Genetic study of an isolated population of adders *(Vipera berus)* founded by historic translocation: implications for conservation

ABSTRACT

Adder (*Vipera berus*) populations in Great Britain have undergone substantial declines in recent decades. Isolation due to habitat fragmentation particularly threatens the demographic and genetic health of small populations. Despite the potential benefit of population supplementation in the conservation management of affected populations, there are currently no consensus guidelines for conservation-motivated translocations of this species. Translocations of adders for ecological mitigation of land development are more frequently undertaken, although these are typically poorly documented and insufficiently monitored, representing a wasted opportunity for strategic learning and improvements. We studied an isolated adder population on a protected site in eastern England, founded in 1999 by the translocation of adders from a development site. With known numbers, age and sex of released individuals, this represented an opportunity to improve our understanding of the genetic outcome of a newly created population. Although apparently thriving despite a low founder number, the finding in 2015 of a stillborn clutch of adders raised the possibility of inbreeding in the population. We sampled adders from the translocated population in 2017, and two individuals from the donor site in 2018. Although we found no increase in homozygosity, relatedness and maximum likelihood sibship analysis revealed high levels of consanguinity, especially within the subgroup of adults. Demographic modelling with Approximate Bayesian Computation supported the known origin of the population, but also a subsequent, undocumented adder release to the site, accounting for the observed

healthy proportion of young adders with lower levels of consanguinity. Despite the protected habitat site, the population remains isolated, and thus demographically and genetically vulnerable. We highlight the importance of careful post-translocation monitoring including targeted genetic analyses. Strategic data gathering coupled with careful management of translocations, whether for ecological mitigation or conservation rescue, could support significant improvements to the conservation management of this species, including reintroduction initiatives.

Keywords

relatedness; inbreeding; stillbirth; foundation bottleneck; population supplementation

INTRODUCTION

Populations of the adder *Vipera berus* (Linnaeus, 1758) are declining in parts of the species' native range across Western Europe (Reading et al., 2010; Guiller et al., 2022), including Great Britain (Arnold, 1995; Baker et al., 2004). Small, spatially confined populations are at risk of becoming demographically non-viable (Lande, 1988; Ball et al., 2020), notably threatened by habitat fragmentation, predation and human disturbance (Gleed-Owen & Langham, 2012; Gardner et al., 2019). Adders, in common with other temperate snakes, are viviparous with low fecundity, low vagility and high philopatry (Madsen & Shine, 1992; Bauwens & Claus, 2019). They are especially vulnerable to stochastic environmental threats (Traill et al., 2010; Wootton & Pfister, 2013), including severe weather events (Reading et al., 2010; Maxwell et al., 2019) and disease (Lorch et al., 2016; Franklinos et al., 2017). Inbreeding depression is another important potential mechanism for the collapse of isolated populations. This is exemplified by a long-isolated adder population in Sweden, in which a fall in numbers was associated with a high rate of stillbirths and deformities (Madsen et al., 1996). Similar findings have been reported in fragmented populations of the congeneric Hungarian meadow viper *V. ursinii rakosiensis* (Ujvárí et al., 2002). Breeding between closely related individuals increases the chance of homozygosity and thus the expression of rare recessive deleterious alleles, contributing to inbreeding depression (Morton et al., 1956; Lynch et al., 1995; Keller & Waller, 2002; Charlesworth & Willis., 2009). A loss of genetic diversity may additionally reduce the adaptive potential of a population, and thus the ability to respond to stress and environmental change (Willi et al., 2006; Kardos et al., 2021), underscoring the importance of active genetic management of small populations (Ralls et al., 2018).

The decline of the well-documented inbred adder population in Sweden was reversed by the introduction of twenty male adders, translocated from a large, outbred population (Madsen et al., 1999; Madsen et al., 2004). In another example of translocations in adder conservation, the species has been successfully reintroduced onto a site in Greater London which had previously supported an adder population (Atkins, 2016; Worthington-Hill., 2016). However, an attempted reintroduction of captive-bred adders in a Bedfordshire, eastern England, did not result in a sustained population, possibly because of predation by a high density of common pheasant (*Phasianus colchicus*), a widely introduced gamebird (Worthington-Hill., 2016). These

contrasting outcomes highlight important considerations in planning translocations, including the origin and demographic composition of the translocated individuals (Willoughby & Christie, 2019), and the identification of exogenous threats to the introduced population (Pérez et al., 2012; Converse et al., 2013; Berger-Tal et al., 2020).

In practice, translocations of adders are more often motivated by mitigation than conservation, with the immediate aim of removing wildlife from a development site, rather than ensuring the long-term survival and genetic health of the translocated individuals and their progeny (Nash et al., 2020; Hunter et al., 2021). In the UK, adders are legally protected under the Wildlife and Countryside Act (1981), but this protection does not encompass their habitat, and individuals may be moved off-site to an alternative area in case of development (Defra, 2021; Natural England, 2022). In addition, UK legislation does not require translocations of adders from development sites to be reported to national recording schemes (Defra, 2021), nor does it include specific provisions for post-translocation monitoring (Nash et al., 2020), thereby losing a potentially valuable source of information for improving the outcome of adder translocations whatever the motivation.

This study was instigated in response to the finding of five dead adder neonates on a nature reserve bordering a large urban site in eastern England in August 2015. The adder population on the reserve is known to have originated from the introduction of seven adders sixteen years previously from a nearby location in response to planned development at the donor site. The population thus provided a valuable opportunity to study the genetic status of a translocated adder population after its known origin from a small number of founders. While the nature reserve supports an apparently healthy population of adders, it is isolated, being partly bounded by large roads and by progressive housing and commercial development, and with no known adder populations in the vicinity. Our initial aim was to investigate the demography and genetic health of the resident adder population using microsatellite markers, with particular focus on the possibility of inbreeding depression. The dead neonates were found as a group still coiled within the amniotic sac, allowing them to be identified as a stillborn clutch, born to the same female parent. This obligate maternal sibship was a useful tool to inform sibship reconstruction and parentage analysis. The clutch was

potentially derived from different fathers, as polyandry is widespread in viviparous snake taxa (Wusterbath et al., 2010), including the adder (Stille et al., 1986; Höggren & Tegelström,1995; Ursenbacher et al., 2009a). Our ultimate objective was to inform the effective conservation of small, isolated adder populations, especially those originating from mitigation translocations or reintroductions.

METHODS

Study site

The study site is a 146 hectare Special Site of Scientific Interest nature reserve in eastern England (exact details withheld as its adder population is already under pressure). An industrial site until the 1990s, it now consists of scrub, grassland, mature woodland, and multiple ponds, bounded by major roads and development. For nearly 20 years it has been actively managed by the wildlife charity Froglife (https://www.froglife.org/). Despite repeated standardised reptile surveys, adders were not detected on the site until after 1999, when there had been a documented introduction of seven adders, comprising three adults (1 male, 2 female), and four subadults (2 male, 2 female), removed as part of development mitigation from a location within 20 km (details withheld to protect residual adders on site). This was the only documented release of adders on to the reserve.

Samples

Tail tip samples were taken from 5 dead newborn adders comprising a stillborn clutch collected from the nature reserve in August 2015, as part of a national wildlife disease surveillance scheme (www.gardenwildlifehealth.org). There was no macroscopic evidence of deformities or trauma. Samples were obtained from 30 live adders on the nature reserve between March and May 2017, avoiding resampling the same spot between visits to reduce repeated captures. Two juvenile adders found at the original translocation donor site were sampled in 2017. Juveniles and subadults were distinguished from adults on the basis of colouring and size (Prestt, 1971; Arnold, 1995; Bauwens & Claus, 2018). Cloacal swabs were collected from adults and sub-adults, and buccal swabs from juveniles (Miller, 2006). Handling was kept to a minimum to reduce capture stress, with the use of transparent plastic tubes for partial immobilisation to safely access the cloaca. Capture of adult females was avoided from

the start of May to reduce disturbance to potentially gravid snakes (Phelps, 2004). Adders were released where they had been found, usually within 3 minutes of capture. DNA was extracted using a QIAmp DNA Minikit (Qiagen), according to manufacturer's protocols.

Mitochondrial DNA (mtDNA) sequencing

A 265-bp portion of mtDNA cytochrome B (Cytb) was amplified using primers L14724Vb and H15914Vb (Ursenbacher et al., 2006). PCR products were cleaned using QIAquick PCR purification kit (Qiagen). Sanger sequencing was undertaken commercially by GATC Biotech (Zurich), using the same primers as for amplification. Results were confirmed by bidirectional resequencing.

Genetic determination of sex

Sex was determined by PCR amplification of the female-specific W-homologue of the snake gametologous gene CTNNB1 (Matsubara et al., 2016; Laopichienpong et al., 2017), as detailed in Supplementary Information.

Microsatellite genotyping

We used eight microsatellite primer sets, of which six had been developed for *V. berus* (Carlsson et al., 2003; Ursenbacher et al., 2009b), and two for the meadow viper (*V. ursinii*) (Metzger et al., 2011), previously evaluated for their cross-genus applicability to *V. berus* (Ball et al., 2020). Protocol details are given in Supplementary Information. Amplified products were resolved by capillary electrophoresis on a 3130xl Genetic Analyser with a LIZ-500 size standard (Applied Biosystems). Replicates and template negative controls were included in each PCR plate to confirm reproducibility of results. Alleles were scored and binned manually, using PeakScanner 1.0 software (Applied Biosystems), without knowledge of the identity of individual samples to avoid subjective bias.

Microsatellite analysis

We used FSTAT v2.9.3.2 (Goudet, 2001) and pegas (Paradis, 2010), implemented in R v3.4.0 (R Core Team 2017), to test for linkage and Hardy-Weinberg equilibrium (HWE), and to estimate allele richness and F statistics (Weir & Cockerham, 1984), using F_{IS} as an indicator of homozygosity (Wright, 1922; Wright, 1965), and expected

heterozygosity (H_S) as a measure of gene diversity. Life history stage groups were compared in FSTAT with respect to allele richness and F-statistics, using 1000 permutations. Confidence intervals for F_{IS} were calculated using the boot.ppfis function of HIERFSTAT v0.04-22 in R (Goudet, 2005). Pairwise relatedness (Rxy) was estimated using a maximum likelihood (ML) method in ML-Relate (Kalinowski et al., 2006). A genetic estimate of the pedigree inbreeding coefficient F (Frankham et al., 2010) was derived using the inbreeding function of adegenet, version 2.0.1 (Jombart, 2008) implemented in R. A Wilcoxon rank sum test, implemented in R, was used to compare estimated values of F and Rxy between subgroups.

To estimate effective population size (Ne) we used two single sample methods. The linkage disequilibrium method (Hill, 1981) was implemented in NeEstimator ver 2.1 (Do et al., 2014), assuming random mating, deriving confidence intervals by jack-knifing (1000 iterations). In the sibship assignment method (Wang, 2009), the frequencies of full and half- sib dyads were used to estimate the current effective breeding size of the population, implemented in COLONY 2.0.6.3 (Jones & Wang, 2010), using the same input parameters as for sibling and parentage analysis. Confidence intervals were obtained by bootstrapping.

For the detection of population bottlenecks we used the BOTTLENECK v 1.2.02, test for significant heterozygosity excess (Piry et al., 1999), applying a one-tailed Wilcoxon test with 1000 iterations, using the two-phase model (90% stepwise mutations, variance=10) (Piry et al., 1999). A mode-shift test for distortion of the allele frequency distribution (Luikart et al., 1998) was also implemented in BOTTLENECK.

For parentage and sibship analysis we used a full-likelihood method, implemented in COLONY 2.0.6.3 (Jones & Wang., 2010). Both male and female polygamy were assumed, with error rate 0.0001, and three medium runs based on a medium sibship prior, not updating allele frequency. The outputs of three independent replicate runs were examined to confirm convergence to the same configuration and log likelihood. The best ML configuration was used to infer parentage and sibship dyads for each offspring.

We used STRUCTURE v2.3 (Pritchard et al., 2000; Falush et al., 2003) to infer genetic clustering, using correlated allele frequencies and admixture models, with or without the locprior option (Hubisz et al., 2009). Results were uploaded to StructureHarvester (Earl &von Holdt, 2012) to derive mean log likelihood and delta-K as a function of K, detecting hierarchical levels of structure (Evanno et al., 2005). Cluster membership coefficients from replicate runs were permuted in CLUMPP (Jakobsson & Rosenberg, 2007). Genetic clustering was further investigated using discriminant analysis of principal components (DAPC) (Jombart et al., 2010) in adegenet version 2.0.1 (Jombart, 2008). The find.clusters function was applied to determine the optimal number of clusters (k) in each population, according to curves of Bayesian Information Criterion (BIC) values as a function of k. The dapc function was applied to the same groupings of sites, using cross-validation and α-score functions to determine the optimum number of principal components to retain in each analysis. The probabilities of assignment of individuals to the different DAPC clusters were visualised using the compoplot function of adegenet.

Demographic modelling

We used Approximate Bayesian Computation (ABC) (Beaumont et al., 2002; Cornuet et al., 2008), implemented in DIY ABC ver 2.1.0 (Cornuet et al., 2014) to investigate the demographic history of the sampled population. ABC is a coalescence-based approach which compares datasets simulated under different competing scenarios, drawing parameters from prior distributions based on available ecological information, and results from genetic testing. The model scenario that best fits the data is identified as that with summary statistics closest to those of the observed dataset. A minimum of 100,000 simulations was performed per scenario. Scenarios were compared using linear discriminant analysis of summary statistics with logistic regression analysis (Beaumont, 2010; Fagundes et al., 2007) for estimation of posterior probability with 95% confidence intervals (Cornuet et al., 2008). Models were evaluated for goodness of fit and potential discrepancies (Gelman et al., 1995), with the inclusion of a range of summary statistics not used for the original simulation process. Parameters were estimated from the posterior parameter distributions of the 1% simulated datasets closest to the observed, using logit transformation. Details of model scenarios, prior settings, summary statistics and model checking are presented in Supplementary Information.

RESULTS

mtDNA haplotypes

All adders sampled on the nature reserve, including the dead neonates, were found to share an identical mtDNA cytb haplotype. The cytb sequence of the two individuals sampled at the donor site differed from this at a single nucleotide, corresponding to position 15828 of the *V. berus* mtDNA sequence accession MF945570 in Genbank (Gao et al., 2017).

Genetic determination of sex

Field-assigned sex of snakes was confirmed, and sex was determined for previously unsexed individuals, including the stillborn clutch (detailed in Supplementary Information).

Microsatellite genotyping

28 of the samples collected from adders at the nature reserve site were genotyped at 8 loci, and 2 at 7 loci. Both donor site samples and all 5 samples from the stillborn clutch were genotyped at 8 loci. There was consistency across PCR replicates. There was no evidence for null alleles, linkage disequilibrium, nor deviation from HWE. Three samples derived from adult males were shown to have an identical 8-locus microsatellite genotype. Another 8-locus genotype was seen in two samples from juvenile males. In both examples of repeated genotypes, samples had been collected on different site visits, which could reflect repeat sampling of the same individual. Records of individual head scale pattern (Bauwens et al., 2018) were not available to exclude replicate sampling. Only one example of each genotype (one adult male and one juvenile male) was therefore included for further analysis. Two other samples with an identical genotype were derived from one adult and one juvenile, evidently different individuals, and both were retained for analysis.

After censoring of possible replicates, the 2017 sample from the nature reserve comprised 27 individuals, including 19 adults (16 male, 3 female), and 8 young (juvenile or subadult snakes) (5 male, 3 female). The stillborn clutch comprised 1 male and 4 females (Table 1). Fig. 1 compares the demographics of the study population,

after censoring for possible replicate sampling, with published results of adder populations of an equivalent size in Great Britain (Ball et al., 2020). Comparable population size was inferred from sample size, equivalent to peak adder counts in the Make the Adder Count (MTAC) study of Gardner et al. (2019). Subadults were excluded from the number of young in the nature reserve study site, as this category was not specified in the published study (Ball et al., 2020).

Microsatellite analysis

Genetic diversity, relatedness and inbreeding (summarised in Table 2). Estimates of allele richness and gene diversity were lower in the sample from the nature reserve than in previously studied sites (Ball et al., 2020). The significance of this could not be formally tested, as the panels of microsatellite loci were only partially overlapping between the two studies. There was no significant difference in allele richness and gene diversity between adult (n=19) and young adders (here defined as juvenile and sub-adult; n=8) in the nature reserve sample.

F_{IS} did not differ significantly from zero in the 2017 sample of adders from the nature reserve, nor in the stillborn clutch analysed separately. There was no significant difference in the mean values of the estimated inbreeding coefficient F between adults, young and stillborn. Mean pairwise relatedness (Rxy) was lower in young adders (0.197) than adults (0.219), but this was not statistically significant ($p > 0.05$). Rxy was higher (0.301) in the stillborn clutch, consistent with a combination of half and full siblings (predicted Rxy 0.25 and 0.5 respectively) as expected in a polyandrous mating system (Stille et al., 1986; Höggren & Tegelstrom, 1995; Ursenbacher et al., 2009a).

Effective population size Ne and bottlenecks (summarised in Table 3). The single sample LDNe method (Waples & Do 2008) failed to deliver finite confidence limits, probably the result of high levels of relatedness (Wang 2018). COLONY results mirrored the number of inferred parents (see below). In bottleneck testing, the 2017 sample from the nature reserve, including both adults and young (n=27), was negative for both heterozygosity excess and for modal shift methods. The subgroup of adults (n=19) was positive for modal shift, but negative for heterozygosity excess. The sample size was too small to allow separate analysis of the young adder subgroup.

Family structure and parentage analysis in COLONY We used the best ML configuration in COLONY to infer half- and full-sibship dyads. Replicate runs converged to equivalent log likelihoods, generating very similar patterns of inferred sibship dyads and assignment to hypothetical parents (not shown). In COLONY each individual is assigned to two inferred parents according to the best ML configuration. Fig. 2a shows inferred parentage charts for the individuals sampled at the nature reserve. In the absence of pedigree data, it is not possible to distinguish between maternal and paternal genotypes, and inferred parents are given an arbitrary number on the x and y axes on parentage charts. Adults (n=19) analysed separately were inferred to all be related at a minimum half sib level, with a total of 5 inferred parents of one sex (parent x) and 7 inferred parents of the opposite sex (parent y), including 9 individuals sharing a single inferred parent. When adults and young (n=27) were analysed together, there was again a group of individuals (n=21) inferred to be related at the half or full sib level, but with a looser parentage plot, including 4 inferred singletons.

We reanalysed the nature reserve site samples to include the stillborn clutch (Fig. 2b). The network of the best ML sibship dyads again revealed a large cluster of individuals inferred to be related at a minimum of half-sib level, with three dominant sibships, one including the stillborn clutch as well as one juvenile and four adults. The obligate maternal sibship of the stillborn clutch defined this cluster as being derived from their inferred mother. By contrast, the other five young adders and six adults were not included in this large cluster, with no inferred first-degree relatives other than a single half-sibship between one adult and a juvenile. The clutch was inferred to have three different fathers. None of the sampled adult females could have been the mother of the clutch, on the basis of incompatible genotypes, each at a minimum of two loci.

Population structure and differentiation Results in STRUCTURE did not indicate significant population substructure or admixture, even with the inclusion of the two individuals sampled at the donor site using the locprior option (not shown). This is consistent with a recent common origin for the adders on the nature reserve and the donor site. In DAPC, which uses a multivariate approach to maximise discrimination between groups, the find.clusters function indicated the likely presence of genetic clusters. Using the dapc function to compare different numbers of clusters (k), we

found that k=3 generated distinct clusters on DAPC scatterplots, and the most clear-cut group membership on compoplot (Jombart et al., 2010). These are illustrated in Fig. 3 for all samples, including the stillborn clutch and the two individuals from the donor site. The scatterplot shows distinct inertia ellipses, with individual group membership on the barplots. The stillborn clutch is assigned across two clusters, one of which also contains one of the two individuals from the donor site, while the other donor site individual is assigned to a cluster composed predominantly of young adders. This apparent difference in group membership of young and adults was not statistically significant ($p>0.05$ on Fisher's exact test in R).

ABC modelling of demographic history

Rationale for model scenarios We first tested the assumption that the documented translocation in 1999 had been the founding event for the population. We then queried whether the documented release of seven adders in 1999 would have been sufficient to account for the apparently thriving population present in 2017, including a high number of young adders relative to previously studied sites of similar abundance (Ball et al., 2020) (Fig. 1). We reasoned that the difference in COLONY results between adults and young was indicative of a temporal pattern (Balloux & Lugon-Moulin, 2002). These observations could be explained by a later additional influx of adders, likely to have been from the same donor site to account for the lack of admixture in STRUCTURE.

Comparison of possible scenarios of demographic history with ABC modelling A scenario in which the nature reserve site 2017 population originated from the donor site population with a founder bottleneck was supported by genetic data (posterior probability 0.6338 [95% CI 0.5990,0.6686]), in comparison with competing models in which the nature reserve site and donor site populations derived independently from a shared ancestral population, with or without a foundation bottleneck (0.1504 [0.1293,0.1716]; 0.2158 [0.1872,0.2444] respectively) (detailed in Supplementary Information). We then used the supported model as the baseline scenario against which to investigate the possibility of an additional later influx of adders from the donor site. A scenario in which the young on the nature reserve site derive from a later admixture between the nature reserve site and the donor site populations was compared with an otherwise identical model with a fixed prior of zero for further input

from the donor site after the foundation event. The admixture model was supported (0.6682 [0.6314,0.7050]).

Estimates of founder size and timing of events The supported model comprising the original foundation event and subsequent additional influx of adders is illustrated in Fig. 4. Model testing of the supported scenarios confirmed goodness-of-fit of the summary statistics of the observed dataset relative to the corresponding posterior predictive distribution (Supplementary Information). In the foundation bottleneck scenario, the posterior distribution modal value for the founder bottleneck size was 7.82, and the modal value of the time of the founder event was 10.4 generations before the time of sampling. The posterior distribution modal value for the timing of the admixture was 3.53 generations before 2017. In the admixture model, the posterior distribution modal value for the proportional contribution of the original nature reserve site population to the admixture was 0.97, with 0.03 from the donor site. Using the recorded release date of 1999 to calibrate the posterior distribution modal value of 10.4 generations pre-2017 for the 1999 foundation event, the subsequent release in the admixture model (posterior modal value 3.53 generations pre-2017) can be estimated to have occurred around 2011.

DISCUSSION

Levels of homozygosity have long been considered the “most natural coefficient of inbreeding” Wright (1922). However, identity by descent may not be associated with an increase in homozygosity for every breeding system (Wright 1922). The observed pattern of retained heterozygosity in the presence of high levels of relatedness in our study population resembles that described in wild adder populations in Great Britain (Ball et al., 2020), and is likely to reflect the polyandrous mating system in the adder (Stille et al., 1986; Höggren & Tegelstrom, 1995; Ursenbacher et al., 2009a).

In addition, mean heterozygosity, whether based on a small panel of neutral genetic markers as in our study, or genome-wide (Schmidt et al., 2021), may not provide a true representation of the extent and patterns of identity by descent. In a recent study, Pozzi et al demonstrated that the genome-wide pattern of heterozygosity in adders is

skewed, with most regions showing low heterozygosity, despite high mean genome-wide heterozygosity. Modelling showed the pattern to be consistent with recent severe bottlenecks (Pozzi et al., 2023), of particular relevance to our study population.

High resolution scans of the distribution of homozygosity within the genome may provide further information on the demographic history of adders. On a finer scale, genomic stretches of consecutive homozygous markers (runs of homozygosity, ROH) may be identified. These represent identical chromosomal segments inherited from a common ancestor, their lengths being determined by the occurrence of recombination events. Longer ROHs are expected where breeding between closely related individuals has occurred within past 10 generations, including recently bottlenecked populations, while shorter ROHs reflect historical inbreeding (McQuillan et al., 2008; Kirin et al., 2010; Keller et al., 2011; Palamara et al., 2012; Ceballos et al., 2018).

Significance of the stillborn clutch

The above discussion indicates that retained heterozygosity does not preclude inbreeding depression as the cause of the stillborn clutch. However, it is unlikely that the loss of the entire clutch can be attributed to the homozygous expression of a recessive deleterious allele, as the clutch was inferred to have multiple paternity. This contrasts with the occurrence of deformities affecting four out seven offspring from a single brood in an inbred population of *V.ursinii rakosiensis* (Ujvárí et al., 2002). It is more likely that the clutch on the nature reserve site was stillborn as a result of maternal fitness, which could be related to inbreeding, or an extrinsic factor. No information was available on the condition of the mother of the clutch.

The background level of stillbirths in healthy adder populations is currently unknown. In a study of 15 adder clutches over 3 years, Ursenbacher et al. (2009a) recorded complete loss of one clutch, and partial mortality in a further eight clutches, although this may have been influenced by the gravid females having been handled (Ursenbacher et al., 2009a). Disturbance of gravid females may contribute to the reported negative effect on adder populations of public disturbance, including dog walking, mountain biking and trampling of vegetation (Gardner et al., 2019). While being generally protected from public access, our study population is likely to be facing

pressure from disturbance and human-linked mortality, due to its proximity to housing developments. Three adult adders were recorded dead on the site in 2014-2017, of which two were likely due to predator attack, based on the type of traumatic injuries observed on post-mortem examination (www.gardenwildlifehealth.org). Weather conditions may also be important; for example, the combination of excessive heat and prolonged drought results in both reduced maternal fitness and increased embryonic mortality in the adder (Dezetter et al., 2021), although these factors are unlikely to apply to the stillborn clutch in our study. The inclusion of adults and juveniles in the same obligate maternal sibship as the stillborn clutch in COLONY does not indicate *de facto* that they represent a minimum of three clutches from the same mother, as inferred sibships are likelihood-based rather than being derived from a true pedigree. Indeed, none of the adult females sampled in 2017 could have been the mother of the 2015 stillborn clutch, on the basis of microsatellite haplotypes. The probability of an inferred parent-offspring dyad will be influenced by the presence of other potentially compatible genotypes, which is especially likely where there is limited diversity and high relatedness.

Irrespective of the cause, the loss of a complete clutch has potentially important genetic and demographic consequences (Stojanovic et al., 2022), especially in a species with low female fecundity. Half the female adders in a Swedish study had only one litter per lifetime (Madsen et al., 1992). Similarly, in a longitudinal study of a large population of adders in Belgium, there was an average of only 1.3 litters per female reproductive lifetime, with 70% of females breeding only once (Bauwens & Claus, 2019). The loss of a single clutch may therefore represent the loss of the entire reproductive output of a female adder, and with it a significant genetic component of the population, especially important in an isolated population with already low genetic diversity. In addition, the reproductive ecology of adders predicts vulnerability to a reduction in breeding females (Madsen & Shine, 1993). The loss of four females, as in the dead clutch in our study, may therefore have demographic implications for a population already showing a relatively low proportion of females.

Inferred demography

We found the study population to have low genetic diversity in comparison with published data from adder populations of equivalent size (Ball et al., 2020), with the

caveat of only partially overlapping microsatellite panels. The observed pattern of low genetic diversity and high relatedness in the nature reserve population is typical for a bottleneck in the demographic history of a population (Nei et al., 1975, Greenbaum et al., 2014; Grossen et al., 2018; Fernández-López et al., 2021). A foundation bottleneck is in keeping with the population on the nature reserve having been founded *de novo* from the documented release of adders eighteen years previously. The low genetic diversity is likely to be the result of the limited number of founders (Stewart et al., 2017), which also provides an explanation for the high level of relatedness and the low number of inferred parents for the adult adders sampled eighteen years after the documented translocation. A founding propagule of seven adders is below the predicted minimum size to ensure the persistence of a genetically healthy population (Shaffer 1981; Lacy, 1989).

We used COLONY in our study to generate a probability-based best ML of parentage and sibships in the sample, an approach which can also provide an estimate of the effective number of breeders in a population (Ackerman et al., 2017; Bacles et al., 2018), especially when relatedness limits the application of a linkage disequilibrium method (Hill, 1981; Wang, 2018), as in our study. Eight microsatellite loci, the number in our study, are sufficient to identify sibship dyads at a 95% probability in COLONY (Jones & Wang, 2010). This approach does not identify actual parentage and sibships but provides an illustration of the extent of relatedness. High relatedness and sibship groupings are also the likely explanation for clustering evident with DAPC, despite the lack of genetic differentiation in STRUCTURE (Ball et al., 2020). The DAPC clusters are thus more likely to reflect allele frequency patterns driven by a polygynandrous mating system in a consanguineous population, rather than discrete panmictic subpopulations. An equivalent phenomenon of clustering in DAPC, but not STRUCTURE, has also been observed in wild adder populations in the UK (Ball et al., 2020), and in the Prairie rattlesnake (*Crotalus viridis*) (Weyer et al 2014).

The pattern of large clusters in the adult subgroup contrasted with the looser network of inferred sibships in the juveniles and subadults, indicative of a temporal genetic structure (Balloux & Lugon-Moulin, 2002). This raised the possibility that there had been an additional undocumented influx of adders, likely by deliberate introduction, as the isolation and lack of connectivity would have precluded natural migration into the

site from wild populations. This would provide an explanation for the apparently high proportion of juvenile adders on the nature reserve in comparison with previously reported wild populations in Great Britain (Fig. 1) (Ball et al., 2020). We investigated this further using coalescent modelling in ABC, comparing simple competing models of equivalent complexity, an approach which in simulation studies was reported to be the most likely to identify the correct scenario (Cabrera & Palsbøll, 2017). While the very small sample size from the donor site in our study clearly necessitates caution, models of the origin of the study population and an additional later influx from the donor site were strongly supported. The accuracy of parameter estimates using approximate computation is lower than for full-likelihood methods (Robert et al., 2011). However, the modal point estimate for the size of the founder bottleneck in our study population was consistent with the documented number of released adders, despite a relatively broad prior range. With respect to estimates of timing, the relation between the inferred number of generations and chronological time is a function of the mutation rate of the genetic markers used (Kimura, 1968*)*. While there are no data on the microsatellite mutation rate in adders, this variable was constant between the different model scenarios. We therefore used the recorded release date of 1999 to calibrate the posterior distribution modal value of the foundation event. By extrapolation, the presumed subsequent release can be calculated to have occurred around 2011, although this may have been influenced the age structure of the population, the survival of females up to the age of each breeding event, and the number of offspring produced at each age class over the entire female life span (Jonasson, 2022), as well as by the possibility that undocumented releases of adders may have occurred on more than one occasion.

Lessons for translocations of adders

Our study highlights important issues with respect to translocations, including the selection of the recipient site, the size and composition of the release group, and the importance of pre- and post-release monitoring (Armstrong & Seddon., 2007; Perez et al., 2012; IUCN/SSC, 2013; Worthington-Hill, 2016; ARG UK, 2020). While an undocumented translocation may have benefited the founder population in our study site, it is important that all translocations are judiciously planned, and are fully documented at all stages. Genetic monitoring is essential following reintroductions

(Marshall et al., 2022), not only for genetic diversity and inbreeding, but also to confirm genetic integration following introductions into sites with a pre-existing population. Our results are consistent with genetic integration between the founding population and adders from a later undocumented introduction, although the extent of this cannot be determined without knowledge of the size and composition of the presumed second release. In addition, in our study mtDNA markers were non-informative, precluding information on sex-specific integration. Genotyping resident and translocated individuals prior to release can provide useful information in this respect. In a study of the parentage of desert tortoise hatchlings (*Gopherus agassizii*) four years post-translocation, hatchlings from both resident and translocated females were all sired by residents (Mulder et al., 2017). Tagging of male adders provides important information on movement patterns of resident and translocated snakes (Reinert & Rupert, 1999; Nash & Griffiths, 2018; Hand, 2018), but is insufficient to quantify paternity in a polyandrous mating system, especially where there may be high variance in individual reproductive success.

Germano et al. (2015), noting that economically motivated mitigation translocations have been less successful than conservation-driven, concluded that "the application of scientific principles and best practices would probably improve the success rate". Undocumented or poorly monitored translocations represent a wasted opportunity to study the process in detail, and thus to inform a systematic, evidence-driven approach to translocations, irrespective of the motivation. Data deficiency in this respect is exemplified by a large-scale mitigation translocation of herpetofauna from a port development site in eastern England in 2011. While legally protected great crested newts had to be relocated locally, the same constraint did not apply to the adders on the site. As a result, a very substantial population of nearly three hundred adders were translocated 140 miles away to a nature reserve with a resident adder population (Williams, 2011; BBC, 2011). Despite the stated intent to monitor the translocated population for 5 years (Williams, 2011), no outcome information is available, whether undocumented, or impenetrably buried within "grey" literature (Bradley et al., 2022). A valuable opportunity to systematically address an evidence shortfall in ecological mitigation practice and guidance (Hunter et al., 2021) was thus lost, especially important given the scale of the translocation. Some potentially useful information may still be available; a genetic survey of adders currently inhabiting the recipient sites

should show the extent of integration of the translocated adders into the original resident population (Stewart et al., 2017), which would be predicted to have divergent mtDNA haplotypes given their original geographic separation (Ball et al., 2020). If there is no residual genetic trace of the translocated animals, it would add to the concern that too many mitigation translocations of snakes simply result in their death (Cornelis et al., 2021).

Future management of study population

Although apparently flourishing, and expanding across the site, with a limited number of founders and no prospect of recruitment from the wild, the adder population on our study site remains demographically and genetically vulnerable. As well as inbreeding, there is the risk of continuing genetic erosion on the background of already limited genetic diversity, such as that described in the pink pigeon (*Nesoenas mayeri*) in Mauritius, where declining populations supplemented by release of captive-bred individuals after a severe population showed loss of genetic variation despite a population rebound (Jackson et al., 2022). Lack of interpopulation movement also results in loss of protection against stochastic events, such as skewed mortality affecting individuals of breeding potential, especially where the number of breeding females is limited. The nature reserve population in our study is thus likely to be dependent on active conservation management, including genetic monitoring and consideration of supplementary introductions, either at the same site or a nearby site with opportunities to generate habitat corridors. Information gained on the trajectory of this population will be relevant not only to the long term demographic and genetic health of reintroductions (Marshall et al., 2022), but also to captive populations (Witzenberger & Hochkirch, 2011), and small populations of adders in the wild, isolated as the result of habitat fragmentation and disturbance, and thus at high risk of extirpation (Gardner et al., 2019).

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Data Accessibility

The datasheet of microsatellite genotypes is included in Supplementary Information.

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FIGURE CAPTIONS

Figure 1 Age and sex composition of study population in comparison with other sites in Great Britain

Bar charts illustrating the relative proportions of male (dark fill) and female (light fill) adders at the Nature reserve site after censoring for possible replicate sampling, in comparison with published data from other sites in mainland Britain, and the number of young individuals included within the total number. The number of young adders does not include sub-adults, as this category was not specified in other sites. On the y-axis, total number of adders refers to the sample size analysed.

NRS: Nature reserve site; UK a-h: sites with >10 samples from Ball et al. (2020).

Figure 2 Sibships and parentage inferred in COLONY

Figure 2a Parentage plots

Each individual is represented as an open circle in a grid according to its two inferred parents. The inferred parents on each axis are given arbitrary numbers, as it is not possible to differentiate which is maternal or paternal in the absence of pedigree data.

The adults when analysed separately (left) are inferred to be related at a minimum half-sib level, and half are inferred to share a single parent (y2). This contrasts with the tail of the parentage plot when juveniles and subadults are included (right).

Figure 2b Networks of inferred sibships

Network of inferred sibships according to the best maximum likelihood configuration on COLONY. Inferred half sib dyads are shown as a single thin line, inferred full sib dyads by thick grey lines.

Top: adults from 2017 sample analysed separately. The network is dominated by a single large inferred sibship. All adults within the sample are inferred to be connected at a minimum half-sib level.

Bottom: adults (filled circles) and young (open squares) from 2017 sample, plus members of the stillborn clutch (open diamonds). The sibship of the clutch is within a dominant cluster with a shared parent, identified by the obligate maternal sibship as being female in origin. The young adders are divided between the main cluster and the group without inferred sibships.

Figure 3 DAPC cluster analysis

DAPC analysis for the NRS dataset, including the stillborn clutch (sb) and the samples from the donor site (DS). The upper panel shows a scatterplot, and the lower panel a barplot of assigned cluster membership for k=3, using the same colour scheme as the scatterplot. The stillborn clutch is divided between two clusters, and both donor site individuals are assigned to clusters, rather than being outliers. All but one of the young are assigned to the same cluster.

Figure 4 Supported model scenario in ABC

Schematic representation of the supported scenarios, showing the derivation of the nature reserve site (NRS) population from the donor site (DS) population with a foundation bottleneck lasting an arbitrary 5 generations (depicted as a thin line), and the subsequent admixture between adults from the NRS site and a further input from the donor site.

The table shows posterior estimates in the supported models for the timing and founder population size at the foundation bottleneck (Nf), and for timing and relative proportions in the admixture event. Results are shown as modal point estimates. Full details of the derivation of the model parameters and testing for goodness of fit are provided in Supplementary Information.