis lupus, Laikre, Ryman & Thompson 1993). In contrast, the few documented examples of potential large-effect recessive alleles expressed in small, wild populations have severe but non-lethal effects. Examples include testicular abnormalities in Florida panthers (Felis concolor coryi, Roelke, Martenson & O’Brien 1993) and South Australian island populations of koala (Phascolarctos cinereus, Seymour et al. 2001; Cristescu et al. 2009), which can effect fertility (Mahmud et al. 2015), and vertebral abnormalities in the Scandinavian wolf (Canis lupus) population (Räikkönen et al. 2006). Furthermore, the evidence that these conditions are genetic is indirect; testicular and vertebral abnormalities are heritable in various domesticated mammals (Kramer et al. 1982; Mahmud et al. 2015), affected panthers were associated with inbred matings (Roelke, Martenson & O’Brien 1993), and testicular abnormalities in koalas are associated with high effective inbreeding coefficients in sequentially founded populations (Seymour et al. 2001).

One reason why impacts of large-effect recessive alleles have not been explicitly documented in small, wild populations might be because such alleles are unlikely to be observed or persist, even given inbreeding. This is because such alleles are often expressed during early development and cause embryo or early-life mortality (e.g. chlorophyll deficiency in plants, Remington & O’Malley 2000; developmental defects in fish, Tiira, Piironen & Primmer 2006; hatching failure in birds, Ortego et al. 2010; Hemmings, Slate & Birkhead 2012). Furthermore, such alleles might be rapidly purged due to strong selection (Hedrick 1994; Husband & Schemske 1996; Wang et al. 1999; Crnokrak & Barrett 2002), meaning that standing large-effect alleles are truly rare. However, purging may be inefficient in small populations where selection may be weak relative to genetic drift (Byers & Waller 1999; Wang et al. 1999; Frankham et al. 2001). Large-effect recessive alleles might also persist if they confer a fitness advantage in heterozygotes (i.e. overdominance, Lacy & Ballou 1998). The current paucity of evidence of large-effect recessive alleles acting in small, wild populations therefore does not exclude the possibility that such alleles might exist and hence exacerbate inbreeding depression and impact population persistence.

When lethal or severe phenotypic disorders are observed in populations of conservation concern, a key first step in management is to identify the aetiology (i.e. form of causation). Genetic aetiologies can be reliably
Lethal recessive allele in a wild population

established through QTL mapping, genomewide association or positional cloning studies that link genotype to phenotype (Peltonen & McKusik 2001; Botstein & Risch 2003; Visscher et al. 2012), or through test-crosses among affected families (Laikre, Ryman & Thompson 1993). However, for many wild populations adequate genomic resources are still unavailable and test-crosses are not feasible. Large-effect alleles can instead be deduced from observed patterns of phenotypic expression across relatives, which are expected to follow recognizable patterns stemming from Mendelian segregation and inheritance (Table 1. Phillips et al. 2007; Tayo et al. 2009). Conversely if an observed disorder had an environmental etiology, for example caused by an infectious disease or abiotic factor, occurrences would be unlikely to match Mendelian expectations but might be spatially or temporally clustered (Hartup et al. 2001; Sabel et al. 2003; Ostfeld, Glass & Keesing 2005; Altizer et al. 2006).

If a genetic etiology is inferred, a second key step is to identify carriers of causal alleles so that the potential for eradication, or selective breeding to reduce phenotypic expression, can be assessed. However, heterozygous carriers of recessive alleles do not express associated phenotypes and cannot be readily identified without sufficient genomic resources to develop diagnostic tests (Romanov et al. 2006; Allendorf, Hohenlohe & Luikart 2010), or sufficient pedigree data to calculate individual carrier probabilities (Laikre, Ryman & Thompson 1993). When these resources are unavailable, as is typical for wild populations, the likely distribution of carriers can only be inferred indirectly. One approach is to quantify relatedness of affected individuals relative to unaffected individuals. If affected individuals are more closely related to each other than to unaffected individuals, then the causal allele may have arisen recently such that all carriers are restricted to a particular family, which could potentially then receive targeted management action (Fig. 1a,b). However, if affected individuals are no more related to each other than to unaffected individuals, then the causal allele may have arisen further back in time such that carriers are now relatively widely distributed in the contemporary population (Fig. 1d,e). Management targeted at particular affected families might then have limited efficacy at eradicating the causal allele, or have unacceptable collateral effects. Such comparisons of relatedness can therefore indicate the potential feasibility and desirability of removing carriers, and inform managers of the value of investing in developing diagnostic molecular markers to definitively identify individual carriers.

Such analyses of relatedness can be achieved using molecular genetic estimators that are relatively easily implemented in populations with limited genomic resources and sampling. Furthermore, estimates of multilocus heterozygosity (a proxy for individual inbreeding level, Chapman et al. 2009; Szulkin, Biene & David 2010; Forstmeier et al. 2012) may allow further inference of the likely contemporary distribution of carriers. If all carriers are clustered within the same family then affected individuals, which must all be the progeny of two closely related carriers, will all be highly inbred and hence have lower mean and variance in heterozygosity than unaffected individuals (Fig. 1c). However, if carriers are widely distributed in the population then relatively unrelated carriers may also mate and produce relatively outbred affected offspring. Affected individuals would then be only slightly less heterozygous on average than unaffected individuals, with little difference in the variance in heterozygosity (Fig. 1f).

Once the likely distribution of carriers in a population has been inferred, a third key step is to investigate whether a focal allele is likely to persist in the population or go extinct without management intervention. Allelic

| Aetiology                                      | Phenotypic expression/occurrence                                      | Expectation                                                                 |
|-----------------------------------------------|-----------------------------------------------------------------------|----------------------------------------------------------------------------|
| Autosomal recessive                           | Expressed by homozygous carriers only (Campbell & Reece 2005)         | Expressed in offspring of unaffected parents. Expect 0.25 rate of occurrence within affected families |
| Autosomal or allosomal dominant               | Expressed by all carriers (Campbell & Reece 2005)                     | Expressed in offspring of affected parents, which is not possible for a pre-reproductive lethal trait |
| Sex-linked (z-linked) recessive                | Females are the heterogametic sex in birds (ZW), meaning that males must be homozygous and females hemizygous for the z-linked allele to express the phenotype (Buckley 1989) | Expressed in females only. Males could be carriers. Male expression would require an affected mother, which is not possible for a pre-reproductive lethal trait |
| Mitochondrial                                 | Expressed matrilineally (Campbell & Reece 2005)                       | Affected offspring would have an affected mother, which is not possible for a pre-reproductive lethal trait |
| Environmental (e.g. infectious or abiotic causative agent) | Occurs in individuals infected by, or exposed to, the causative agent | No consistent pattern of occurrence across and within affected families. However, occurrences might be clustered spatially or temporally and affect whole broods |

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persistence will depend partly on any expression of overdominance and resulting increased fitness of heterozygous carriers. For example, carriers could have high fecundity, which could compensate for the fitness cost of producing affected offspring. Such compensation would decrease purging and increase the probability that a deleterious allele will persist (Lacy & Ballou 1998). The relative fitness of carriers and non-carriers must therefore be estimated to understand the natural dynamics of deleterious or lethal recessive alleles and associated management implications. However, few studies have demonstrated the phenotypic expression of a lethal recessive allele in a wild population, or hence inferred the likely within-population distribution and dynamics of any such allele.

Here, we provide evidence for the phenotypic expression of a large-effect (lethal) recessive allele causing blindness in a small, insular red-billed chough (Pyrrhocorax pyrrhocorax Linnaeus, hereafter ‘chough’) population of conservation concern in Scotland, UK. Blindness, characterized by severe corneal opacity (Appendix S1, Supporting information), was first observed in a chough nestling in 1998 and has been regularly observed subsequently (Fig. 2). Blind nestlings have occurred in the same nest sites, reared by the same parent pairs, across multiple years suggesting either a genetic or localized site-specific environmental aetiology. Veterinary post-mortems suggest that blindness is caused by an early-stage developmental abnormality of the anterior segment of the eye, similar to the human condition ‘Peters’ anomaly’. Peters’ anomaly has a genetic aetiology, with multiple genes that function in a common eye developmental pathway implicated (Gould & John 2002; Bhandari et al. 2011; Reis & Semina 2011). Furthermore, domestic chicken, mouse and zebrafish gene knockout studies have created analogous phenotypes (Hsieh et al. 2002; Wurm et al. 2008; McKeone et al. 2011; Morris 2011; Zhao et al. 2012). We therefore hypothesized that blindness might have a genetic aetiology in choughs. Blindness is inevitably a pre-reproductive lethal trait in a wild bird; although affected (i.e. blind) nestlings can grow normally, they inevitably die at fledging. As breeding adults cannot be blind, causal alleles cannot be dominant. We therefore hypothesized that blindness constitutes a notable example of the phenotypic expression of a lethal recessive allele in a small, inbred population of conservation concern.

To infer the aetiology of blindness in choughs, and assess the potential for targeted management, we first quantified the pattern of occurrence of blindness across years and broods. We show that this pattern matched that expected given a single-locus autosomal lethal recessive
allele, and did not match that expected given other postulated aetiologies for a pre-reproductive lethal trait (Table 1). Secondly, to infer whether the causal allele is likely to have emerged recently and hence be restricted to a particular family, or is older and hence more widely distributed across contemporary families, we compared relatedness and multilocus heterozygosity among affected and unaffected individuals (e.g. Fig. 1). Finally, we tested whether brood sizes differed between affected and unaffected families, and thereby assessed the potential for overdominance and associated constraints on purging.

Materials and methods

STUDY SYSTEM AND SAMPLING

Choughs have experienced substantial reductions in range and population size across Europe since 1800 (Holloway & Gibbons 1996; Eaton et al. 2009). They are consequently listed on Annex 1 of the EU Wild Birds Directive and ‘Amber listed’ in the UK (Eaton et al. 2009). In Scotland, choughs are restricted to the islands of Islay and Colonsay, totalling ca. 53 breeding pairs in 2014 (Hayhow et al. 2015). These populations are isolated and genetically depauperate relative to other British and continental European populations, as evidenced by low microsatellite diversity and high between population differentiation (Wenzel et al. 2012). The small population size and apparent lack of immigration mean that inbreeding is inevitable.

Scottish choughs form socially monogamous breeding pairs and are highly territorial (Bignal, Bignal & McCracken 1997). Pairs breed once per year and nest in cavity sites in caves and farm buildings (Bignal, Bignal & McCracken 1997; Reid et al. 2006). Both sexes contribute to nestling provisioning (Bignal, Bignal & McCracken 1997) and can fledge up to five chicks per brood (mean on Islay 1·99 ± 0·15 SD, Reid et al. 2004). Each year since 1981, 30–80% of active nest sites on Islay were visited 2–3 weeks after nestlings hatched to count and uniquely colour-ring nestlings (Reid et al. 2003, 2004). The proportion of active nest sites visited increased across years (Fig. 2a). Colour-ringed adults breeding at each nest site were identified, but unringed adults were not captured and hence remained unmarked. Mean annual adult survival rate was 0·83, meaning that most adults survive to breed in multiple years (Reid et al. 2003, 2004). Across all years, one or both attending adults were colour-ringed in ca. 57% of all monitored broods (increasing to ca. 63% during
1997–2014. Observations of colour-ringed adults demonstrate very high mate and nest-site philopatry (as also observed in Spanish choughs, Banda & Blanco 2014) and show that a change of nest site is typically associated with a change in at least one member of the breeding pair due to mortality.

PATTERN OF OCCURRENCE OF BLINDNESS

During nest-site visits, nestlings were checked for blindness, presenting as severe corneal opacity (Appendix S1). Corneal opacity is readily observable in 2- to 3-week-old nestlings, meaning that blind individuals in visited broods that survived to this age are unlikely to have gone unnoticed. Corvidae nestlings are altricial and typically open their eyes 4–12 days post-hatch (Bateman & Balda 1973; Woolfenden 1978; Whitmore & Marzluff 1998). Permanently blind nestlings are therefore unlikely to be directly disadvantaged prior to this time. Furthermore, observed blind nestlings grow normally and survive to fledging at ca. 6 weeks post-hatch (Appendix S2). There is consequently no clear expectation that blind nestlings will have higher mortality than their normally sighted brood mates prior to nest visits.

To quantify the overall frequency of occurrence of blindness, we calculated the proportion of nestlings checked in each year that were blind, and the proportion of all checked broods that contained at least one blind nestling. To compare the observed pattern of occurrence of blindness to the patterns expected given specific aetiologies (Table 1), we quantified the numbers of blind and non-blind nestlings produced by ‘affected families’. Affected families were defined as nuclear families where parent pairs were observed to produce blind nestlings in at least one brood (i.e. a standard ‘case-family’ design, Hopper, Bishop & Easton 2005). All broods produced by parent pairs that were known or assumed to comprise the same adults were included. Parent pairs where both adults were colour-ringed were known with certainty. As choughs show extremely high mate philopatry and site philopatry (see above, Banda & Blanco 2014), unringed pairs that bred repeatedly at the same nest site were assumed to comprise the same parent pair (Appendix S3).

Quantitative comparisons between observed and expected patterns of occurrence of blindness could potentially be confounded by extra-pair parentage within affected families, as affected or unaffected nestlings would be attributed to incorrect parents. However, the rate of extra-pair parentage in choughs (~5%) is too low to cause substantial biases (Appendix S4).

We used simulations to compare the observed distribution of blind nestlings within broods of affected families to that expected given a 0.25 probability of occurrence in each nestling (as expected given a single-locus autosomal recessive allele, Table 1). Nestlings within each observed brood were randomly assigned as blind (vs. not blind) through independent Bernoulli trials with probability 0.25. The numbers of broods that contained zero to five blind nestlings were summed across the real and simulated data. The observed frequency of each count was compared to the mean and 95% confidence interval calculated across 1000 simulated frequencies, based on observed brood sizes.

GENOTYPING AND MOLECULAR SEXING

Molecular genetic analyses were used to sex blind nestlings, estimate pairwise relatedness between individuals from affected and unaffected families and quantify multilocus heterozygosity. In 2012–2014, non-blind nestlings at accessible nest sites were blood sampled via brachial venipuncture, and blood was stored in EDTA tubes. Although adults were not captured, chough moult coincides with breeding. DNA from adults attending accessible nest sites was therefore non-invasively sampled by collecting moulted feathers. Tissue samples from adults and non-blind nestlings were also opportunistically collected from carcasses after natural mortalities. Tissue samples from blind nestlings were collected during veterinary post-mortem, following natural mortality or humane euthanasia (Scottish Natural Heritage licence 19354).

DNA was extracted from <5 μL of blood, small pieces of liver or muscle tissue or 3–5 mm clippings of the lower feather calamus, using standard ammonium acetate precipitation (Hogan et al. 2008). All individuals were initially genotyped at 13 microsatellite loci developed for choughs and polymorphic in the Islay population (Wenzel et al. 2011, Appendix S5). To increase power for estimating relatedness and heterozygosity, individuals sampled in 2014 and all blind individuals sampled were genotyped at a further five polymorphic microsatellite loci cross-amplified from other passerine species (Appendix S5). After tests for scoring errors and conformity to Hardy–Weinberg and linkage equilibrium, 17 microsatellite loci were used for further analysis. Details of genotyping and descriptive statistics of microsatellite loci are provided in Appendix S6.

To compare the sex ratio of blind nestlings to that expected given a sex-linked recessive causal allele vs. other aetiologies (Table 1), blind nestlings were sexed using the 2550/2718 primer pair (Fridolfsson & Ellegren 1999) and the P2/P8 primer pair (Griffiths et al. 1998) for the CHD-1 gene. The presence of blind males, which would refute a z-linked mode of inheritance (Table 1), was then ascertained. The sex ratio of blind nestlings was also compared to the expected 1 : 1 ratio given an autosomal mode of inheritance, using chi-square goodness-of-fit tests.

RELATEDNESS AND HETEROZYGOSITY

To identify and calculate the most appropriate relatedness estimator for the study population, we used simulations of different pairwise relatedness estimators based on observed allele frequencies, implemented in COANCESTRY v.1.0.1.5 (Wang 2011). The dyadic maximum likelihood (DyadML, Milligan 2003) was selected as it yielded the highest correlation between simulated and true relatedness estimates (Appendix S7). DyadML can account for inbreeding and genotyping error and is constrained within the biologically meaningful range of 0–1 for the probability of identity by descent (IBD, Milligan 2003).

Relatedness estimates can be used as a continuous measure of relative IBD between dyads, or to assign dyads to relationship categories (Blouin 2003; Weir, Anderson & Hepler 2006). However, studies of wild populations often lack sufficient power from available marker loci to reliably assign relationship categories. Instead, power can be maximized by comparing mean relatedness between groups of interest (e.g. Vangestel et al. 2011; Mattila et al. 2012). Accordingly, DyadML relatedness calculated between dyads of individuals from affected vs. unaffected families was compared to relatedness calculated between dyads of individuals from different affected families, thereby quantifying the relative distributions of estimated relatedness (e.g. Fig. 1.b.e). Truly ‘unaffected families’ are challenging to identify as heterozygous carriers of recessive alleles cannot be identified phenotypically. Furthermore, as the ‘affected’ phenotype is only expressed probabilistically, an absence of observed affected (i.e. blind) nestlings

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does not mean that a family is truly 'unaffected'. We consequently defined 'functionally unaffected families' as parent pairs with ≥10 observed non-blind nestlings, giving a <0.06 probability that the family was actually affected. Furthermore, we excluded parent pairs with a known first-degree relationship to an affected family, as informed by available colour-ringing data.

To ensure that comparisons of relatedness between individuals from affected and unaffected families were not confounded by including multiple first-degree relatives (i.e. parents and offspring, or multiple siblings), analyses were restricted to either both parents or one randomly selected nestling from each affected family, but not both generations simultaneously. Equivalent numbers of genotyped individuals were randomly selected from the defined unaffected families. The distribution of relatedness estimates between dyads of individuals drawn from affected families was compared to the distribution of relatedness estimates between dyads of individuals drawn from affected and unaffected families. To maximize use of all available genotypic data, calculations were repeated by resampling parents or a nestling from each affected and unaffected family across 1000 iterations. As relatedness estimates have considerable associated uncertainty, we divided the estimates into 10 equal categories from 0 to 1 and recorded the frequency of dyads in each category. We thereby compared the overall distributions of relative relatedness between individuals from different affected families and individuals from affected vs. unaffected families (e.g. Fig. 1b,c).

We tested whether standardized multilocus heterozygosity (sMLH, Colman et al. 1999) differed between blind and non-blind individuals (e.g. Fig. 1c,f). The blind group included all genotyped blind nestlings. The non-blind group included individuals from genotyped unaffected families. For each unaffected family, a single genotyped nestling or both genotyped parents, but not both generations, were included to ensure independence of sMLH values given unequal allele frequencies (Nietlisbach, Keller & Postma 2016). Linear-mixed models were used to test whether sMLH differed between the blind and non-blind groups, including random family effects to account for non-independence among multiple blind nestlings sampled from the same family. Homogeneity of variance in sMLH estimates between blind and non-blind groups was tested using Levene's test.

To examine how well individual sMLH estimated from the available marker data reflects genomewide levels of heterozygosity, the identity disequilibrium measure $g_2$ and the probability that $g_2$ differed from zero were estimated using the software Robust Multilocus Estimate of Selfing (RMES, David et al. 2007; Szulkin, Bierne & David 2010), with 1000 iterations. Additionally, local effects of genotyped microsatellite loci were examined by jackknifing across loci and estimating sMLH from the reduced data sets. Mean sMLH of blind and non-blind individuals was then compared using the jackknifed data sets.

**Carriers in Fecundity**

Relative fecundity of known carriers of the blindness allele (i.e. parents of affected nestlings) and parents from 'functionally unaffected families' (which are unlikely to both be carriers) was quantified by comparing brood sizes (i.e. number of nestlings at ringing) between affected and unaffected families. All broods produced by known affected families during 1997–2014 were included, irrespective of whether blind nestlings were observed in a given brood. For 'functionally unaffected families', all observed broods spanning 1997–2014 were initially included. However, using 'functionally unaffected families', where parent pairs have ≥10 observed non-blind nestlings, might bias the sample towards high-fecundity parent pairs, potentially upwardly biasing brood size estimates. This analysis was therefore repeated using all apparently unaffected families where blind nestlings were never observed, irrespective of the total number of non-blind nestlings observed. Generalized linear-mixed models with Poisson error distributions were used to test whether brood size differed between affected and unaffected families, with random family and year effects to account for non-independence of broods from the same family and breeding season.

Analyses were run in R v.2.15.2 (R Development Core Team 2012) unless otherwise stated, using packages GENHET (Coulon 2010) and lme4 (Bates et al. 2015). Likelihood ratio tests were used to assess the significance of effects in linear and generalized linear-mixed models, verified by additionally fitting maximum likelihood as well as restricted maximum-likelihood models.

**Results**

**Pattern of Occurrence of Blindness**

During 1981–2014, 1791 chough nestlings were observed across 1043 broods on Islay. In total, 29 nestlings (1.6%) were blind. Since the first observed case in 1998 (Fig. 2), the number of blind nestlings observed per year ranged from 0 to 6 (median = 1, representing a median of 3% of nestlings checked per year). Blind nestlings were observed in 19 broods (1.8% of all monitored broods), produced by nine parent pairs across eight different nest sites whose locations spanned Islay. In total across all observed broods produced by known affected families, there were 85 non-blind nestlings. Blindness therefore occurred at a rate of exactly 0.25 (29/(29 + 85)), matching the rate expected if blindness was caused by a single-locus autosomal recessive allele. Furthermore, the rate of occurrence did not differ significantly from 0.25 within any of the affected families (Appendix S3). The observed distribution of blind nestlings within broods of affected families also did not differ from that expected if each nestling within the brood had a 0.25 probability of being blind, as expected for a recessive allele segregating under simple Mendelian inheritance (Fig. 3).

**Sex of Blind Nestlings**

Molecular sexing of 15 DNA-sampled blind nestlings revealed nine males and six females. The presence of blind males precludes an allosomal z-linked recessive mode of inheritance (Table 1). The sex ratio of blind nestlings did not differ significantly from 1:1, as expected given an autosomal mode of inheritance ($\chi^2 = 0.6, P = 0.44$).

**Relatedness and Heterozygosity**

The distribution of DyadML relatedness estimates between genotyped individuals from functionally unaffect-
fected families (n = 24) and individuals from affected families (n = 19, total ‘normal–blind dyads’ = 456) showed a clear single peak at <0/07 (median 0/07, Fig. 4a). This suggests that most ‘normal–blind’ dyads are relatively unrelated, as the median relatedness of simulated unrelated dyads was 0/07 (Appendix S7). The distribution of relatedness estimates between individuals from affected families (total ‘blind–blind’ dyads = 171) also showed a peak at <0, indicating that most individuals from different affected families are also relatively unrelated to each other, and are no more related to each other than to unaffected individuals. However, a second small peak at ≥0/7 indicates that some dyads of individuals from different affected families are relatively highly related (black arrow, Fig. 4a; median relatedness of simulated first-order relatives was 0.50, Appendix S7). This distribution matches that expected if the blindness allele arose multiple generations ago (e.g. Fig. 1d), such that carrier and affected individuals are not restricted to a single close family lineage.

Mean sMLH tended to be higher across all genotyped non-blind individuals (n = 74, mean 1.03 ± 0.29 SD) than across all genotyped blind individuals (n = 15, mean 0.86 ± 0.26 SD, Fig. 4b), but this difference was marginally non-significant (estimate 0.17 ± 0.09SE, likelihood ratio test: G2 = 3.5, P = 0.06). Furthermore, sMLH estimates of all except one of the blind individuals sampled fell within the range of sMLH estimates for non-blind individuals (Fig. 4b). The variance in sMLH was not significantly lower across blind individuals than across non-blind individuals (0.07 vs. 0.10, Levene’s test: F1 = 1.2, P = 0.27).

Identity disequilibrium significantly exceeded zero (g2 = 0.03, P < 0.01), suggesting that sMLH estimates

![Fig. 3. Frequencies of (a) brood sizes produced by affected families and of (b) numbers of blind nestlings observed in broods of given sizes (BS), and (c) mean expected frequencies (dots, with 95% confidence limits) and observed frequencies (crosses) of blind nestlings across broods of observed sizes of 0–5 nestlings, given a 0.25 probability of blindness.](image)

![Fig. 4. (a) Frequencies of dyadic maximum-likelihood relatedness estimates between individuals from different affected families (grey boxes) and between individuals from affected vs. unaffected families (white boxes), calculated from a resampling simulation. Black arrow indicates second small peak of high dyadic relatedness among individuals from affected families. (b) Standardized multilocus heterozygosity estimates of blind and non-blind individuals. Box-plots show the median (solid line), first and third quartiles (box limits), the highest or lowest data point within 1.5 times the interquartile range (whiskers) and outliers (dots). On (a), the x-axis values denote the upper relatedness category boundaries.](image)
reflect genome-wide heterozygosity, and hence indicate individual inbreeding level, to some degree. Mean sMLH was consistently greater in non-blind than blind individuals after jackknifing to eliminate locus-specific effects (Appendix S8).

**CARRIER FECUNDITY**

Across 35 observed broods from known affected families, mean brood size was $3.3 \pm 1.1$ SD nestlings (median = 4). Across 194 observed broods from defined 'functionally unaffected families', mean brood size was $2.7 \pm 1.3$ SD nestlings (median = 3). Brood size in affected families was significantly larger than in unaffected families (likelihood ratio test: $\chi^2 = 3.8, P = 0.05$, back-transformed effect size: 0.82, Appendix S9). This difference remained significant when analyses were repeated including all 427 observed broods from apparently unaffected families ($\chi^2 = 9.7, P = 0.002$, back-transformed effect size: 0.67, Appendix S9).

**Discussion**

Inbreeding depression in fitness occurs widely in wild populations and can increase extinction risk (O’Grady *et al.* 2006; Grueber *et al.* 2010; Mattila *et al.* 2012). However, few studies have demonstrated the phenotypic expression of lethal recessive alleles in wild populations of conservation concern, meaning that the contribution of such alleles to inbreeding depression remains unclear and is consequently rarely considered in the context of population management. Here, we demonstrated that the pattern of blindness observed in Scottish choughs matches that expected for an autosomal single-locus lethal recessive allele, as evidenced by the 0.25 rate of occurrence within and across affected families, and by the distribution of blind nestlings within broods of affected families. Other potential modes of inheritance can be rejected (Table 1). Dominant autosomal or allosomal inheritance with complete penetrance would mean that all carriers would be blind, but as blindness in a wild bird is pre-reproductively lethal then parents of blind nestlings cannot be blind. Likewise, mitochondrial inheritance can be excluded because mothers of blind nestlings cannot be blind. Similarly, allosomal recessive inheritance is also precluded given the presence of blind males, as this would require blind mothers (Table 1). Blind nestlings occurred at the same nest sites and to the same putative parent pairs across years, which could indicate a genetic or site-specific environmental aetiology. However, as blindness affected partial broods and cases occurred across multiple years and locations, an environmental aetiology appears less likely. Although a dominant allele with reduced penetrance cannot be definitively excluded, the most parsimonious explanation for the observed pattern of blindness is that it reflects the phenotypic expression of an autosomal single-locus lethal recessive allele in a wild population.

**ALLELE ORIGIN AND DISTRIBUTION**

When a large-effect recessive allele is suspected to act in a population of conservation concern, knowledge of the distribution of carriers is required to evaluate the feasibility and necessity of management action to eliminate the allele or reduce expression (Laikre, Ryman & Thompson 1993; Ralls *et al.* 2000; Hammerly, Morrow & Johnson 2013). However, heterozygous carriers of recessive alleles do not express associated phenotypes and cannot be identified directly without molecular diagnostic tests. A useful approach is then to examine whether carriers are widely distributed in the contemporary population, as could be the case if the allele originated multiple generations ago and is present as standing genetic variation, or whether carriers are restricted to a particular family lineage, as would be the case if the allele arose recently through migration or *de novo* mutation. Mutational origins can be inferred using a formal coalescence approach (Rosenberg & Nordborg 2002), but coalescent times are hard to estimate and can be downwardly biased given inbreeding. Instead, we illustrate a means of indirectly inferring allele and carrier distributions from dyadic relatedness and multilocus heterozygosity estimates (Fig. 1), which can be relatively readily obtained from wild populations of non-model organisms using modest numbers of molecular markers. Within the Scottish chough population, the distribution of dyadic relatedness estimates suggests that most individuals from different affected families are relatively unrelated. Furthermore, while blind individuals tended to have lower sMLH estimates than non-blind individuals, they did not have significantly lower variance in sMLH. These lines of evidence both suggest that the putative ‘blindness allele’ originated multiple generations ago and is now relatively widely distributed in the population rather than clustered within a single close family lineage. Blindness can consequently be expressed when relatively distantly related carriers mate as well as following inbreeding between closely related carriers. This conclusion is not necessarily contradicted by the fact that blindness was first observed in the population in 1998 (i.e. equating to ca. two chough generations ago). As relatively small proportions of nestlings were checked in previous years (Fig. 2), earlier occurrences of blindness could have gone unobserved.

**ALLELE FREQUENCY, MANAGEMENT AND DYNAMICS**

The inference that the ‘blindness allele’ is probably widely distributed within the Scottish chough population implies that eliminating the allele by removing known affected families may have limited efficacy, as the allele is probably not restricted to these families. Furthermore, the observed frequency of blindness (affecting 1.6% and 1.8% of observed nestlings and broods since 1981) implies that the underlying allele had a long-term frequency of $q \approx 0.13$ in the population (assuming Hardy–Weinberg proportions with $q^2 \approx 0.016–0.018$), which is not trivial. Removing all
actual or potential carriers would therefore have the undesirable concomitant effect of substantially decreasing the number of available breeders, and hence effective population size and contemporary genetic variation (e.g. Laikre 1999; Ralls et al. 2000). Meanwhile, the relatively low resulting frequency of blindness (median of 3% of ringed nestlings per year since 1998) might suggest that eradicating the blindness allele should not be the highest priority for short-term chough conservation compared to managing causes of post-fledging mortality (Reid et al. 2011). Furthermore, chough population growth rate is relatively insensitive to small reductions in mean population-wide breeding success (i.e. chicks fledged per breeding attempt), such as that caused by the current frequency of blindness (Reid et al. 2004). A long-term management strategy may be to translocate choughs to Islay and Colonsay from other populations, thereby reducing the overall degree of inbreeding and inbreeding depression (e.g. Vilà et al. 2003; Hogg et al. 2006), reducing the relative frequency of the blindness allele and therefore indirectly reducing future expression.

Population management decisions in general, and for choughs in particular, will depend partly on the probability that a large-effect recessive allele will persist in the population, or go extinct without management intervention. Large-effect recessive alleles might be expected to be rapidly purged from a population (Hedrick 1994; Husband & Schemske 1996), implying that the blindness allele might naturally go extinct in the Scottish chough population. However, in populations with a small effective size, the effects of selection and consequent purging may be weak relative to genetic drift (Byers & Waller 1999; Frankham et al. 2001; Keller & Waller 2002). Additionally, large-effect deleterious alleles that confer increased fitness when heterozygous, due to overdominance or associative overdominance, may experience reduced purging and be maintained (Wang et al. 1999; Crnokrak & Barrett 2002). Interestingly, in choughs, mean brood sizes of affected families were larger than mean brood sizes of unaffected families, raising the unexpected possibility that the blindness allele may display some form of overdominance. In affected families, the cost of producing a blind nestling with a probability of 0.25 was balanced by the larger initial brood size. Specifically, the mean brood size of affected families after mortality of blind nestlings would be 2.5 [mean pre-mortality brood size of 3.3 × (1−0.25)], which is similar to the observed mean brood size of unaffected families of 2.7. Purging may consequently be weakened, as any reduction in carrier fitness due to mortality of blind nestlings is compensated by increased fecundity. Indeed, a number of genes implicated in causing the human disorder ‘Peter’s anomaly’ have known pleiotropic effects (e.g. Mxs2, Satokata et al. 2000; Pax6, Simpson & Price 2002). In particular, Pax6 is involved in pituitary gland development (Bentley et al. 1999; Zhu, Gleiberman & Rosenfeld 2007), and the pituitary gland produces a variety of hormones that regulate avian reproduction (Scanes 1999). Knowledge of the causal mutation and genomic architecture underlying blindness in choughs is required before overdominance or associative overdominance can be conclusively established.

Overall, however, the long-term dynamics of the blindness allele, and hence its long-term impacts on population persistence, cannot yet be readily predicted. First, the current population-wide allele frequency, and its temporal dynamics during 1981–2014, cannot be definitively inferred from the frequency of blindness observed across ringed nestlings and broods. This is because the chough’s relatively long life span and lifelong monogamy cause intrinsically restricted and highly autocorrelated biological sampling of pairings within and across years. The true population-wide allele frequency could consequently differ substantially from the $q = 0.13$ calculated from the observed frequency of blindness, simply due to chance pairing and sampling effects in a small population (akin to demographic stochasticity). Furthermore, as observed blind nestlings tended to have slightly lower sMLH than nestlings from apparently unaffected families, the population-wide frequency of blindness could potentially be underestimated due to increased inbreeding depression in pre-fledging survival of all nestlings from affected families. However, any such unobserved affected families would not bias the observed 0.25 frequency of blindness within known affected families, or hence affect conclusions regarding the mode of inheritance. Secondly, predictions of allele dynamics requires estimation of the effective population size, which is itself not straightforward in age-structured populations with overlapping generations and substantial variance in reproductive success (Engen et al. 2010), as in choughs (Reid et al. 2003, 2006). Thirdly, population projections would require the relative survival and hence lifetime fitness of carriers vs. non-carriers, rather than solely their relative brood sizes, to be estimated. Such estimations and predictions should become feasible given further years of demographic monitoring.

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**Data accessibility**

Data on occurrences of blindness in affected families are available in the Supporting Information. All other data associated with this article are available at the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.57t89 (Trask et al. 2016).
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**Supporting Information**

Additional Supporting Information may be found in the online version of this article.

**Appendix S1.** Red-billed chough nestling showing severe corneal opacity.

**Appendix S2.** Biometrics of blind and non-blind nestlings.

**Appendix S3.** Occurrences of blindness in affected families.

**Appendix S4.** Extra-pair parentage in Scottish red-billed choughs.

**Appendix S5.** Descriptive statistics of microsatellite markers.

**Appendix S6.** Genotyping procedures and summary statistics of microsatellite markers.

**Appendix S7.** Relatedness estimators.

**Appendix S8.** Jackknifed multilocus heterozygosity estimates.

**Appendix S9.** Model comparing brood sizes between affected and unaffected families.