RESEARCH ARTICLE

Genetic and demographic vulnerability of adder populations: Results of a genetic study in mainland Britain

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Abstract

Genetic factors are often overlooked in conservation planning, despite their importance in small isolated populations. We used mitochondrial and microsatellite markers to investigate population genetics of the adder (Vipera berus) in southern Britain, where numbers are declining. We found no evidence for loss of heterozygosity in any of the populations studied. Genetic diversity was comparable across sites, in line with published levels for mainland Europe. However, further analysis revealed a striking level of relatedness. Genetic networks constructed from inferred first degree relationships suggested a high proportion of individuals to be related at a level equivalent to that of half-siblings, with rare inferred full-sib dyads. These patterns of relatedness can be attributed to the high philopatry and low vagility of adders, which creates high local relatedness, in combination with the polyandrous breeding system in the adder, which may offset the risk of inbreeding in closed populations. We suggest that reliance on standard genetic indicators of inbreeding and diversity may underestimate demographic and genetic factors that make adder populations vulnerable to extirpation. We stress the importance of an integrated genetic and demographic approach in the conservation of adders, and other taxa of similar ecology.

Introduction

Population genetics remain overlooked in conservation planning [1], although genetic factors may lead to population extinction even after other threats have been addressed [2]. Loss of genetic diversity and inbreeding depression represent the primary genetic threats [3], with the potential to contribute to an extinction vortex [4].

Tingley et al [5] have stressed the importance of addressing the optimal genetic management of small isolated reptile populations. The adder Vipera berus (Linnaeus, 1758) is a terrestrial snake with an extremely wide geographic range [6], which accounts for its IUCN Red List
status as “Least Concern”, although with a decreasing population trend [7, 8]. Adders, like many other temperate snakes, are viviparous with low fecundity, low vagility and high philopatry [9, 10], life-history traits that render them vulnerable to local extinction [11, 12]. The negative outlook for adder populations is exacerbated by snakes being among the least popular terrestrial vertebrates, more likely to be targets of intentional killing than of conservation management [13]. The potential risk of inbreeding depression in adders is highlighted by an isolated adder population in Sweden, in which a decline in numbers was associated with stillbirths and deformities, and a reduction in genetic diversity, all of which responded to the introduction of adult males from a large outbred population [14–16]. Újvári et al [17] have similarly reported low juvenile survival and birth deformities with reduced genetic diversity and increased homozygosity in fragmented populations of the congeneric Hungarian meadow viper, V. ursinii rakosiensis.

Small population size is an important factor in loss of genetic diversity, exacerbated by bottleneck events. This has led to the concept of a minimum viable population size [18, 19], based on the inverse relationship between the effective population size (\( N_e \)) [20] and the rate of erosion of genetic variation by drift, which is supported by studies of wild populations [21, 22]. \( N_e \) estimates tend to be low in relation to census population size in natural populations [23, 24], influenced by demographic fluctuation and life-history traits [25, 26]. Both small population size and genetic erosion render populations more susceptible to stochastic environmental and demographic adverse events, such as climate change or disease [19, 27, 28]. Small populations isolated by habitat fragmentation are also at increased risk of inbreeding. However, several important questions regarding genetic variation and inbreeding depression in natural populations remain largely unanswered. In particular, it is unclear to what extent mating between close relatives and loss of genetic diversity contribute to population decline and extinction in the wild, and thus to how results of genetic studies should influence their conservation management [29].

We report the results of the UK Adder Genetic Project (UKAGP), a study into the genetic status of lowland adder populations in southern mainland Britain, where national distribution surveys have indicated a decline in comparison with historic records [30–33]. A national questionnaire-based investigation survey of adder populations showed that declines were more likely to be reported in small sites with fewer than ten adders, whether based on systematic surveys or anecdotal evidence [31]. The subsequent Make the Adder Count (MTAC) initiative, based on peak springtime adder counts over sequential years, further underscored the increased risk of decline in small populations [34], flagging threats of habitat loss, public disturbance and predation, especially by cats and birds. To these threats should be added the potential risk of disease caused by the release of captive non-native snakes onto adder habitat, especially in view of the recent finding of the causative agent for snake fungal disease (Ophidiomyces ophiodiicola) in UK adders in the wild [35]. This consistently emerging picture of habitat fragmentation and local decline forms the background for our study, in which we have used a combination of mitochondrial DNA (mtDNA) and microsatellite markers to investigate the potential role of genetic factors in their decline of adders in mainland Britain.

The aim of this study was to document population genetic structure and differentiation, and to estimate indicators of inbreeding and genetic diversity in lowland adder populations in southern mainland Britain. To assess the ecological and conservation significance of our study, we interpret our results in comparison with published studies of adder populations in mainland Europe, and with reference to the size, and thus the likely risk status, of the study populations.
Methods

UKAGP study sites and samples

Ethics and animal welfare: the project was reviewed prospectively and approved by the ZSL Ethics Committee. Sampling was undertaken in concordance with ZSL ethical guidelines. Cloacal swabs were collected without anaesthetic from adult snakes, and buccal swabs from juveniles [36], by ecologists experienced in snake handling. All snakes were released at the site of capture. Handling of adult females was avoided after mid-May, to minimise disturbance to gravid snakes. No study animal was subjected to euthanasia.

Samples were collected from 220 adders at 16 sites in southern mainland Britain between March and May 2011 (Table 1). No permits were required, as there were no restrictions on site access, and the adder in the UK has no specific protection status other than against deliberate injury or killing, or collection for trade (Wildlife and Countryside Act 1981, as amended 1991). For each site, adders were caught over a one to two-day period. Dates and optimal weather conditions for sampling were determined according to local ecological expertise.

DNA was extracted using a DNeasy blood and tissue kit (Qiagen), following the manufacturer’s protocol for swabs.

Mitochondrial DNA (mtDNA) sequencing

We designed primers from a 918-bp mtDNA control region (CR) sequence and a 1043-bp mtDNA cytochrome b sequence (Cytb) [37], based on high levels of variability across 40 European adder haplotypes, and on consistent flanking region stability. The primer sequences selected for this study are shown in Table 2A. PCR for both loci was performed in HotStarTaq-Plus (Qiagen), with an annealing temperature of 55˚C. The same primers were used for

Table 1. Details of sites and samples.

| Site                  | Lat/long | Alt | Size  | Total (young) | Male | Female | mtDNA | msat |
|-----------------------|----------|-----|-------|---------------|------|--------|-------|------|
| Woodbury Common       | WC 50.68 N 3.37 W 174 large | 15 (3) | 2 | 13 | 13 | 11 |
| Blackmoor Reserve     | BM 51.30 N 2.71 W 247 large | 26 | 15 | 11 | 12 | 20 |
| Cranham Common        | CC 51.81 N 2.15 W 200 small | 4 | 0 | 4 | 4 | 4 |
| Crickley Hill         | CH 51.82 N 2.12 W 274 small | 10 | 7 | 3 | 6 | 10 |
| Ewyas Harold          | EH 51.96 N 2.89 W 124 large | 11 | 4 | 7 | 7 | 10 |
| Malvern Hills Swinyard| MHS 52.09 N 2.34 W 251 large | 20 | 12 | 8 | 5 | 17 |
| Bradnor Hill          | BH 52.22 N 3.06 W 292 small | 5 | 1 | 4 | 5 | 7 |
| Bircher Common        | BC 52.30 N 2.78 W 199 small | 9 | 8 | 1 | 6 | 6 |
| Mortimer Forest       | MF 52.36 N 2.74 W 87 large | 18 (1) | 12 | 6 | 6 | 13 |
| Wyre Forest           | WF 52.41 N 2.32 W 139 large | 19 | 18 | 1 | 5 | 16 |
| Pounds Green Coppice  | PGC 52.41 N 2.36 W 97 small | 7 | 4 | 3 | 5 | 6 |
| Kinver Edge           | KE 52.44 N 2.25 W 143 small | 6 | 4 | 2 | 4 | 6 |
| Thundry Meadow        | TM 51.19 N 0.72 W 55 small | 5 | 2 | 3 | 3 | 5 |
| Holt Lowes            | HL 52.89 N 1.10 E 66 large | 27 | 24 | 3 | 5 | 20 |
| Martlesham Heath      | MH 52.06 N 1.26 E 27 small | 9 (1) | 7 | 2 | 6 | 9 |
| Dunwich               | DUN 52.26 N 1.62 E 18 large | 25 | 28 | 1 | 5 | 25 |

The total number of samples for each site is given; the number of samples from young adders (juveniles or subadults) is shown in brackets (included in total).
Lat/long: point GPS coordinates latitude and longitude for site (not for sampling of individual adders); Alt: altitude (metres above sea level) derived from google map for site coordinates.
mtDNA: number of individuals genotyped for mtDNA; msat: number of samples genotyped for microsatellites at minimum of 6 of 8 loci.
size: allocation to MTAC category [34], according to peak springtime time count > 10 = large, ≤ 10 = small.

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sequencing reactions. PCR products were cleaned using a QIAquick PCR purification kit (Qia-
gen, UK), and sequenced using ABI BigDye® chemistry and 3130XL sequencer, following manu-
facturers’ protocols. We generated alignments of concatenated Cytb/CR consensus sequences using MEGA 6 [38]. Individual haplotypes were identified using the haplotype func-
tion of pegas package v0.10 [39], implemented in R v3.4.0 [40]. A haplotype network [41] was con-
structed using the median-joining method [42] in NETWORK v4.6.1 (www.fluxus-
eering.com). Phylogenetic analysis was carried out as described in Supporting Informa-
tion (S1 Table, S1 Fig).

Microsatellite genotyping

In preliminary studies we tested published microsatellite primers that had been developed for adders (V. berus) [43, 44], selecting five polymorphic loci that we found to amplify consistently, with a minimum of stutter bands. To increase the number of loci, we also evaluated 15 congeneric microsatellite markers which had been developed for meadow vipers (V. ursinii) [45], selecting three that successfully amplified and demonstrated polymorphism in adder samples (results not shown). PCR was performed in 10 μl volumes with 20–100 ng DNA, 5 μl mastermix (HotStarTaq Plus or Multiplex; Qiagen), 5 μmol/L unlabelled reverse primer and 5 μmol/L fluorophore-labelled forward primer (Applied Biosystems). Amplification was per-
formed in simplex with initial denaturation 95˚C 5 min, 60 cycles of 94˚C 60 sec, 57–59˚C 60 sec, 72˚C 60 sec, and final extension 72˚C 7 min, optimized in preliminary studies for each primer pair. Primer sequences and locus-specific PCR conditions are summarized in Table 3. Amplified products were resolved by capillary electrophoresis on a 3130xl Genetic Analyser with a LIZ-500 size standard (Applied Biosystems). Alleles were scored and binned manually, using PeakScanner 1.0 software (Applied Biosystems).

Microsatellite data analysis

Quality control. Replicates and template negative controls were included in all plates to confirm reproducibility of results. Results were analysed for genotyping errors and null alleles in Micro-Checker v 2.2.0.3 [46]. We used FSTAT v2.9.3.2 [47] and pegas [39], implemented in R, to test for Hardy-Weinberg equilibrium (HWE), and to exclude linkage disequilibrium.

Measures of population structure and differentiation. In this study, we use the term population to refer to all individuals sampled at a single study site in the sampling time period. Pairwise FST values between populations were estimated in FSTAT.

To test for isolation by distance [48] we applied the mantel.rtest function of ade4 v1.7–13 [49], implemented in R, with 999 repetitions, using pairwise FST to estimate genetic distance. Geographic distance was estimated at https://andrew.hedg es.name/experiments/haversine

Table 2. Primers used in mtDNA PCR and sequencing.

| Locus | Primer | Amplicon |
|-------|--------|----------|
| CR    | 5’-TGC CCC ATG ATT AAG CCG GA-3’ | 349 bp |
|       | 5’-AAC CAG CGG CCT TGG AAA GGA -3’ |
| Cytb  | 5’-CCA AAC CAT TAC TGG ATT CTT CC-3’ | 265 bp |
|       | 5’-ATA GCC GAA GAA GCC TGT TG-3’ |

Primers were designed for this study as described in methods, based on original sequences from Ursenbacher et al (2006) [37]. Amplicon: size of PCR product for each locus.

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To investigate genetic differentiation, we used STRUCTURE v2.3 [51, 52], using correlated allele frequencies and admixture models, with or without the locprior option [53]. The initial alpha was set at 1/n, where n is the number of sample locations, to allow for variation in sample sizes between populations [54]. We used burn-in of $10^5$, followed by $10^6$ iterations for 10 independent replicate runs for values of K from 1 to the number of populations being studied. Results were uploaded to StructureHarvester [55] to derive mean log likelihood and delta-K as a function of K, detecting hierarchical levels of structure [56]. Results across replicate runs were permuted using the greedy function of CLUMPP [57] to derive proportional assignments to each cluster for supported values of K. We also studied population structure using discriminant analysis of principal components (DAPC) [58] in adegenet version 2.0.1 [59] implemented in R. DAPC is a multivariate method to identify clusters of genetically related individuals, which is not based on a predefined model, and makes no assumptions of HWE.

The find.clusters function was applied to determine the optimal number of clusters (k) in each population, independent of the number of sampling sites. The dapc function was then applied, using the $\alpha$-score function to determine the optimum number of principal components to retain in each analysis. Probabilities of assignment of individuals to each of the different clusters were visualised using the compoplot function of adegenet [59].

**Microsatellite summary statistics: Baseline indicators of genetic diversity and inbreeding.** For each population, we estimated F-statistics [60] and allele richness in FSTAT. Confidence intervals for F_{IS}, a measure of intrapopulation heterozygote deficiency due to inbreeding, were calculated using the boot.ppfis function of hierfstat package v0.04–22 in R [61]. Mean allele richness was determined using a rarefaction method [62] in PopGenReport [63], implemented in R. We used FSTAT to compare populations with respect to allele richness and F-statistics, using 1000 permutations.

**Detection of population bottlenecks.** We used BOTTLENECK v 1.2.02 to test for significant heterozygosity excess, applying a one-tailed Wilcoxon test with 1000 iterations, using the two-phase model (TPM) (90% stepwise mutations, variance 10) [64]. A mode-shift test for distortion of the allele frequency distribution [65] was also implemented in BOTTLENECK. We

| Locus | Ref | Origin | Repeat | PCR | n° alleles |
|-------|-----|--------|--------|-----|------------|
| Vu57  | a   | V.ursinii | 2      | 60 MP | 8          |
| Vu4   | a   | V.ursinii | 3      | 60 HS | 11         |
| CA71  | c   | V.berus  | 2      | 60 HS | 11         |
| Vb-B’2 | b  | V.berus  | 2      | 58 HS | 24         |
| CA11  | c   | V.berus  | 2      | 60 MP | 31         |
| CA3   | c   | V.berus  | 2      | 58 HS | 19         |
| Vb-B’10 | b  | V.berus  | 2      | 55 MP | 9          |
| Vu18  | a   | V.ursinii| 2      | 55 MP | 9          |

origin: species for which locus had been developed.
PCR: annealing temperature ºC and PCR buffer system.
MP: Multiplex mastermix (Qiagen), HS HotStarTaqPlus mastermix (Qiagen).
repeat: size of microsatellite repeat motif in nucleotides.
n° alleles: number of alleles for relevant locus in total study dataset.
ref: a) Metzger et al (2011) [45]; b) Ursenbacher et al (2009) [44]; c) Carlsson et al (2003) [43].

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(accessed 23 April 2108) using the Haversine great circle method, a measure of the shortest distance between two points on a sphere [50].
derived M-ratio statistics [66] using the mRatio function of the strataG package v 2.0.2 [67], implemented in R.

**Effective population size (Nₑ).** Two single sample methods were used for estimation of Nₑ. The linkage disequilibrium method [68, 69] was implemented in NeEstimator ver 2.1 [70], assuming random mating, deriving confidence intervals by jack-knifing (1000 iterations). We also used the sibship assignment method [71], which estimates the current effective breeding size of the population, implemented in COLONY 2.0.6.3 [72], using the same input parameters as detailed below for sibship and parentage analysis. Confidence intervals were obtained by bootstrapping.

**Further investigation of breeding between relatives.** We applied the inbreeding function of adegenet, version 2.0.1 [59] in R, to derive genetic estimates of the pedigree inbreeding coefficient FPED, which denotes the probability that both alleles at a single locus are identical by descent from a single ancestor [73]. Pairwise relatedness (Rxy) was estimated using a maximum likelihood method in ML-Relate [74]. To calibrate Rxy values with first degree family relationships in our data, we simulated genotypes for pairs of individuals with defined relationships (100 pairs for each category of unrelated, half-sibling, full-sibling and parent-offspring), using the familysim function of the package related v1.0 [75], implemented in R, based on allele frequency data of observed datasets. Pairwise Rxy between each pair of simulated genotypes of defined relationships was measured using ML-Relate. Means and confidence intervals were derived in R. Significance of within-population relatedness was tested using the grouprel function of related v1.0.

For sibship and parentage analysis we used a full-likelihood method, implemented in COLONY 2.0.6.4 [72], assuming both male and female polygamy. In the absence of known pedigree structure, all individuals for each study population were treated as a single offspring group. We used default settings for sibship priors, including small sibship size, with the aim of reducing false sibship assignments [76]. The outputs of three independent replicate runs, using independent seeds for random number generation, were examined to confirm convergence to the same configuration and log likelihood. The best maximum likelihood cluster configuration was used to infer half- and full sib dyads and inferred parentage.

**Interpretation of results.** We compared our results for F-statistics and allele richness from UKAGP study populations with summary statistics from two published studies of adder populations in mainland Europe [44, 77], and from a site containing a very large (n > 500) population of lowland adders in northern Belgium ([78], Mergey & Bauwens unpublished data). Direct statistical comparison was precluded by only partially overlapping microsatellite panels between the studies (2/8 loci of our study were in common with Ursenbacher et al [44, 77]; 3/8 loci in common with Bauwens et al [78]).

In the UK, the MTAC survey demonstrated opposite average population trends between sites with small and large adder populations, the threshold being a mean normalised peak count of 10 adders, below which there was significant decline over time [34]. This provides an approach of demonstrated demographic relevance with which to classify and compare populations according to their likely risk of decline. At eight sites in our study, more than 10 individuals had been sampled on a single visit (range 11–29). The number of adders sampled at the other eight sites was lower (range 4–10) (Table 1), despite equivalent or higher sampling effort by an experienced ecologist familiar with the sites. We therefore applied an equivalent threshold to categorise UKAGP study populations as large (presumed lower risk) (count > 10, n = 8), or small (presumed higher risk) (count ≤ 10, n = 8) (Table 1), based on the number of adders sampled, as an approximation of the MTAC criteria. We compared first-line summary statistics, FPED and R_xy between small and large populations defined in this way, using a Wilcoxon rank sum test implemented in R.
Although exceeding the MTAC peak count threshold, the springtime counts for the UKAGP populations in the “large” category were still relatively low, with a maximum of 27. In the absence of a very large well-characterised UK population for comparison, we analysed 50 genotypes from the Belgian site [78], focusing primarily on indices of relatedness and inbreeding. This 1570 ha site (“Groot Schietveld”, N 51˚ 20–22’–E 4˚ 32–37’), has been used as a military exercise zone since 1893, and is separated from neighbouring adder populations by a minimum of 18km of unsuitable agricultural habitat. The site is transected by a road, constructed in 1875 as a narrow cobble road, then transformed in 1982 to its present state as a two-lane asphalt trunk road, flanked by two asphalt cycle tracks (total width ca. 20 m). In capture-mark-recapture studies, the road was shown to constitute an effective hard barrier to adder migration (Claus & Bauwens, unpublished data). The sample of 50 Groot Schietveld genotypes (GS50) was selected randomly from the total dataset of microsatellite genotypes of individual adders, which had been sampled from 14 locations within the Groot Schietveld site between 2011 and 2013, and genotyped for 9 microsatellite loci as detailed in Bauwens et al 2018 [78]. The sample included 18 individuals from the north east (NE) segment of the site, and 32 from the south west (SW), relative to the transecting road. No mtDNA sequence data were available for the GS50 sample.

Results

mtDNA haplotypes

Eight different Cytb/CR haplotypes were identified in 97 individuals across the 16 sites (Fig 1). Six haplotypes were unique to single sites. Three sites had more than one Cytb/CR haplotype. All 53 individual samples sequenced from a cluster of ten sites in the West Midlands/South West had the same Cytb/CR haplotype (WMids Haplogroup). As predicted, the UKAGP haplotypes broadly clustered with the mainland Europe Northern phylogenetic clade of V. berus, as described by Ursenbacher et al 2006 [37] (S1 Fig).

Microsatellites

Quality control. There was no evidence for null alleles, allele drop out or linkage disequilibrium between loci. No two samples had identical genotypes. Results from duplicate samples confirmed consistency of genotyping. Samples that failed to amplify at a minimum of 6/8 microsatellite loci were excluded from analysis. In all, 186 samples (84.5%) were retained for downstream analysis. There was no significant divergence from HWE. Two small populations TM (n = 5) and CC (n = 4) had missing data for more than one individual at a single locus (CA71 and Vu4 respectively). For these, genetic diversity was calculated with the omission of the relevant locus, which had little impact on summary statistics for the other populations (S2 Table).

Genetic substructure of WMids Haplogroup.

The WMids Haplogroup includes ten geographically neighbouring sites, separated by up to 100km (Fig 1, Table 4). All individuals tested in these sites shared the same mtDNA haplotype, consistent with their origin from a common ancestor. We therefore investigated this group for evidence of more recent differentiation, using microsatellite markers. Although this haplotype was found two in other study sites, they were not included in this analysis, as both showed evidence for additional haplotypes, and were from less intensively sampled regions, with larger distances between sites.

Genetic differentiation within the WMids Haplogroup is reflected in the pairwise FST matrix between these sites (Table 4). Mantel testing for isolation by distance was negative (r = 0.0042; simulated p value = 0.538). In STRUCTURE there was support for hierarchical clustering [56], both with and without applying the locprior option (Fig 2; S2 Fig). DAPC
analysis also showed clustering within the WMids Haplogroup, optimal at four clusters (Fig 2). To evaluate concordance between STRUCTURE and DAPC results we also applied the `dapc` function for three and six clusters, the optimum values for K in STRUCTURE using the loc-prior option. The population proportional assignments to each cluster by each of the two methods was very similar at the higher hierarchical level of clustering (K = 3), but more divergent for K = 6 (S3 Fig).

**Genetic diversity and inbreeding (F<sub>IS</sub>).** F<sub>IS</sub> values did not differ significantly from zero in any of the UK study populations (Table 5). This is in line with the findings of the mainland Europe study, where only 2/16 sites had been reported to have raised levels of F<sub>IS</sub>, in both cases attributable to high homozygosity at a single locus [44]. Estimates of genetic diversity (H<sub>S</sub> or H<sub>E</sub>) were at similar levels across the study sites, and broadly equivalent to published results from European populations [44, 77], including the large Belgium lowland population [78] (Table 6). Allele richness was in a similar range to that of the large lowland Belgian population, and of the Belgian, Netherlands and northern France lowland populations in the studies of Ursenbacher et al [44, 77], although with the caveat of only partially overlapping microsatellite panels. We found mean allelic richness was lower (p<0.05) in small populations. This was the only statistic for which small and large populations differed significantly, other than for size (Table 7).

**Effective population size N<sub>e</sub>.** The single sample LDNe method [69] failed to deliver plausible results, which may reflect small sample size and high levels of relatedness [79]. Results derived using the sibship assignment method are shown in Table 8. The small populations again gave very wide confidence intervals. Results for SS50 SW and NE by the sibship assignment method are also shown, generating lower results than expected for the very large number of adders on the site, discussed further below. Unfortunately, the combination of small sample size and high relatedness thus prevented us from deriving reliable estimates for effective population size, a significant disadvantage in the study of wild populations.

### Table 4. Genetic differentiation between populations in WMids Haplogroup.

|       | EH  | CH  | KE  | MF  | WF  | PGC | MHS | BC  | BH  |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| EH (11) | 55  | 69  | 45  | 66  | 61  | 43  | 39  | 32  |
| CH (10) | 0.113 | 0.079 | 0.03 | 0.055 | 0.35 | 0.28 | 0.38 | 0.24 |
| KE (6)  | 0.153 | 0.085 | 0.14 | 0.11 | 0.082 | 0.8  | 0.31 | 0.38 | 0.58 |
| MF (13) | 0.015 | 0.049 | 0.071 | 0.091 | 0.119 | 1.04 | 0.136 | 0.35 | 0.50 |
| WF (16) | 0.167 | 0.164 | 0.169 | 0.09 | 0.109 | 0.188 | 0.108 | 0.168 | 0.129 |
| PGC (6) | 0.155 | 0.098 | 0.101 | 0.052 | 0.096 | 0.109 | 0.168 | 0.129 |
| MHS (17) | 0.155 | 0.142 | 0.09 | 0.109 | 0.188 | 0.108 | 0.168 | 0.129 |
| BC (6)  | 0.155 | 0.098 | 0.101 | 0.052 | 0.096 | 0.109 | 0.168 | 0.129 |
| BH (7)  | 0.155 | 0.142 | 0.09 | 0.109 | 0.188 | 0.108 | 0.168 | 0.129 |

Pairwise FST (genetic distance) below diagonal, geographic distance (km) above diagonal. Sample sizes are shown in brackets. Shaded values are significant at p < 0.05 level after 12000 permutations, with Bonferroni correction. Site abbreviations as in Table 1, geographic locations as in Fig 1. Site CC is excluded, due to its small size and missing data.
Population bottlenecks. As small sample size may give rise to false positives in bottleneck tests, we tested different sample sizes randomly selected from the simulated population of 100 unrelated pairs. A sample of 5 genotypes generated a positive result for both heterozygosity excess and modal shift. Results for both tests were negative for simulated samples of 10 or 20 (Table 9). In two of the UKAGP study populations with a sample size of \( \geq 10 \), both heterozygosity excess and allele frequency modal shift tests generated positive bottleneck results (EH, WF). Two (BM, CH) was positive for modal shift only. MRatio results did not discriminate between any of the simulated or study populations, irrespective of size, or results of other bottleneck tests (Table 9).

Further investigation of breeding between relatives. Estimated inbreeding coefficient FPED results showed little variation between populations, irrespective of size (Table 7). The mean population FPED was 0.233 (95% CI 0.218–0.248), although some populations had individual outliers with FPED >0.50 (Fig 3). We found a similar pattern of FPED results for the GS50 sample, again with occasional outliers FPED >0.50. FPED results did not differentiate between simulated populations of 100 pairs of defined relationship, whether unrelated, half- or full sibling (Fig 3).

Intra-population mean \( R_{xy} \) estimates ranged from 0.135 to 0.377 (mean 0.220, 95% CI 0.188–0.252), with no significant difference between large and small populations (Fig 3; Table 7). All UKAGP study populations showed significant within-population relatedness (S3 Table). For the GS50 sample, the mean \( R_{xy} \) between individual samples from the same side of
the transecting road (intra-SW, intra-NE) was significantly higher than for pairwise Rxy across the road (SW-NE) (Fig 4). As expected, and in contrast to FPED, mean Rxy differed significantly between simulated pairs of defined relationship (Fig 3).

Rxy frequency distribution curves of simulated pairs show clear differences between the defined relationships, with a dominant density peak at Rxy = 0 in the unrelated pairs, and a progressive right shift of the curve for the simulated half-sib and full-sib pairs (Fig 5). The Rxy frequency distribution curves for the individual UKAGP study populations showed variable right shift of the curve, in association with and blunting or loss of the unrelated peak at Rxy = 0. An equivalent pattern was apparent in the GS50 SW and NE populations (Fig 5). The GS50 populations were analysed separately, as mean Rxy is affected by genetic structure within a sample.

**Family structure and parentage analysis in COLONY.** Fig 5 illustrates networks of individuals linked by first degree relationships (inferred full or half-sibship) in representative UKAGP study populations, based on the best maximum likelihood configurations in COLONY, shown with their respective Rxy frequency distribution curves. Rxy distribution curves for simulated unrelated, half-sib and full-sib pairs are shown for comparison. These networks are characterised by extensive linkage at the inferred half-sib level in all populations. In some study populations, especially those with a right shift of the Rxy frequency distribution curve, networks show dominant inferred half-sibships, some very large, sharing the same inferred parent.

When the GS50 sample was analysed as a single group in COLONY, the patterns of inferred parentage differed between individuals from the SW and NE sampling sites (Fig 4), providing further evidence that the dividing road acts as a barrier to gene flow. The network of COLONY-derived sibships in the GS50 NE and SW samples also showed a loose pedigree linked at

| Table 5. Summary statistics for individual study populations. |
|-------------|-----|-----|-----|-----|-----|-----|
|             | n   | Ar  | Ho  | Hs  | FIS | FST |
| EH          | 11  | 2.42| 0.63| 0.64| 0.02| 0.03|
| CH          | 10  | 2.75| 0.70| 0.73| 0.05| -0.03|
| WC          | 11  | 2.79| 0.79| 0.73| -0.08| 0.04|
| KE          | 6   | 2.67| 0.67| 0.76| 0.11| -0.10|
| MF          | 13  | 2.73| 0.66| 0.73| 0.09| 0.02|
| BM          | 20  | 2.61| 0.71| 0.69| -0.03| 0.00|
| WF          | 16  | 2.75| 0.75| 0.73| -0.02| 0.04|
| MI          | 9   | 2.60| 0.61| 0.69| 0.12| 0.03|
| PGC         | 6   | 2.61| 0.65| 0.72| 0.10| -0.02|
| DUN         | 25  | 2.60| 0.70| 0.69| -0.02| 0.00|
| HL          | 20  | 2.72| 0.73| 0.72| -0.02| 0.01|
| MHS         | 17  | 2.60| 0.62| 0.69| 0.10| -0.03|
| BC          | 6   | 2.43| 0.65| 0.67| 0.04| -0.03|
| BH          | 7   | 2.61| 0.77| 0.68| -0.13| 0.08|
| TM*         | 5   | 1.99| 0.46| 0.50| 0.09| 0.16|
| CC**        | 4   | 2.36| 0.68| 0.59| -0.15| 0.06|

n: number of samples genotyped at a minimum of 6/8 loci.
TM*: 7 loci, excluding CA71; CC**: 7 loci excluding Vu4.
Ar: allele richness; Ho: observed heterozygosity; Hs: gene diversity (expected heterozygosity); n: sample size.

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the half-sib level, with occasional larger inferred shared-parent sibships, and rare inferred full-sib dyads (Fig 5).

Concordance between COLONY and DAPC cluster membership. We found no evidence for intra-population substructure or admixture on STRUCTURE analysis of the individual UKAGP populations (not shown), nor in the GS50 SW or NE samples, consistent with the low $F_{ST}$ results for individual sites. By contrast, DAPC analysis revealed clustering within the all individual study populations, including GS50 (S4 Fig), despite the low $F_{ST}$ results. We hypothesised that patterns of relatedness might underlie this within-population clustering.

Table 6. Comparison of mean summary statistics between UK study and sites from mainland Europe.

|                     | UK AGP | Ursenbacher 2009 [44] | Ursenbacher 2015 [77] |
|---------------------|--------|----------------------|-----------------------|
|                     | mean   | no                   | mean                  |
|                     | 11.63  | 24.50                | 16.60                 |
|                     | SE     | 1.58                 | 4.09                  |
|                     | Ar     | 2.58                 | 2.98                  |
|                     | Ho     | 0.67                 | 0.50                  |
|                     | Hs     | 0.69                 | 0.52                  |
|                     | FIS    | 0.02                 | 0.01                  |
|                     | FST    | 0.02                 | 0.14                  |
|                     | Comment| current study        | Mainland Europe       |
|                     |        | 16 pops; lowland     | 16 pops; montane      |

Means and standard errors (SE) for equivalent summary statistics between groups of populations from different studies, except Bauwens 2018, where results are from a single large population. Shaded cells denote no comparable information available.

NeA = number of effective alleles; Ho: observed heterozygosity; Hs: gene diversity (expected heterozygosity); F = fixation index.

MC: Massif Central; Jk: Jura Mountains; AC: Atlantic coast; NE: north east France, Belgium and Netherlands (NL); na: not applicable.

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Concordance between COLONY and DAPC cluster membership. We found no evidence for intra-population substructure or admixture on STRUCTURE analysis of the individual UKAGP populations (not shown), nor in the GS50 SW or NE samples, consistent with the low $F_{ST}$ results for individual sites. By contrast, DAPC analysis revealed clustering within the all individual study populations, including GS50 (S4 Fig), despite the low $F_{ST}$ results. We hypothesised that patterns of relatedness might underlie this within-population clustering.

Table 7. Comparison of results between small and large study populations.

| Size | mAR | Ar | Hs (He) | Ho | HoHe | FIS | FST | Rxy | $F_{PED}$ |
|------|-----|----|---------|----|------|-----|-----|-----|-----------|
|      | Small | Large | Small | Large | Small | Large | Small | Large | Small | Large | Small | Large | Small | Large | Small | Large | Small | Large |
| min  | 4    | 11  | 2.45    | 2.74| 1.99 | 2.42| 0.50 | 0.64| 0.46 | 0.62 | 0.88 | 0.90| -0.15| -0.08| -0.10| -0.03| 0.17 | 0.14| 0.18 | 0.19|
| max  | 10   | 25  | 2.97    | 3.03| 2.67 | 2.79| 0.76 | 0.73| 0.77 | 0.79| 1.15 | 1.08| 0.12 | 0.10| 0.16 | 0.01| 0.38 | 0.27| 0.28 | 0.27|
| mean | 6.14 | 15.89| 2.72    | 2.92| 2.47 | 2.66| 0.66 | 0.71| 0.64 | 0.70| 0.97 | 0.99| 0.03 | 0.01| 0.03 | 0.01| 0.25 | 0.19| 0.24 | 0.23|
| SE   | 0.72 | 1.74| 0.05    | 0.04| 0.04 | 0.04| 0.01 | 0.01| 0.02 | 0.02| 0.04 | 0.02| 0.04 | 0.02| 0.03 | 0.01| 0.02 | 0.02| 0.01 | 0.01|
| wcox p| <0.001| <0.05| 0.09 ns| 0.24 ns| 0.17 ns| 0.35 ns| 0.41 ns| 0.53 ns| 0.10 ns| 0.54 ns|

Comparison of results between the eight small and eight large study populations (as detailed in Table 1). mAR: mean allele richness; Ar: allele richness; Hs: observed heterozygosity; Ho: gene diversity; He: expected heterozygosity.

Rxy pairwise relatedness within population; F$_{PED}$: genetic estimate of inbreeding coefficient.

wcox p: p value from comparison between small and large using Wilcoxon test in R; ns: not significant (p value >0.05).

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therefore compared individual cluster membership in DAPC with inferred sibship and parent-age results from COLONY, shown in S5 Fig. The individual membership of large, dominant half-sibships sharing the same inferred parent in COLONY showed striking concordance with that of DAPC clusters. By contrast, for larger populations with looser first-degree relationship networks in COLONY there was poor concordance between COLONY and DAPC cluster membership, illustrated by the results for the large HL population.

**Discussion**

The aim of our study was to investigate the genetic status of lowland adders in the UK, in response to concerns about declining numbers, especially affecting small, fragmented populations. We initially adopted a standard panel of microsatellite-based summary statistics, including genetic diversity and the standard F<sub>IS</sub> measure of inbreeding, to allow comparison with published studies of adders in mainland Europe. We also applied the MTAC criterion of a threshold peak count to categorise study sites into small or large, predicted to be at high or low risk of decline respectively [34]. This initial panel of genetic tests generated a similar pattern of results across all the UKAGP study sites, irrespective of size, although there was a modest decrease in mean allele richness in small populations relative to large. This is likely to have been influenced by the inevitably small sample size, illustrating the difficulty inherent in analysing the unavoidably small sample sizes of the most vulnerable populations.

The interpretation of genetic results requires a biologically relevant comparator, especially for single time-point samples. Estimates of allele richness in the UKAGP study populations

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**Table 8. Estimate of effective population size by sibship assignment method.**

|            | Assuming random mating |                  | Assuming non-random mating |                  |
|------------|------------------------|------------------|---------------------------|------------------|
|            | n          | Ne  | CI 95(L) | CI 95 (H) | Ne   | CI 95(L) | CI 95 (H) | alpha  |
| all        | 186       | 186 | 150      | 240      | 120  | 93       | 154      | 0.19   |
| WMHg       | 96        | 86  | 62       | 116      | 53   | 37       | 80       | 0.2    |
| BC         | 6         | 15  | 3        | 26       | 12   | 5        | >>>      | 0.08   |
| BH         | 7         | 7   | 3        | 26       | 7    | 2        | 30       | 0.01   |
| BM         | 20        | 14  | 7        | 33       | 11   | 5        | 28       | 0.12   |
| CC         | 4         | 8   | 2        | >>>      | 19   | 6        | >>>      | -0.19  |
| CH         | 10        | 4   | 8        | 25       | 6    | 2        | 21       | 0.13   |
| DUN        | 25        | 18  | 9        | 36       | 13   | 7        | 31       | 0.15   |
| EH         | 11        | 10  | 5        | 30       | 7    | 3        | 28       | 0.14   |
| HL         | 20        | 25  | 14       | 30       | 19   | 11       | 42       | 0.09   |
| KE         | 6         | 9   | 4        | 96       | 7    | 2        | 487      | 0.08   |
| MF         | 13        | 12  | 6        | 30       | 8    | 3        | 28       | 0.16   |
| MH         | 9         | 16  | 7        | 64       | 10   | 4        | 43       | 0.2    |
| MHS        | 17        | 16  | 8        | 35       | 10   | 5        | 27       | 0.22   |
| PGC        | 6         | 9   | 4        | 56       | 6    | 2        | 49       | 0.13   |
| TM         | 5         | 40  | 8        | >>>      | 26   | 5        | >>>      | 0.17   |
| WC         | 11        | 10  | 5        | 28       | 9    | 4        | 26       | 0.03   |
| WF         | 16        | 22  | 11       | 54       | 17   | 8        | 41       | 0.1    |
| GS50 SW    | 32        | 14  | 8        | 30       | 12   | 6        | 26       | 0.07   |
| GS50 NE    | 18        | 12  | 6        | 30       | 9    | 4        | 24       | 0.12   |

Effective population size using sibship assignment full likelihood method, with 95% confidence intervals.

all: entire UKAGP dataset; WMHg: WMids Haplogroup; n: sample size; >>>: > 10^9.

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were similar to those of the large Belgian population [78], and of populations of lowland adders in Belgium, NE France, and the Netherlands [77], despite the only partially overlapping panels of microsatellite markers used in the different studies. The mtDNA haplotypes of our study populations places them within the Northern phylogenetic clade of European adders [37]. They are therefore likely to have been part of the same post-glacial recolonization process as their counterparts in north-eastern France, in which a central-marginal effect on genetic diversity has been described [77].

Table 9. Testing for population bottlenecks.

| Individual | Heterozygote excess | Mratio |
|------------|---------------------|--------|
|            | Populations | TPM 10/90 | SMM | Mode shift | Mean | 95% L | 95% H |
| CH         | 10         | 0.32      | 0.37 | shift     | 0.43 | 0.26 | 1.00 |
| EH         | 11         | 0.03      | 0.19 | shift     | 0.63 | 0.25 | 0.99 |
| MF         | 13         | 0.27      | 0.32 | normal    | 0.43 | 0.20 | 0.66 |
| MHS        | 17         | 0.37      | 0.77 | normal    | 0.53 | 0.29 | 0.77 |
| BM         | 20         | 0.16      | 0.27 | shift     | 0.49 | 0.27 | 0.71 |
| WC         | 11         | 0.32      | 0.42 | normal    | 0.48 | 0.27 | 0.69 |
| DUN        | 25         | 0.32      | 0.42 | normal    | 0.43 | 0.27 | 0.60 |
| HL         | 20         | 0.63      | 0.84 | normal    | 0.42 | 0.27 | 0.57 |
| WF         | 16         | 0.00      | 0.01 | shift     | 0.44 | 0.30 | 0.58 |
| sim UR 200 | 200        | 0.77      | 0.99 | normal    | 0.44 | 0.23 | 0.74 |
| sim UR 20  | 20         | 0.41      | 0.81 | normal    | 0.49 | 0.38 | 0.61 |
| sim UR 10  | 10         | 0.53      | 0.68 | normal    | 0.36 | 0.20 | 0.61 |
| sim UR 5   | 5          | 0.10      | 0.27 | shift     | 0.32 | 0.16 | 0.51 |

Top panel: results of tests using three methods (heterozygote excess, mode shift of allele frequency distribution curve, and Mratio) for populations with sample size \( \geq 10 \). Positive results are shaded.

TPM: two phase model; SMM: stepwise mutation model (see methods for details). Results for heterozygote excess are shown as p values (one-tailed Wilcoxon, 1000 iterations).

Bottom panel: results of tests applied to different sample sizes of simulated populations, comprising individual genotypes drawn randomly from 100 simulated unrelated pairs, based on the total UKAGP dataset (for which bottleneck tests were negative with all methods), showing false positive results for sample size of 5.

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Fig 3. Inbreeding and pairwise relatedness. Box and whisker plots of genetic estimates of FPED (A), and for pairwise relatedness Rxy (B) for the study populations (open box) GS50 SW and NE (light shading), and for 100 simulated pairs of genotypes of defined relationship (dark shading). Individual outliers are shown as open circles.

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Notably, F<sub>IS</sub> levels were not significantly above zero in any of the UKAGP study populations, irrespective of size, indicating retention of heterozygosity, despite the clear risk of inbreeding depression in fragmented, isolated populations. Our findings are again consistent with published studies of remnant adder populations of varying sizes in mainland Europe [44, 77]. In small isolated populations of species with high philopatry and low vagility, like the adder, mating opportunities will inevitably be biased towards relatives [80, 81], and total avoidance of consanguineous mating could rapidly lead to population extinction [82]. We therefore investigated other measures of consanguinity, a very valuable comparison for the effect of size being provided by the Belgian adder population, isolated but thriving, well-studied, and very much larger than the UKAGP study populations [78].

Identity by descent: Genetic legacy of population demographic history

In all the UK study populations, genetic estimates of the mean inbreeding coefficient FPED were at a level consistent with the degree of genetic identity expected in the offspring of a one-off mating between half siblings [73]. However, the nature of the defined relationship of simulated pairs of genotypes, whether unrelated, full-sibling or half-sibling, did not appreciably influence FPED estimates. This is in keeping with FPED estimates being a measure of cumulative identity by descent, the embedded genetic legacy of long-standing consanguineous breeding. In the WMids Haplogroup, for example, the shared mtDNA haplotype provides evidence for a historic common ancestor, which could date back to as early as post-glacial colonisation.

Fig 4. GS50: Dividing road acts as barrier to gene flow. (A) Bar chart of inferred parentage of individuals of GS50 sampled from NE and SW of the transecting road. The best-supported cluster in COLONY for the full GS50 sample inferred a total of 10 parents of one sex (parent A), and 12 of the other sex (parent B). The bar charts show the number of individuals (“offspring”) from the NE (blue) and SW (orange) assigned to each inferred parent. The difference in inferred parentage is in keeping with the road acting as a barrier to gene flow. (B) Significantly lower mean pairwise R<sub>xy</sub> sampled from different sides of the dividing road (SW to NE (shaded)) than between individuals sampled from the same side of the road (intra SW, intra NE), again consistent with the road acting as barrier to gene flow.
The Belgian site similarly represents a relict population, probably isolated for more than a century [78].

**Identity by state: Reflection of contemporary relatedness**

By contrast pairwise relatedness in simulated pairs of genotypes was significantly and predictably influenced by the defined relationship, providing a very useful template against which to interpret Rxy results for the study populations. We found a range of patterns of relatedness, with variable loss or blunting of the modal unrelated peak seen in the simulated unrelated pairs of genotypes, and right shift of the Rxy distribution curve seen in the half- or full-sibling simulated pairs. Pairwise Rxy thus generated the most informative results in our study, with the potential caveat that results may be influenced by cryptic genetic differentiation within the sample. Repeat studies will be necessary to determine trends and timescales in changing patterns of relatedness.

COLONY results, like Rxy, provide a snapshot estimate of contemporary relatedness between individuals within the sample, but in the format of best maximum likelihood combinations of inferred genotypes of sibling and parent-offspring dyads. This approach generated dramatic networks of inferred half-sibships in our study populations. However, in the absence of pedigree data to inform COLONY analysis, loose networks of inferred half sibships may
simply reflect identity by state, rather than true first-degree relationships, especially for inferred half sibships in larger populations [76]. While we sought to minimise this phenomenon by applying stringent parameters for sibship assignment in COLONY, it is likely to have been exacerbated by the presence of a high level of background relatedness, as well as by the relatively limited number of genetic markers. This high level of relatedness is also the likely explanation for the unexpectedly low estimates of Ne by the sibship method for the GS50 populations.

We found the membership of inferred dominant large sibships in small populations to be concordant with the assignment of individuals to clusters in DAPC, in keeping with DAPC clusters reflecting allele frequency patterns driven by a polygynandrous mating system in a small population, rather than discrete panmictic subpopulations. An equivalent phenomenon of clustering in DAPC, but not STRUCTURE, has also been described in the Prairie rattlesnake (Crotalus viridis) [83]. This is an important consideration when DAPC is used to investigate cryptic genetic structure within consanguineous populations.

**Inbreeding depression: Protective effect of polyandry**

As a single year snapshot, our study is only a starting point, and inevitably limited by population size, and thus sample size, especially in the study of the most vulnerable populations. We have nevertheless exposed a previously undocumented degree of consanguinity in wild adder populations, despite their showing no increase in homozygosity to suggest inbreeding depression. In models of inbreeding, a system of half-sib mating is more likely to maintain heterozygosity than one of maximum avoidance of inbreeding [84, 85]. Polyandry, which is widespread in taxa of live-bearing snakes [86], including the adder [87–89], may thus represent a protective mechanism against inbreeding [82, 90]. Interestingly, Mourier et al (2013) described a very similar network pattern of extensive pairwise relatedness between individuals of the sicklefin lemon shark (Negaprion acutidens) in French Polynesia [91]. Despite the biological differences, there are clear similarities in the reproductive ecology between the taxa, the lemon shark also being viviparous, of low female fecundity, with a polyandrous mating system and limited distribution [91].

**Inbreeding depression: Size matters**

While polyandry may delay, it will not prevent the eventual loss of heterozygosity in isolated populations, where movement of adders is prevented by loss of connectivity between patches of fragmented habitat. It is therefore interesting to compare the large, thriving Belgian site, with high levels of relatedness but no loss of heterozygosity, with the well-documented Swedish population of adders with unequivocal evidence for inbreeding depression [14–16]. Both sites have been isolated for more than a century by agricultural landscape, are situated some 20km from neighbouring adder populations [15, 78], and both would fulfil the MTAC definition of a large population [34]. However, at 1570ha, the Belgian site is significantly larger than its 20ha Swedish counterpart, which provides the likely explanation for the difference in inbreeding. As the Belgian site has more recently been subjected to asymmetric fragmentation by the truncating road, it will be especially interesting to monitor the genetic status of the smaller NE fragment in comparison with its larger SW counterpart unless gene flow can be restored across the truncating road.

**Safety in numbers: Demographic vulnerability of smaller adder populations**

“Unfortunately, the best way to find tipping points so far has been to cross them—a dangerous proposition” [92]. Our findings suggest that genetic factors are unlikely to be the direct cause...
of the observed decline in small populations of adders. Instead, small populations may already be “doomed to extinction by demographic factors before genetic effects act strongly” [93], representing the “living dead” [94, 95], where continuation of a population or metapopulation becomes demographically impossible. For example, the reproductive ecology of adders renders small populations profoundly vulnerable to stochastic sex bias [96–98]. In adders, males are the actively mate-seeking sex [99, 100], with only a short interval of female sexual receptiveness, and thus limited time available for mating, while female adders have low lifetime fecundity, with high fitness costs of reproduction [101, 9, 10], suggesting particular vulnerability of small adder populations to a limiting number of females. Conversely, a relative reduction in males would be predicted to impact on any protective effect of polyandry against inbreeding. Sampling over a limited period precluded an accurate field assessment of sex ratios in our study, but we are addressing this question of breeding sex ratios in ongoing work.

Future studies
We are using radio-telemetry of adult snakes to inform habitat management, especially with respect to connectivity [100], in combination with ongoing genetic monitoring of study populations. This will allow us to investigate the reproductive success of potential mating connections, generating very interesting data with respect to the breeding system in this secretive species, including assortative mate choice, overt or cryptic. Pedigree information will also enhance the interpretation of results in COLONY, including estimates of the effective numbers of breeding adults.

We plan to use genomic sequencing to increase the number of informative markers available, especially important for genetic diversity and pedigree studies. In addition, the development of a genomic SNP panel will help to increase consistency and comparability across sites and laboratories, currently hampered by the limited numbers of microsatellite markers, and potential inconsistencies in their application [102–104]. This will be especially important in decision making and post-release monitoring in any future adder translocations, whether for reasons of mitigation or conservation [105–107]. A panel of genomic SNPs will also allow the investigation of heterozygosity affecting different loci [104], facilitating the study of inbreeding at the whole genome level. In addition, the pattern of runs of homozygosity [108–112] will help to elucidate the demographic history of this fascinating species, as well as the identification of potential targets of selection [113].

Conclusions
Our results suggest that the most immediate threat to small adder populations is demographic rather than genetic. For larger populations high levels of relatedness indicate that genetic factors are likely to represent a real threat, albeit less imminent, but also less visible and thus more insidious [22]. Continuing monitoring will be essential to determine the urgency and nature of intervention. Our study thus underscores the need for a systematic, evidence-driven approach in conservation planning for adder populations, whether healthy or declining, integrating population genetics and traditional ecology [33]. In this the “true cost of loss and degradation of habitat” [13] should not be neglected, including public engagement to reduce persecution by changing the public perception of snakes [114]. Attempts at genetic or demographic rescue may be similarly doomed unless such underlying factors can be addressed [115].

Supporting information
S1 Fig. Phylogenetic tree of UKAGP showing mtDNA haplotypes relative to European clades. Bootstrap consensus tree of UKAGP concatenated Cytb/CR haplotypes (500 replicates)
relative to homologous sequences in Genbank.

(PPTX)

**S2 Fig. Effect of locprior on STRUCTURE results.** Bar charts showing proportional membership coefficients of individuals to each of the inferred clusters for $K = 3$–6, grouped according to their study population, with locprior option (left) and without (right). Hierarchical clustering is apparent with both approaches. The colour schemes differ between bar charts.

(PPTX)

**S3 Fig. Concordance between STRUCTURE and DAPC.** Pie charts for each population of the WMids Haplogroup, showing the proportion of group membership assigned probabilistically to $K = 3$ or $K = 6$ clusters in analysis applying the locprior option. In each panel the populations are superimposed on a Venn diagram of overlapping circles according to their broad proportional membership of the three clusters inferred in STRUCTURE for $K = 3$. The colour schemes are independent for STRUCTURE and DAPC. Top left panel: pie charts for $K = 3$ in STRUCTURE. Top right panel: pie charts for $K = 3$ in DAPC. Bottom left panel: pie charts for $K = 6$ in STRUCTURE. Top right panel: pie charts for $K = 6$ in DAPC.

(PPTX)

**S4 Fig. DAPC clustering in individual populations.** DAPC scatterplots ($K = 3$) for individual study populations with sample size $\geq 10$, including GS50 SW and NE, showing clearly separated clusters.

(PPTX)

**S5 Fig. Concordance between COLONY and DAPC.** For each population, the network of COLONY-inferred sibship dyads is shown, together with a table (upper) of inferred parentage for each individual, and their assignment to one of the clusters of individuals linked at a minimum of half-sibling level. Dominant hypothetical parents are highlighted in the parentage table and network. The lower table for each population shows bar plots of the probability of assignment of each individual (in same order as in COLONY) to DAPC clusters. For population CH (top left), the assignment of individuals to $K = 2$ clusters in DAPC is concordant with the COLONY-defined clusters, assignment of individuals to the largest of $K = 3$ clusters in DAPC is concordant with the hypothetical dominant parent of the inferred family structure in COLONY. Different patterns of concordance are evident in populations MHS, BM, EH and MF. By contrast, for the larger population HL (bottom right), the membership of DAPC clusters shows poor concordance with the inferred parentage of the larger, looser COLONY network.

(PPTX)

**S1 Table. Genbank sequences used in alignment for phylogenetic tree.**

(PPTX)

**S2 Table. Effect on summary statistics of removal of loci with missing data.**

(DOCX)

**S3 Table. Matrix of mean pairwise relatedness within and between populations.**

(DOCX)

**S4 Table. Excel spreadsheet of UKAGP microsatellite genotypes.**

(XLSX)

**S1 Text. Alignment of UKAGP concatenated Cytb/CR haplotypes (fas format).**

(TXT)
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References

1. Ralls K, Ballou JD, Dudash MR, Eldridge MDB, Fenster CB, Lacy RC, et al. Call for a Paradigm Shift in the Genetic Management of Fragmented Populations. Conservation Letters. 2017; 11(April): 1–6. http://doi.org/10.1111/conl.12412

2. Frankham R. Genetics and extinction. Biological Conservation. 2005; 126(2): 131–140. https://doi.org/10.1016/j.biocon.2005.05.002

3. Amos W, Balmford A. When does conservation genetics matter? Heredity. 2001; 87(3): 257–265. https://doi.org/10.1046/j.1365-2540.2001.00940.x

4. Gilpin ME, Soule M. Minimum viable populations: Processes of species extinction. In Soule ME, editor. Conservation Biology: The Science of Scarcity and Diversity. Sinauer Associates, Inc., Sunderland, MA, USA, 1986. p.19–34.

5. Tingley R, Meiri S, Chapple DG. Addressing knowledge gaps in reptile conservation. Biological Conservation. 2016; 204: 1–5. http://doi.org/10.1016/j.biocon.2016.07.021
6. Sillero N, Campos J, Bonardi A, Corti C, Creemers R, Crochet PA, et al. (2014). Updated distribution and biogeography of amphibians and reptiles of Europe. Amphibia Reptilia. 2014; https://doi.org/10.1163/15685381-0002940

7. Isailovic JC, Vogrin M, Corti C, Sá-Sousa P, Cheylan M, Pleguezuelos JM, et al. Vipera berus. The IUCN Red List of Threatened Species 2009: e.T157248A5059709. Downloaded on 11 November 2019.

8. Cooke AS, Arnold R. National changes in the status of the common British amphibians and reptiles before 1974. British Journal of Herpetology. 1982; 6:206–207.

9. Madsen T, Shine R. Determinants of reproductive success in female adders, Vipera berus. Oecologia, 1992; 92(1): 40–47. https://doi.org/10.1007/BF00317260 PMID: 28311810

10. Bauwens D, Claus K. Intermittent reproduction, mortality patterns and lifetime breeding frequency of females in a population of the adder (Vipera berus). Peer J. 2019; 7(1): 99–112.

11. Reading C, Luiselli J, Akani LMM, Bonnet GC, Amori X, Ballouard G, et al. Are snake populations in widespread decline? Biology Letters. 2010; 6(6): 777–780. https://doi.org/10.1098/rsbl.2010.0373 PMID: 20534600

12. Todd BD, Nowakowski AJ, Rose JP, Price SJ. Species traits explaining sensitivity of snakes to human land use estimated from citizen science data. Biological Conservation. 2017; 206, 31–36. https://doi.org/10.1016/j.biocon.2016.12.013

13. Clemann N. Cold-blooded indifference: A case study of the worsening status of threatened reptiles from Victoria, Australia. Pacific Conservation Biology. 2015; 21(1): 15–26. http://doi.org/10.1071/PC14901

14. Madsen T, Still B, Shine R. Inbreeding depression in an isolated population of adders Vipera berus. Biological Conservation. 1996; 75(2):113–118. http://doi.org/10.1016/0006-3207(95)00067-4

15. Madsen T, Shine R, Olsson M, Wittzell HH, Reynolds AJ, Lawrence C, et al. Restoration of an inbred adder population. Nature. 1999; 402:34–35. https://doi.org/10.1038/46941

16. Madsen T, Újvári B, Olsson M. Novel genes continue to enhance population growth in adders (Vipera berus). Biological Conservation. 2004; 120(1):145–147. http://doi.org/10.1016/j.biocon.2004.01.022

17. Újvári B, Madsen T, Kotenko T, Olsson M, Shine R, Wittzell H. Low genetic diversity threatens imminent extinction for the Hungarian meadow viper (Vipera ursinii rakosiensis). Biological Conservation, 2002; 105(1):127–130. https://doi.org/10.1016/S0006-3207(01)00176-8

18. Shaffer ML. Minimum Population Sizes for Species Conservation. BioScience, 1981; 31(2):131–134. https://doi.org/10.2307/1308256

19. Traili LW, Brook BW, Frankham RR, Bradshaw CJA. Pragmatic population viability targets in a rapidly changing world. Biological Conservation, 2010; 143(1): 28–34. https://doi.org/10.1016/j.biocon.2009.09.001

20. Wright S. Evolution in mendelian populations. Genetics. 1931; 16:97–159. https://doi.org/10.1007/BF02459575 PMID: 17246615

21. Berger J. Persistence of Different-Sized Populations: An empirical assessment of rapid extinctions in bighorn sheep. Conservation Biology. 1990; 4(1):91–98.

22. Taylor HR, Colbourne RM, Robertson HA, Nelson NJ, Allendorf FW, Ramstad KM. Cryptic inbreeding depression in a growing population of a long-lived species. Molecular Ecology., 2017; 26(3):799–813. https://doi.org/10.1111/mec.13977 PMID: 28093817

23. Frankham R. Effective population size/adult population size ratios in wildlife: A review. Genetics Research. 2008; 89(5–6):491–503. https://doi.org/10.1017/S0016672308009695

24. Palstra FP, Fraser DJ. Effective/census population size ratio estimation: A compendium and appraisal. Ecology and Evolution. 2012; 2(9):2357–2365. https://doi.org/10.1002/ece3.329 PMID: 23139893

25. Álvarez D, Lourenço A, Oro D, Velo-Antón G. Assessment of census (N) and effective population size (Ne) reveals consistency of Ne single-sample estimators and a high Ne/N ratio in an urban and isolated population of fire salamanders. Conservation Genetics Resources. 2015; 7(3):705–712. https://doi.org/10.1007/s12686-015-0480-0

26. Wang J, Santiago E, Caballero A. Prediction and estimation of effective population size. Heredity. 2016; 117(4):193–206. https://doi.org/10.1038/hdy.2016.43 PMID: 27353047

27. Hoffmann AA, Szegö CM. Climate change and evolutionary adaptation. Nature. 2011; 470:479–485. https://doi.org/10.1038/nature09670 PMID: 21350480

28. Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, et al. Emerging fungal threats to animal, plant and ecosystem health. Nature. 2013; 484(7393):1–18. https://doi.org/10.1038/nature10947.
29. Lande R. Genetics and demography in biological conservation. Science. 1988; 241(4872):1455–1460. https://doi.org/10.1126/science.3420403 PMID: 3420403

30. Arnold HR. Atlas of amphibians and reptiles in Britain. Institute of Terrestrial Ecology Research Publication no. 10. Natural Environment Research. 1995.

31. Baker J, Suckling J, Carey R. Status of the adder Vipera berus and slow-worm Anguis fragilis in England. English Nature Research Reports, report 546. 2004.

32. Wilkinson JW, Arnell AP. NARRS Report 2007–2008: Interim results of the UK National Amphibian and Reptile Recording Scheme Widespread Species Surveys. ARC Research Report 11/01. 2011.

33. Gleed-Owen C, Langham S. The Adder Status Project—a conservation condition assessment of the adder (Vipera berus) in England, with recommendations for future monitoring and conservation policy. Report to Amphibian and Reptile Conservation. ARC, Bournemouth, UK. 2012.

34. Gardner E, Julian A, Monk C, Baker J. Make the adder count: population trends from a citizen science survey of UK adders. Herpetological Journal. 2019; 29:57–70.

35. Franklinos LHV, Lorch JM, Bohuski E, Fernandez JRR, Wright ON, Fitzpatrick L, et al. Emerging fungal pathogen Ophidiomyces ophiodiicola in wild European snakes. Scientific Reports. 2017; 7(1). https://doi.org/10.1038/s41598-017-03352-1.

36. Miller HC. Cloacal and buccal swabs are a reliable source of DNA for microsatellite genotyping of reptiles. Conservation Genetics. 2006; 7(6):1001–1003. http://doi.org/10.1007/s10592-006-9120-2.

37. Ursenbacher S, Carlsson M. Helpers V, Tegelström H, Fumagalli L. Phylogeography and Pleistocene refugia of the adder (Vipera berus) as inferred from mitochondrial DNA sequence data. Molecular Ecology. 2006; 15(11):3425–3437. https://doi.org/10.1111/j.1365-294X.2006.03031.x PMID: 16968280

38. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 2013; 30(12):2725–2729. https://doi.org/10.1093/molbev/msv059.

39. Paradis E. Pegas: An R package for population genetics with an integrated-modular approach. Bioinformatics. 2010. http://doi.org/10.1093/bioinformatics/btp696

40. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2017. https://www.R-project.org/.

41. Posada D, Crandall KA. Intraspecific gene genealogies: Trees grafting into networks. Trends in Ecology & Evolution. 2001; 16:37–45.

42. Bandelt HJ, Forster P, Röhl A. Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution. 1999; 16, 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036 PMID: 10331250

43. Carlsson M, Isaksson M, Höggren M, Tegelström H. Characterization of polymorphic microsatellite markers in the adder, Vipera berus. Molecular Ecology Notes. 2003; 3:73–75.

44. Ursenbacher S, Monney JC, Fumagalli L. Limited genetic diversity and high differentiation among the remnant adder (Vipera berus) populations in the Swiss and French Jura Mountains. Conservation Genetics, 2009; 10(2), 303–315. http://doi.org/10.1007/s10592-008-9580-7.

45. Metzger C, Ferchaud AL, Geiser C, Ursenbacher S. New polymorphic microsatellite markers of the endangered meadow viper (Vipera ursinii) identified by 454 high-throughput sequencing: when innovation meets conservation. Conserv Genet Resour. 2011; 3:589–592

46. Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P. Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes. 2004; 4:535–538.

47. Goudet J. FSTAT, version 2.9.3. A program to estimate and test gene diversities and fixation indices. 2001. Lausanne University, Lausanne, Switzerland.

48. Mantel N. The detection of disease clustering and a generalized regression approach. Cancer Research.1967; 27:209–220 PMID: 6018555

49. Dray S, Dufour A. The ade4 Package: Implementing the Duality Diagram for Ecologists. Journal of Statistical Software, 2007; 22(4):1–20 https://doi.org/10.18637/jss.v022.i04

50. Sinnott RW. Virtues of the Haversine. Sky and Telescope, 1984; 68(2).

51. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000; 155: 945–959. https://doi.org/10.1111/j.1471-8286.2000.01759.x PMID: 10835412

52. Falush D, Stephens M, Pritchard JK. Inference of population structure using multilocus genotype data: Dominant markers and null alleles. Molecular Ecology Notes. 2007; 7(4):574–578. https://doi.org/10.1111/j.1471-8286.2007.01758.x PMID: 18784791
53. Hubisz MJ, Falush D, Stephens M, & Pritchard JK. Inferring weak population structure with the assistance of sample group information. Molecular Ecology Resources, 2009; 9(5):1322–1332. https://doi.org/10.1111/j.1755-0998.2009.02591.x PMID: 21564903

54. Wang J. The computer programme STRUCTURE for assigning individuals to populations: easy to use but easier to misuse. Molecular Ecology. 2017; 17(5): 981–990. https://doi.10.1111/1755-0998.12650

55. Earl DA, von Holdt BM. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources. 2012; 4(2):359–361. https://doi.org/10.1007/s12686-011-9548-7

56. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. Molecular Ecology. 2005; 14(8):2611 –2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x PMID: 15969739

57. Jakobsson M, Rosenberg NA. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics. 2007; 23:1801–1806. https://doi.org/10.1093/bioinformatics/btm233 PMID: 17465489

58. Jombart T, Devillard S, Balloux F. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genetics. 2010; 11(1):94. http://doi.org/10.1186/1471-2156-11-94

59. Jombart T. Adegenet: A R package for the multivariate analysis of genetic markers. Bioinformatics. 2008 24(11):1403–1405. https://doi.org/10.1093/bioinformatics/btn129 PMID: 18397895

60. Weir BS, Cockerham CC. Estimating F-Statistics for the Analysis of Population Structure. Evolution. 1984; 38(6):1358 –70. https://doi.org/10.1111/j.1558-5646.1984.tb05657.x PMID: 28563791

61. El Mousadik A, Petit RJ. High level of genetic differentiation for allelic richness among populations of the argan tree [Argania spinosa (L.) Skeels] endemic to Morocco. Theoretical and Applied Genetics. 1996; 92(7):832–839. https://doi.org/10.1007/BF00221895 PMID: 24166548

62. Joly DA, Gruber B. PopGenReport: Simplifying basic population genetic analyses in R. Methods in Ecology and Evolution. 2014; 5(4):384–387. https://doi.org/10.1111/2041-210X.12158

63. Piry S, Luikart G. BOTTLENECK: A Computer Program for Detecting Recent Reductions in the Effective Population Size Using Allele Frequency Data. Journal of Heredity. 1999; 90(4):502–503.

64. Luikart G, Allendorf FW, Cornuet J, Sherwin WB. Distortion of Allele Frequency Distributions Provides a Test for Recent Population Bottlenecks. Journal of Heredity. 1998; 89(3):238–247. https://doi.org/10.1093/jhered/89.3.238 PMID: 9656466

65. Garza JC, Williamson EG. Detection of reduction in population size using data from microsatellite loci. Molecular Ecology. 2001; 10:305–318. https://doi.org/10.1046/j.1365-294X.2001.01190.x PMID: 11298947

66. Archer FI, Adams PE, Schneiders BB. strataG: An R package for manipulating, summarizing and analysing population genetic data. Mol Ecol Resour. 2016; https://doi.org/10.1111/1755-0998.12559 PMID: 27327208

67. Hill WG. Estimation of effective population size from data on linkage disequilibrium. Genetics Research, 1981; 38(3), 209–216. https://doi.org/10.1017/S0016672300020553

68. Waples RS, Do C. LDNE: A program for estimating effective population size from data on linkage disequilibrium. Molecular Ecology Resources. 2008; 8(4):753–756. https://doi.org/10.1111/j.1755-0998.2007.02061.x PMID: 18585883

69. Do C, Waples RS, Peel D, Macbeth GM, Tillet BJ, Ovenden JR. NeEstimator v2: Re-implementation of software for the estimation of contemporary effective population size (Ne) from genetic data. Molecular Ecology Resources. 2014; 14(1):209–214. https://doi.org/10.1111/1755-0998.12157 PMID: 23992227

70. Wang J. A new method for estimating effective population sizes from a single sample of multilocus genotypes. Molecular Ecology. 2009; 18(10):2148–2164. https://doi.org/10.1111/j.1365-294X.2009.04175.x PMID: 19389175

71. Jones OR, Wang J. COLONY: A program for parentage and sibship inference from multilocus genotype data. Molecular Ecology Resources. 2010; 10(3):551–555. https://doi.org/10.1111/j.1755-0998.2009.02787.x PMID: 21565056

72. Frankham R, Ballou JD, Briscoe DA. Introduction to conservation genetics ( 2nd ed.). Cambridge, UK: Cambridge University Press. 2010

73. Kalinowski ST, Wagner AP, Taper ML. ML-RELATE: A computer program for maximum likelihood estimation of relatedness and relationship. Molecular Ecology Notes. 2006; 6(2):576–579. https://doi.org/10.1111/j.1471-8286.2006.01296.x
75. Pew J, Muir PH, Wang J, Frasier TR. related: an R package for analysing pairwise relatedness from codominant molecular markers. Mol Ecol Resour. 2015; 15: 557–561. https://doi.org/10.1111/1755-0998.12323 PMID: 25186958

76. Wang J. Users’ guide for software COLONY Version 2.0.6.4 Software. 2017. http://www.zsl.org/science/research-projects/software/

77. Ursenbacher S, Guillon M, Cubizolle H, Dupoué A, Blouin-Demers G, Lourdais O. Postglacial recolonization in a cold climate specialist in western Europe: Patterns of genetic diversity in the adder (Vipera berus) support the central-marginal hypothesis. Molecular Ecology. 2015; 24:3639–51. https://doi.org/10.1111/mec.12325 PMID: 26053307

78. Bauwens D, Claus K, Mergeay J. Genotyping validates photo-identification by the head scale pattern in a large population of the European adder (Vipera berus). Ecology and Evolution. 2018; 8(5):2985–2992 https://doi.org/10.1002/ece3.3917 PMID: 29531711

79. Wang J. Effects of sampling close relatives on some elementary population genetics analyses. Molecular Ecology Resources. 2018; 18(1):41–54. https://doi.org/10.1111/1755-0998.12708 PMID: 28776944

80. Brouwer L, Van De Pol M, Atema E, Cockburn A. Strategic promiscuity helps avoid inbreeding at multiple levels in a cooperative breeder where both sexes are philopatric. Molecular Ecology, 2011; 20(22):4796–4807. https://doi.org/10.1111/j.1365-294X.2011.05325.x PMID: 22008256

81. Duthie AB, Bocedi G, Reid JM. When does female multiple mating evolve to adjust inbreeding? Effects of inbreeding depression, direct costs, mating constraints, and polyandry as a threshold trait. Evolution; International Journal of Organic Evolution. 2016; 70(9):1927–1943. https://doi.org/10.1111/evol.13005 PMID: 27464756

82. Stockley P, Searle J, Macdonald DW, Jones C. Female multiple mating behaviour in the common shrew as a strategy to reduce inbreeding. Proceedings. Biological Sciences. 1993; 254(1341):173–179. https://doi.org/10.1098/rspb.1993.0143 PMID: 8108451

83. Stockley P, Searle J, Macdonald DW, Jones C. Female multiple mating behaviour in the common shrew as a strategy to reduce inbreeding. Proceedings. Biological Sciences. 1993; 254(1341):173–179. https://doi.org/10.1098/rspb.1993.0143 PMID: 8108451

84. Weyer J, Jørgensen D, Schmitt T, Maxwell TJ, Anderson CD. Lack of detectable genetic differentiation between den populations of the Prairie Rattlesnake (Crotalus viridis) in a fragmented landscape. Canadian Journal of Zoology. 2014; 92(10):837–846. https://doi.org/10.1139/cjz-2014-0025

85. Theodorou K, Couvet D. The efficiency of close inbreeding to reduce genetic adaptation to captivity. Heredity. 2015; 114(1):38–47. https://doi.org/10.1038/hdy.2014.63 PMID: 25052417

86. Wusterbarth TL, King RB, Duvall MR, Grayburn WS, Burghardt GM. Phylogenetically widespread multiple paternity in New World natricine snakes. Herpetological Conservation and Biology. 2010; 5:86–93.

87. Stille B, Madsen T, Niklasson M. Multiple paternity in the adder, Vipera berus. Oikos, 1986; 47(2):173–175. http://doi.org/10.2307/3566042

88. Höggren M, Tegelstrom H. DNA fingerprinting shows within-season multiple paternity in the Adder (Vipera berus). Copeia. 1995; 2:271–277.

89. Ursenbacher S, Erny, Fumagalli L. Male reproductive success and multiple paternity in wild, low-density populations of the Adder (Vipera berus). Journal of Heredity. 2009; 100(3):365–370. https://doi.org/10.1093/jhered/esn104 PMID: 19074755

90. Tregenza T, Wedell N. Polyandrous females avoid costs of inbreeding. Nature. 2002; 415:71–73. https://doi.org/10.1038/415071a PMID: 11780118

91. Mourier J, Buray N, Schultz JK, Clua E, Planes S. Genetic Network and Breeding Patterns of a Sickle-fin Lemon Shark (Negaprion acutidens) Population in the Society Islands, French Polynesia. PLoS ONE, 2013; 8(8):1–11. https://doi.org/10.1371/journal.pone.0073899

92. Strange CJ. Facing the Brink without Crossing It. BioScience. 2007; 57(11):920. http://doi.org/10.1641/B571103

93. Wootton TJ, Pfister CA. Experimental separation of genetic and demographic factors on extinction risk in wild populations. Ecology. 2013; 94(10):2117–2123. https://doi.org/10.1890/12-1828.1 PMID: 24358695

94. Janzen DH. Latent Extinction- The Living Dead. In Encyclopedia of Biodiversity: Second Edition. 2001. https://doi.org/10.1016/B978-0-12-384719-5.00085-X

95. Hanski I, Ovaskainen O. Extinction debt at extinction threshold. Conservation Biology. 2002; 16:666–673 https://doi.org/10.1046/j.1523-1739.2002.00342.x

96. Legendre S, Clobert J, Meller AP, Sorci G. Demographic Stochasticity and Social Mating System in the Process of Extinction of Small Populations: The Case of Passerines Introduc ted to New Zealand. The American Naturalist, 1999; 153(5):449–463. https://doi.org/10.1086/303195 PMID: 29578793
97. Lee AM, Sæther BE, Engen S. Demographic Stochasticity, Allee Effects, and Extinction: The Influence of Mating System and Sex Ratio. The American Naturalist, 2011; 177(3):301–313. https://doi.org/10.1086/658344 PMID: 21460539

98. Boukal DS, Berec L. Single-species models of the Allee effect: Extinction boundaries, sex ratios and mate encounters. Journal of Theoretical Biology. 2002; 218(3):375–394. https://doi.org/10.1006/jtbi.2002.3084 PMID: 12381437

99. Madsen T, Shine R, Loman J, Håkansson T. Determinants of mating success in male adders, Vipera berus. Animal Behaviour. 1993; 45(3):491–499. https://doi.org/10.1006/anbe.1993.1060

100. Hand, N. The secret life of the adder (Vipera berus) revealed through telemetry. The Glasgow Naturalist. 2018. The Amphibians and Reptiles of Scotland, 27, supplement.

101. Prestt I. An ecological study of the viper Vipera berus in southern Britain. Journal of Zoology. 1971; 164:373–418.

102. Hoffman JL, Amos W. Microsatellite genotyping errors: Detection approaches, common sources and consequences for paternal exclusion. Molecular Ecology. 2005; 14(2):599–612. https://doi.org/10.1111/j.1365-294X.2004.02419.x PMID: 15660949

103. Guichoux E, Lagache L, Wagner S, Chaumet P, Léger P, Lepais O, et al. Current trends in microsatellite genotyping. Molecular Ecology Resources. 2011; 11(4):591–611. https://doi.org/10.1111/j.1755-0998.2011.03014.x PMID: 21565126

104. McLennan E A, Wright BR, Belov K, Hogg CJ, Grueber CE. Too much of a good thing? Finding the most informative genetic data set to answer conservation questions. Molecular Ecology Resources. 2019; 19(3):659–671 https://doi.org/10.1111/1755-0998.12997 PMID: 3068296

105. Nash DJ, Griffiths RA. Ranging behaviour of adders (Vipera berus) translocated from a development site. Herpetological Conservation and Biology. 2018; 28(October), 155–159

106. Germano JM, Field KJ, Griffiths RA, Clulow S, Foster J, Harding G, et al. Mitigation-driven translocations: Are we moving wildlife in the right direction? Frontiers in Ecology and the Environment. 2015; 13(2):100–105. http://doi.org/10.1890/140137

107. Natural England. Moving reptiles as mitigation https://www.gov.uk/guidance/reptiles-protection-surveys-and-licences#avoidance-mitigation-and-compensation-methods Oct 2015 update Natural England and Defra. Accessed 1 Nov 2018

108. McQuillan R, Leutenegger AL, Abdel-Rahman R, Franklin CS, Pericic M, Barac-Lauc L, et al. Runs of homozygosity in European populations. Am. J. Hum. Genet. 2008; 83:359–372. https://doi.org/10.1016/j.ajhg.2008.08.007 PMID: 18760389

109. Kirin M, McQuillan R, Franklin CS, Campbell H, McKeigue PM, et al. Genomic runs of homozygosity record population history and consanguinity. PLoS One 2010. 5: e13996. https://doi.org/10.1371/journal.pone.0013996

110. Keller M. C., Visscher P. M. & Goddard M. E. Quantification of inbreeding due to distant ancestors and its detection using dense single nucleotide polymorphism data. Genetics. 2011; 189:237–249. https://doi.org/10.1534/genetics.111.130922 PMID: 21705750

111. Palamara PF, Lencz T, Darvasi A, Pe’er I. Length distributions of identity by descent reveal fine-scale demographic history. Am. J. Hum. Genet. 2012; 91, 809–822. https://doi.org/10.1016/j.ajhg.2012.08.030 PMID: 23103233

112. Ceballos FC, Joshi PK, Clark DW, Ramsay M, Wilson JF. Runs of homozygosity: windows into population history and trait architecture. Nat. Rev. Genet. 2018. 19: 220–234. https://doi.org/10.1038/nrg.2017.109 PMID: 29355644

113. Nielsen R. Molecular Signatures of Natural Selection. Annu. Rev. Genet. 2005; 39:197–218 https://doi.org/10.1146/annurev.genet.39.073003.112420 PMID: 16285858

114. Bonnet X, Lecq T, Darvasi A, Pe’er I. Length distributions of identity by descent reveal fine-scale demographic history. Am. J. Hum. Genet. 2012; 91, 809–822. https://doi.org/10.1016/j.ajhg.2012.08.030 PMID: 23103233

115. Menkhorst P, Clemann N, Sumner J. (2016). Fauna-rescue programs highlight unresolved scientific, ethical and animal welfare issues. Pacific Conservation Biology. 2016. http://doi.org/10.1071/PC16007