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Unexpected high genetic diversity in small populations suggests maintenance by associative overdominance

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Abstract

The effective population size (N_e) is a central factor in determining maintenance of genetic variation. The neutral theory predicts that loss of variation depends on N_e, with less genetic drift in larger populations. We monitored genetic drift in 42 *Drosophila melanogaster* populations of different adult census population sizes (10, 50 or 500) using pooled RAD sequencing. In small populations, variation was lost at a substantially lower rate than expected. This observation was consistent across two ecological relevant thermal regimes, one stable and one with a stressful increase in temperature across generations. Estimated ratios between N_e and adult census size were consistently higher in small than in larger populations. The finding provides evidence for a slower than expected loss of genetic diversity and consequently a higher than expected long-term evolutionary potential in small fragmented populations. More genetic diversity was retained in areas of low recombination, suggesting that associative overdominance, driven by disfavoured homozygosity of recessive deleterious alleles, is responsible for the maintenance of genetic diversity in smaller populations. Consistent with this hypothesis, the X-chromosome, which is largely free of recessive deleterious alleles due to hemizygosity in males, fits neutral expectations even in small populations. Our experiments provide experimental answers to a range of unexpected patterns in natural populations, ranging from variable diversity on X-chromosomes and autosomes to surprisingly high levels of nucleotide diversity in small populations.

KEYWORDS

effective population size, evolutionary potential, genetic diversity, genetic drift, nucleotide diversity, small populations

1 | INTRODUCTION

Identifying the ecological and genetic determinants of levels of standing genetic variation in natural populations is fundamentally important for understanding the ability of populations to adapt to their current and future environments (Hoffmann & Willi, 2008; Willi, Van Buskirk, & Hoffmann, 2006). Neutral theory of molecular evolution predicts that the equilibrium level of genetic variation is given by the balance between random genetic drift and mutation rate (Crow & Kimura, 1970). Levels of genetic drift are typically described by estimates of the effective population size (N_e), which in conservation biology has resulted in the recommendation that N_e should be larger than 50–100 to avoid inbreeding depression (Frankham, Bradshaw, & Brook, 2014; Franklin, 1980). Determining the required adult census population size (N) to fulfil this guideline is challenging, but has typically relied on standard assumptions of N_e/N between 0.1 and 0.2 (Frankham, 1995; Frankham et al., 2014; Palstra & Fraser, 2012). In combination with estimates of N, this ratio is

also widely used in evolutionary biology to estimate the evolutionary potential of natural populations (Hedrick, 2005; Nunney, 1996). It is well known that N_e/N is species specific; it is influenced by components of the mating system, for example, the effective sex ratio or the sex-specific reproductive variance (Nunney, 1993; Ruzzante et al., 2016). But N_e/N may also be affected by life history. It has, for example, been suggested that high fecundity combined with high juvenile mortality can decrease N_e/N (Palstra & Ruzzante, 2008). Furthermore, age at maturity has been shown to be important for N_e/N, especially in insects (Waples, Luikart, Faulkner, & Tallmon, 2013).

In natural populations, N_e/N is not necessarily constant within a species (Ferchaud et al., 2016; Palstra & Fraser, 2012), for example, due to changes in the reproductive skew/reproductive variance across habitats (Whiteley et al., 2015) or differences in the selective landscape (Frankham, 2012). The idea that selection may retain genetic variation is somewhat controversial given the low efficacy of selection in small populations; nevertheless, it is frequently proposed that balancing selection may conserve some genetic variation in small natural populations (Demontis et al., 2009; Fraser, Debes, Bernatchez, & Hutchings, 2014; Funk et al., 2016; Hedrick, 2012; Kaeuffer, Coltman, Chapuis, Pontier, & Reale, 2007). Studies on allozyme markers and quantitative variation have suggested that associative overdominance (i.e., neutral loci becoming effectively overdominant as a result of disequilibrium with a locus under selection; Frydenberg, 1963) may conserve genetic variation in small populations compared to expectations from neutral theory (Gilligan, Briscoe, & Frankham, 2005; Rumball, Franklin, Frankham, & Sheldon, 1994). As an alternative to a single selected locus, simulations suggest that multiple deleterious recessive alleles distributed across different haplotypes will, in the presence of long distance linkage disequilibrium, result in a fitness advantage of the heterozygote and thus the maintenance of genetic variation across the genome (Bersabé, Caballero, Pérez-Figueroa, & García-Dorado, 2016; Ohta, 1973; Pamilo & Pálsson, 1998; Zhao & Charlesworth, 2016). If such a mechanism is effective in populations going through a bottleneck, levels of genetic variation may be considerable higher than under neutral expectations. Initially, this will not increase evolutionary potential due to linkage effects leading to Hill-Robertson interference (Hill & Robertson, 1966). However, in the long term, after recovery from the bottleneck, recombination will increase the genetic variation readily available to selection, leading to an increased evolutionary potential (Castellano, Coronado-Zamora, Campos, Barbadilla, & Eyre-Walker, 2016; Frydenberg, 1963). As opposed to different forms of balancing selection, directional selection can result in lower genetic variation than expected by demography, as sections of the genome hitchhike with the causative loci (Huang, Wright, & Agrawal, 2014; Smith & Haigh, 1974). The extent of directional selection depends among others on the specific habitat, and it is therefore likely that rates of loss of genetic diversity and N_e/N ratios are influenced by the characteristics of a given environment.

For species with heterogametic sex determination, where the X-chromosome resides in only one copy in the male, effects of selection and drift at the X-chromosome are very distinct from that on the autosomes (see, e.g., Ellegren, 2009). Everything else being equal, this decreases N_e and the X-chromosome to autosome (X to A) diversity ratio to three-fourths of that of the autosomes and disables functional overdominance in males (see, e.g., Ellegren, 2009). In species with no recombination in males, such as *Drosophila melanogaster*, the rate of recombination is higher on the X-chromosome than on the autosomes, resulting in less influence of hitchhiking effects due to background selection in the X-chromosome, potentially increasing the X to A diversity ratio (Charlesworth, 2012). One may also predict that the X to A diversity is decreased in the presence of associative overdominance. At the X-chromosome, recessive deleterious alleles are exposed in males and thereby removed by purifying selection, limiting the potential for associative overdominance during a bottleneck. Bottlenecks decrease the X to A diversity ratio as a consequence of the lower N_e of the X-chromosome (Pool & Nielsen, 2007). This pattern is observed in several species undergoing habitat expansion with associated founder events (Pool & Nielsen, 2007), but has never been tested experimentally.

Here we use time-series of pooled restriction site-associated DNA (RAD) sequencing to investigate the population genetic dynamics in 42 experimental populations reared for 20 generations at adult census population sizes of $N = 10$ (N10), $N = 50$ (N50) or $N = 500$ (N500) and assess how distinct thermal environments may affect these dynamics (Figure 1). To characterize and compare fitness of flies from the two thermal environments as well as to survey the overall conditions of the experimental populations, we estimated egg-to-adult viability in both thermal environments. We worked with the null hypothesis that N_e is a fraction of N, due to deviations from the Wright-Fisher idealized population, and that N_e/N is constant across different N and thermal environments. We found N_e/N to be highest in the smallest populations, and the loss of nucleotide diversity to be slower than expected in small populations. Generally, the rates of loss of nucleotide diversity on the X-chromosomes followed theoretical predictions and were faster than on the autosomes. We observed no strong influence of the thermal environment on the maintenance of nucleotide diversity, except on the X-chromosome in the smallest populations, where nucleotide diversity was lost faster in the stable (benign) compared to the ramping temperature environment. Based on this observation and a lower loss of nucleotide diversity in areas of low recombination, we suggest that maintenance of nucleotide diversity at the autosomes in small populations is driven by associative overdominance.

2 | MATERIALS AND METHODS

2.1 | Replicate populations and thermal regimes

A mass-bred population of *D. melanogaster* was established from 589 field-caught inseminated females (Schou, Kristensen, Kellermann, Schlötterer, & Loeschcke, 2014) and maintained for two generations ($N \sim 6{,}000$) at 25 ± 1°C and a 12:12-hr light:dark (L:D) photocycle (Figure 1). Hereafter, we established three breeding

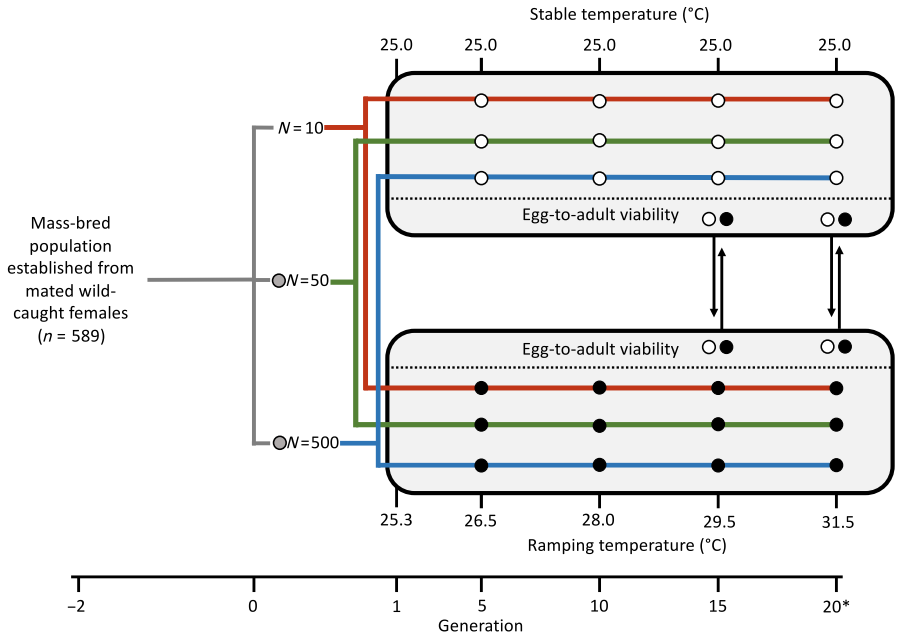

FIGURE 1 Schematic representation of the experimental design. Two generations after the establishment of the mass-bred population we established three breeding regimes, N10, N50 and N500, in which populations were reared at population sizes 10, 50 and 500. We initiated eleven N10 base populations (red), seven N50 base populations (green) and three N500 base populations (blue). Offspring from each of the 21 populations were split into two replicate populations. One set was reared in the stable thermal regime (upper section) where the mean and diurnal temperature fluctuations across generations were unchanged and one in the ramping regime (lower section), where the mean, min and max temperatures of the diurnal fluctuations were increased by 0.3°C every generation. Samples for sequencing were obtained at five different time points (generations 0, 5, 10, 15 and 20), as indicated by the grey (base populations), open (stable populations) and closed (ramping populations) circles on the coloured lines. Prior to assessing egg-to-adult viability, we created test replicates of all replicate populations, which were reared in a common garden for two generations. Egg-to-adult viability of test replicates originating from the stable and ramping regimes was then assessed at the temperature regimes reached in the ramping and stable regimes in generations 15 and 20. *In generation 20, it was particularly difficult to obtain sufficient individuals for freezing due to increased environmental stress in the ramping regime and high levels of inbreeding, and the test replicates were therefore used for freezing [Colour figure can be viewed at wileyonlinelibrary.com]

regimes from virgin flies: (i) N10: 11 replicate populations of 10 adult individuals; (ii) N50: seven replicate populations of 50 adult individuals; (iii) N500: three replicate populations of 500 adult individuals. The increasing replication of smaller sized populations was chosen as we expected a stronger influence of the stochastic process of genetic drift with smaller population sizes. All replicate populations were established with equal sex ratios. Sexing was performed by the use of CO_2 anaesthesia. The 21 base populations were duplicated by two consecutive 24-hr egg-laying periods. The two duplicates were distributed into two different thermal regimes (stable and ramping), where daily variation in temperature and light followed a Gaussian function. The stable regime, and generation 0 of the ramping regime, had a mean temperature of 24.8°C, a night-time temperature of 23.5°C and a daily temperature peak of 27.5°C. In each generation, the mean, night-time and peak temperatures of the ramping regime were increased by 0.3°C (Figure 1). Replicate populations were maintained with equal sex ratios. Across the three breeding regimes, the replicate populations were reared on approximately equal medium surface area per individual as well as adult and developmental rearing densities. To equalize the surface area and adult density among breeding regimes, it was necessary to split the N500 replicate populations into ten separate bottles, which were carefully mixed when initiating a new generation to avoid population structuring. Each of the replicate N10 populations was maintained in a 27-ml vial containing 7-ml medium (a horizontal medium surface of exactly 3.5 cm^2, resulting in 0.35 cm^2/individual), while groups of 50 flies from each of the replicate N50 and N500 populations were maintained in 100-ml glass bottles containing 35-ml medium (a horizontal medium surface of 19.63 cm^2, corresponding to 0.39 cm^2/individual). The flies were reared on a standard oatmeal-sugar-yeast-agar *Drosophila* medium throughout the experiment. To establish a new generation and to keep developmental density within each vial/bottle approximately constant within and across generations, 5–8 days old flies laid eggs in a 12-hr period in one set of vials/bottles followed by a 24-hr laying period in another set of vials/bottles. From these two sets of vials/bottles, the low-density vials/bottles were used to establish the next generation. In this way, density was approximately controlled. As soon as approximately 95% of all flies of a replicate population had emerged, flies were sexed under CO_2 anaesthesia to establish equal sex ratios when setting up the new generation. A potential caveat in this study is the possibility of females to mate prior to the random selection of individuals for the subsequent generation. However, this is unlikely to account for the results, as females were given at least 4 days with the selected

males before egg production, whereby a secondary mating and more than 80% sperm displacement is expected to have taken place (Prout & Bundgaard, 1977). A replicate population was considered extinct when it could no longer produce enough offspring to establish the required males and females needed to set up the next generation. To estimate egg-to-adult viability in all surviving pairs of replicate populations in generations 15 and 20, we established test replicates of each replicate population. Test replicates were reared at 25°C for two generations and propagated to an *N* of approximately 500, after which groups of 20 eggs were transferred to vials with 7-ml food. Either these eggs developed at the stable regime or the ramping regime under the temperature reached in the generation of translocation. The number of emerging adult flies was counted. Thus, for each replicate population, we obtained estimates of egg-to-adult viability when reared in the stable and in the ramping regime after two generations of common garden. The genomic and phenotypic data of the N500 populations have been used in a previous publication to investigate the ability of these populations to adapt to the increasing temperatures of the ramping regime (Schou et al., 2014).

2.2 | Analysis of egg-to-adult viability

Egg-to-adult viability data were analysed separately for each generation, with models containing the fixed effects breeding regime, test regime (the thermal regime in which the egg-to-adult viability was assessed) and thermal regime (the thermal regime at which a given replicate population was reared throughout the experimental evolution prior to the test), as well as respective two-way interactions. The population size of each breeding regime was log transformed to fulfil assumptions of linearity of predictors and modelled as a continuous variable. All models were mixed logistic regressions performed using the package LME4 (Bates, Mächler, Bolker, & Walker, 2015) in R (v. 3.3.2; R Core Team 2017) with replicate population as a random effect. We detected overdispersion in all models and included an observational level random effect to address this issue. We performed sequential model reductions and compared full and reduced models using likelihood ratio tests. In case of a significant interaction, further model reduction in the involved predictors was halted. As both generations 15 and 20 had significant interactions between breeding regime and test regime, we split the data set according to the test regime and tested for an effect of breeding regime.

2.3 | DNA extraction and sequencing

Pooled samples of 25 females were acquired prior to the establishment of the next generation for each surviving replicate population in generations 0, 5, 10, 15 and 20, resulting in a total of 166 samples. However, this preferred number of females was not always acquired (Table S1), and N10 base populations (generation 0) were not included as only 10 flies could be obtained per population. At generation 20, it was particularly difficult to obtain enough individuals due to increased environmental stress in the ramping regime and high levels of inbreeding, and the test replicates were therefore used. Genomic results from this generation are excluded from statistical analyses. Genomic DNA was extracted using a CTAB-based method (Junghans & Metzlaff, 1990) or by a modified salting out procedure (Miller, Dykes, & Polesky, 1988) in the clinical laboratory of BGI (Beijing Genomics Institute, Hong Kong). RAD libraries were created by following the standard protocol (Baird et al., 2008) and digesting the DNA with the 6-cutter enzyme *Eco*RI (New England Biolabs, Ipswich, MA, USA) for 1 hr, and 12 cycles of PCR after ligation of adaptors. Sequencing of RAD libraries was performed by BGI Hong Kong Co, Limited with a 45-bp single-end Illumina sequencing strategy. Only reads containing barcodes with a perfect match were accepted. Using POPOOLATION (Kofler et al., 2011), all reads were trimmed at the 3'-end until average phred quality score was at least 18 or discarded if read length was below 40. Alignment to the flybase *D. melanogaster* reference genome (v. 5.38, later adjusted to v. 6.02), as well as the reference genomes of *Wolbachia* (AE017196.1), *Acetobacter* (AP011121.1) and *Lactobacillus* (CP000416.1), was performed using POPOOLATION (Kofler et al., 2011) and BWA (v0.5.8c; -n 0.01 (error rate), -o 1 (max. no. of gap openings), -d 5 and -e -5 (max. gap length)) with DISTMAP (Li & Durbin, 2009; Pandey & Schlötterer, 2013). Local realignment was performed with GATK (v. 2.4-7) (McKenna et al., 2010). The three N500 base population were realigned using REALIGNERTARGETCREATOR (-mismatch 0.04, -window 5, -maxInterval 200) followed by INDELREALIGNER (-LOD 5, -maxConsensus 500, -greedy 1000, -entropy 0.15). The UNIFIEDGENOTYPER (-stand_call_conf 80, -stand_emit_conf 80, -glm GENERALPLOIDYINDEL, -ploidy 20, -max_alternate_alleles 2, -minIndelCnt 5, -minIndelFrac 0.01) of GATK was then used to call indels present in the three realigned samples. Hereafter, all indels present in at least two of the three realigned samples were used as priors to realign all samples with INDELREALIGNER setting KNOWNS_ONLY. The produced SAM files were converted into BAM files using SAMTOOLS (v. 1.1; Li et al., 2009) while removing reads with lower mapping quality than 20. Pileup files were created with SAMTOOLS (v.1.1; Li et al., 2009) while only retaining nucleotides with a minimum phred quality score of 36 (Boitard, Schlötterer, Nolte, Pandey, & Futschik, 2012). RAD-loci with a mapping pattern deviating from the expected (two stacks overlapping with four nucleotides) or a consistent high coverage across samples were discarded. Consistent high coverage was defined as when the sum of the standardized coverage of a locus across all samples exceeded three standard deviations above the median sum. Indels and their flanking regions (5 bp), as well as simple sequence repeats and transposable elements, were identified and removed using POPOOLATION2 and REPEATMASKER (v. 3.29). All restriction sites were removed from the data set. We used a minimum coverage of 100 and down-sampled all sites to 100 without replacement using POPOOLATION (Kofler et al., 2011). The presence of a segregating site in the restriction site may bias estimates of nucleotide diversity at the RAD-locus (Gautier et al., 2013). However, only RAD-loci present in all samples were retained in the data set, whereby the majority of RAD-loci with segregating restriction sites were removed, as these are likely to be lost via genetic drift in at least one of the replicate N10 or N50 populations. Using SNP markers of common inversions in *D. melanogaster* (Kapun, van Schalkwyk, McAllister, Flatt, &

Schlötterer, 2014), we excluded the presence of *In(3L)P* and *In(3R)C*. One replicate N10 population from generation 20 was removed from the data set as it showed strong indications of contamination (increase in π from 0.002 in generation 15 to 0.0036 in generation 20, and a small genetic distance to replicate N500 populations in generation 20).

2.4 | Estimation of population genetic parameters

After all filtering steps, the data covered 0.75% of the genome and distributed across 14211 RAD-loci of which 34% were in exons, 42% in introns and 24% in intergenic regions. Nucleotide diversity (π, Tajima, 1983) was calculated in each generation and for each chromosomal arm with POPOOLATION (Kofler et al., 2011) and a minimum calling frequency of 4%. We estimated N_e from generation 0 to 15, as generation 15 represents the largest genomic evolution, while disregarding generation 20 where several replicate ramping populations had gone extinct. Three methods were used to estimate N_e: (i) a standard Wright-Fisher model which utilizes the fraction of autosomal estimates of π retained across a number of generations (Hein, Schierup, & Wiuf, 2005). Nonlinear models for each replicate population across generations 5, 10 and 15 were used to obtain a least-squares estimate of N_e. Methods 2 and 3 utilize changes in variant frequencies across generations; (ii) WFABC (Foll, Shim, & Jensen, 2015) includes frequency estimates from several different time points (generations 0, 5, 10 and 15) allowing inference of temporal variation in allele frequency when obtaining estimates of N_e; (iii) The R-package NEST (vs. 1.1.2; Jonas, Taus, Kosiol, Schlötterer, & Futschik, 2016) only allows for two sampling time points, but is developed specifically for Pool-Seq (sequencing of DNA from pooled individuals) and allows for the incorporation of the number of individuals contributing to the gene frequency estimates (a second binomial sampling using the method "P_AJ_planII"). Only SNPs fulfilling the following calling criteria (1463) were used for WFABC and NEST: (i) situated in intergenic regions on the autosomes; (ii) minor alleles present in at least 4% of the reads from the ten base population replicates combined; (iii) minimum distance between SNPs of 10,000 bp. The expected loss of nucleotide diversity, when assuming populations are behaving as an idealized population and given our Pool-Seq sampling scheme, was assessed by simulating the demographic history with FASTSIMCOAL2 (Excoffier, Dupanloup, Huerta-Sánchez, Sousa, & Foll, 2013). Recombination rates vary across the genome and across lineages (Comeron, Ratnappan, & Bailin, 2012; Fiston-Lavier, Singh, Lipatov, & Petrov, 2010). To address this variation in recombination rate, we simulated 1,000 linkage blocks of 0.1 Mb and 100 linkage blocks of 1 Mb; each block was simulated with a recombination rate sampled from a log-uniform distribution with a minimum recombination rate of 10e-6 cM/Mb and a maximum of 9 cM/Mb. We did not include any form of selection as a parameter in the simulations, whereby deviations of the observed from the expected loss of nucleotide diversity may be a consequence thereof. We simulated a starting N_e leading to the same π as the base populations using a mutation rate of 2.8e-9 (Keightley, Ness, Halligan, & Haddrill, 2014). We then simulated bottlenecks of N_e of 10, 50 and 500 ($N_e/N = 1$) as well as 2, 10 and 100 ($N_e/N = 0.2$) lasting 0, 5, 10, 15 and 20 generations. We estimated the confidence limits of the expected π by bootstrapping ($n = 10{,}000$) the 1,000 linkage blocks (0.1 Mb) or the 100 linkage blocks (1 Mb) at each time point. We used two different block lengths to assess the sensitivity of the simulated results to linkage block length.

2.5 | Statistical analyses of population genetic parameters

We analysed the effect of breeding regime and thermal regime on autosomal π across generations 5–15 in a general linear mixed model with replicate population as a random effect. To fulfil assumptions of normality of residuals and homogeneity of variance, π, was squared. We log transformed *N* to fulfil the assumption of linearity of predictor variables. The same analytical approach was used to analyse the effect of thermal regime and *N* on the X to A diversity ratio across generations. The X to A diversity ratio was squared to fulfil assumptions of parametric analysis. Using the replicate population N_e estimates from the Wright-Fisher model, WFABC and NEST, we calculated average N_e/N for each breeding regime. However, analyses of the relationship between N_e/N and *N* are problematic as the correlation of a ratio with its denominator may be spurious. We therefore investigated the relationship between N_e and *N*, and assessed whether the rate by which N_e increased with *N* at small *N* was higher than the rate of change at larger *N*. Such a result would represent a higher N_e/N in small populations compared to larger populations. For all three methods of N_e estimation, we observed a nonlinear relationship between N_e and *N*. As opposed to addressing this by transformation or by fitting a nonlinear curve based on three predictor values, we chose to estimate and compare the slopes from N10 to N50 and the slopes from N50 to N500. We did not detect any effect of thermal regime on loss of nucleotide diversity, and we therefore disregarded thermal regime in this analysis. We obtained the slopes from linear models for each N_e estimation method and slope comparisons were done using parametric 95% confidence intervals obtained from the models.

2.6 | Correlating the loss of nucleotide diversity with recombination rate

Directional selection is expected to reduce nucleotide diversity in the segments of the genome where there is little recombination due to hitchhiking (Smith & Haigh, 1974). Conversely, the potential for different forms of balancing selection, such as associative overdominance to conserve genetic variation, is likely to be increased when recombination rate is low and linkage extends further (Charlesworth, 2006; Frydenberg, 1963). Recombination rates are known to be population-specific and may evolve as a consequence of inbreeding (Comeron et al., 2012; Fiston-Lavier et al., 2010; Hunter, Huang, Mackay, & Singh, 2016); however, the RADseq data used in this study preclude estimating population-specific recombination rates.

Instead, we used published estimates of recombination rates (Comeron et al., 2012). We correlated the recombination rates with the average fraction of nucleotide diversity (π, Tajima, 1983) retained across replicate populations in generation 15. We estimated π in nonoverlapping windows of 300 kb (minimum calling frequency of 4%) using POPOOLATION (Kofler et al., 2011) for each replicate population. We then estimated the fraction of π retained in each replicate population in generation 15 using the average π in generation 0 (N50 and N500 populations) in each window as the initial π. We calculated the average fraction of π retained at each window within each thermal regime and breeding regime combination for the autosomes and for the X-chromosome. A general linear mixed model was used to detect differences between thermal regimes and breeding regimes in their relationship between fraction of π retained and recombination rate (see Table S2 for further details). Genomic windows with recombination rates higher than seven (n = 3) were found to be highly influential and were excluded from the analyses.

3 | RESULTS

3.1 | Extinctions and egg-to-adult viability

The increasing temperature in the ramping regime (Figure 1) resulted in several extinctions after generation 15 and few remaining replicates in generation 20 (n_{N10} = 3; n_{N50} = 4; n_{N500} = 3), while only one N10 extinction occurred in the stable regime. Thus, results from generation 20 should be interpreted with caution and were therefore excluded from genomic analyses. For egg-to-adult viability, we found no effect of the thermal regime of origin (stable and ramping thermal regimes) on the performance when translocated and tested in either of the two thermal regimes (test regimes; Figure 2; Table 1; see also Schou et al. (2014). Egg-to-adult viability decreased with decreasing N in both test generations (Figure 2; Table 1), and this effect was exacerbated when replicate populations were tested in the ramping regime compared to the stable regime, as illustrated by a significant interaction between test regime and N (Table 1). The decreased relative fitness for small populations in the ramping regime in comparison with the stable regime (i.e., inbreeding by environment interaction) provides evidence that the ramping regime is more stressful (Fox & Reed, 2011; Schou, Loeschcke, & Kristensen, 2015). For this reason, we investigated the effect of N in each test regime separately and found the detrimental effect of lower N to be present in all regimes (ramping generation 15: $\chi^2_{(1)} = 20.23$, $p < .001$; ramping generation 20: $\chi^2_{(1)} = 17.77$, $p < .001$; stable generation 15: $\chi^2_{(1)} = 12.73$, $p < .001$; stable generation 20: $\chi^2_{(1)} = 16.42$, $p < .001$).

3.2 | Nucleotide diversity

Autosomal nucleotide diversity decreased across generations in all breeding regimes (N10, N50 and N500), but with a faster rate at low N (Figure 3; Table 2). The rate of loss of nucleotide diversity across breeding regimes was not affected by the thermal regime at which they were reared (Table 2). Simulations of the loss of nucleotide diversity under different N_e/N and without selection show that replicate N10 populations lost nucleotide diversity corresponding to N_e/N = 1, and thus deviated from the loss of nucleotide diversity expected when N_e/N = 0.2 (Figure 3).

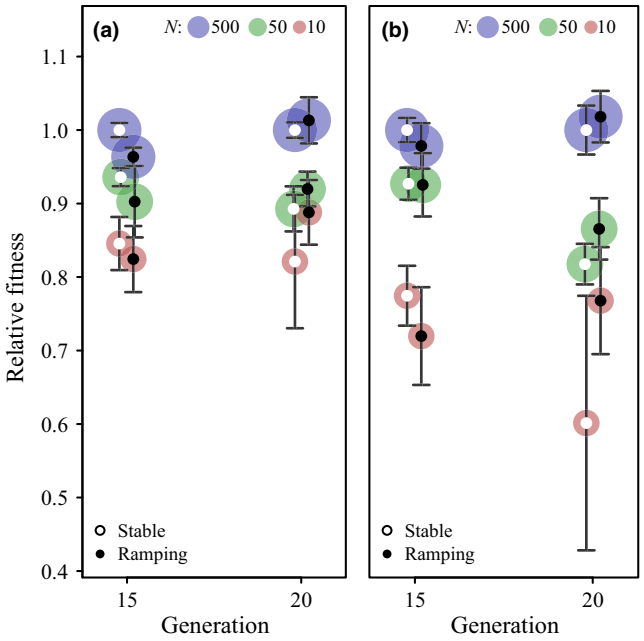

FIGURE 2 Relative fitness measured in the two thermal test regimes. Egg-to-adult viability of all replicate populations (i.e., both from the ramping, black dots and the stable regime, white dots) were assessed in the stable test regime (a), and in the ramping test regime (b) after two generations of common garden rearing (see Figure 1). We estimated the relative fitness to enable comparison of N10 and N50 breeding regimes across the two test regimes. This was done by dividing the egg-to-adult viability of each replicate population by the mean of the three replicate N500 populations from the stable thermal regime. Relative fitness decreased with decreasing population size at all test time points. When tested in the ramping regime, replicate N10 populations had a lower relative fitness than when tested in the stable regime (Table 1), suggesting higher stress levels leading to inbreeding by environment interactions (Fox & Reed, 2011; Schou et al., 2015). The egg-to-adult viability within test regimes did not differ between replicate populations from different thermal regimes (Table 1; see also (Schou et al., 2014)). All values are means ± *SE*. The number of replicate populations assessed was the same for ramping and stable regimes. N10: n = 11 and 3 in generations 15 and 20, respectively. N50: n = 7 and 4 in generations 15 and 20, respectively. N500: n = 3 and 3 in both generations 15 and 20. For raw egg-to-adult viability data see Fig. S1 [Colour figure can be viewed at wileyonlinelibrary.com]

The average X to A diversity ratio ± *SD* in the base populations, representing the mass-bred population, was 0.69 ± 0.01 (Figure 4). We found a significant three-way interaction between breeding regime, generation and thermal regime on the X to A diversity ratio, with lower N resulting in a faster decrease across generations (Figure 4; Table 3). Analysing each breeding regime separately revealed no interaction between generation and thermal regime in neither N500 nor N50 breeding regimes (Table 3), but a faster decrease in replicate N10 populations from the stable regime than from the

TABLE 1 Analysis of egg-to-adult viability. Egg-to-adult viability data were analysed separately for each generation. All models were mixed logistic regressions with replicate population as a random effect. We performed sequential model reductions and compared models using maximum-likelihood ratio tests. In case of a significant interaction, further model reduction of the involved predictors was halted. Model coefficients are given for the minimal adequate model and represent the ramping regime

	Generation 15			Generation 20		
Source of variation	Estimate (*SE*)	$\chi^2_{(1)}$	*p*	Estimate (*SE*)	$\chi^2_{(1)}$	*p*
Intercept	−0.85 (0.26)	–	–	−0.63 (0.33)	–	–
Test regime	0.83 (0.12)	–	–	1.24 (0.20)	–	–
Thermal regime	–	0.32	.57	–	2.26	.13
Breeding regime	−0.27 (0.07)	–	–	−0.32 (0.08)	–	–
Thermal regime*Breeding regime	–	0.03	.86	–	0.39	.53
Thermal regime*Test regime	–	0.01	.91	–	0.94	.33
Breeding regime*Test regime	−0.12 (0.03)	12.11	<.001	−0.12 (0.04)	7.10	<.01

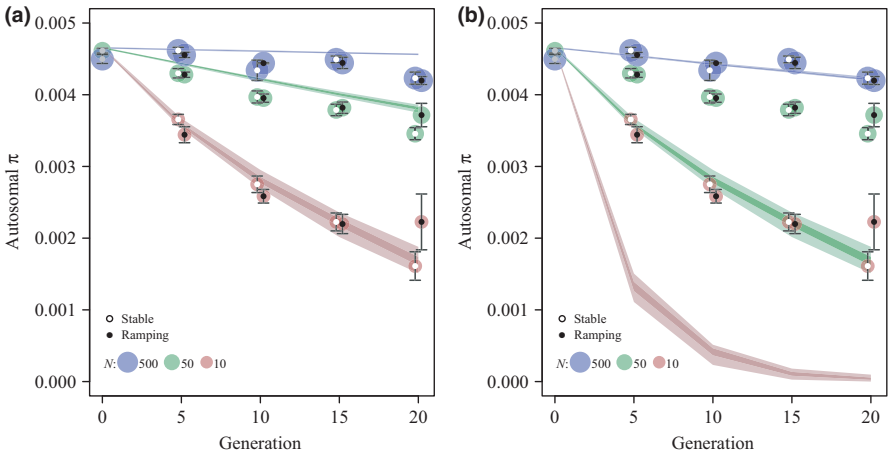

FIGURE 3 Observed and expected nucleotide diversity across generations under $N_e/N = 1$ and $N_e/N = 0.2$. The decrease in nucleotide diversity was highly dependent on *N*, but with no apparent difference between groups from the stable and ramping thermal regimes. All values are means ± *SE*. The corresponding coloured lines display the 95% confidence interval (strong colours: blocks of 0.1 Mb; transparent colours: blocks of 1 Mb) of the expected loss of nucleotide diversity across generations for the breeding regimes under the assumption of $N_e/N = 1$ (a) and $N_e/N = 0.2$ (b), under neutrality. In both regimes, the number of replicates was 11, 7 and 3 (N10, N50 and N500, respectively) in generations 5, 10 and 15. In generation 0, no N10 were sequenced. In generation 20 the number of replicates for the ramping regime was 3, 4 and 3, while it was 9, 7 and 3 for the stable regime (N10, N50 and N500, respectively). For the change in nucleotide diversity on specific chromosome arms, see Fig. S2. Results from the statistical analysis are available in Table 2 [Colour figure can be viewed at wileyonlinelibrary.com]

ramping regime (Table 3). To further investigate this pattern, we simulated the expected loss of nucleotide diversity in replicate N10 populations assuming $N_e = N$ for the autosomes and that the N_e of X-chromosomes is three-fourths of that of the autosomes. The simulation revealed that in the ramping regime, the loss of nucleotide diversity of replicate N10 populations in the X-chromosome corresponds to the expectation given the loss at the autosomes. Conversely, in the stable regime, replicate N10 populations were experiencing a faster loss of nucleotide diversity in the X-chromosome than expected from the loss at the autosomes (Figure 4).

3.3 | Effective and census population size

We estimated N_e of each replicate population using frequency changes in intergenic regions from generation 0 to 15. Estimating N_e is challenging for small populations, and we therefore employed three different methods. The mean $N_e \pm SD$ across replicate populations within breeding regimes based on WFABC was 38 ± 14 for the N500 regime, 18 ± 5 for the N50 regime and 9 ± 1 for the N10 regime, while estimates from Nest were 127 ± 21 for the N500 regime, 40 ± 13 for the N50 regime and 16 ± 2 for the N10 regime (Fig. S3). Finally, estimates obtained from the Wright-Fisher model showed the same pattern, with N_e for N500 being estimated to 268 ± 124, while the estimate for N50 was 40 ± 10 and 10 ± 2 for N10 (Fig. S3). In summary, all three methods showed a consistent increase in N_e/N with lower *N* (Figure 5), despite the expected variation in the absolute estimates. This result was also consistent at each chromosome arm (Figs S4 and S5) and across different sets of generations (Figs S6 and S7). To investigate whether the pattern shown in Figure 5 truly results from a faster increase of N_e with *N* in small

TABLE 2 Analysis of autosomal nucleotide diversity. Results from the analysis of the effects of breeding regime (N10, N50 or N500) and thermal regime (stable or ramping) on change in the autosomal π across generations 5, 10 and 15. Generation 20 was omitted from the analysis as only a subset of replicate populations survived to this time point. Model comparisons were done with likelihood ratio tests. We found a significant interaction between breeding regime and generation and therefore halted further model reductions with regard to these two variables. Model coefficients are given for the minimal adequate model

Source of variation	Estimate (*SE*) $*10^{-5}$	$\chi^2_{(1)}$	*p*
Intercept	1.29 (0.12)	–	–
Breeding regime	0.15 (0.03)	–	–
Generation	−0.11 (0.01)	–	–
Thermal regime	–	0.66	.42
Breeding regime*Thermal regime	–	0.35	.55
Generation*Thermal regime	–	1.57	.21
Breeding regime*Generation	0.02 (0.003)	38.25	<.001
Breeding regime* Generation*Thermal regime	–	0.46	.50

populations than in larger populations, we investigated the change in slope of N_e across N. All slope estimates were significantly larger than zero with $p < .001$, and all of the confidence intervals (see below) were positioned between zero and one, showing that the increase in N_e with increased population size is less than the increase in N. When comparing the slope ±95CI across N, we found a steeper slope at small N than at larger N using all three methods (WFABC: N10 vs. N50 = 0.22 ± 0.05 and N50 vs. N500 = 0.04 ± 0.01; Nest: N10 vs. N50 = 0.59 ± 14 and N50 vs. N500 = 0.19 ± 03; Wright-Fisher: N10 vs. N50 = 0.76 ± 10 and N50 vs. N500 = 0.50 ± 14). This relationship is the driver of the high N_e/N in small populations and a smaller N_e/N in larger populations as observed in Figure 5.

3.4 | Correlating the retained fraction of nucleotide diversity with recombination rate

When assessing the fraction of retained nucleotide diversity in each window (300 kb) across the genome and correlating it with the recombination rate of the given window, we found no significant effect of thermal regime but a significant interaction between recombination rate and breeding regime ($\chi^2_{(1)} = 34.58$, $p < .001$; for further details see Table S2). We therefore recomputed the average retained fraction of nucleotide diversity in each window across replicate populations, this time disregarding the thermal regime of origin of the replicate populations, and fitted separate linear models to each breeding regime. We found a significant decrease in the fraction of retained nucleotide diversity with increasing rate of recombination in the N10 breeding regime ($F_{1,303} = 46.38$, $p < .001$, b ± *SE* = −0.016 ± 0.002; Figure 6a). The same significant pattern was found for the N50 breeding regime, but not as strong ($F_{1,303} = 12.89$, $p < .001$, b ± *SE* = −0.007 ± 0.002; Figure 6a), while there was no significant effect of recombination rate in the N500 breeding regime ($F_{1,303} = 0.18$, $p = .66$, Figure 6a). When performing the same analysis on the X-chromosome, we found no significant effects of recombination rate for any population size (Figure 6b, Table S2).

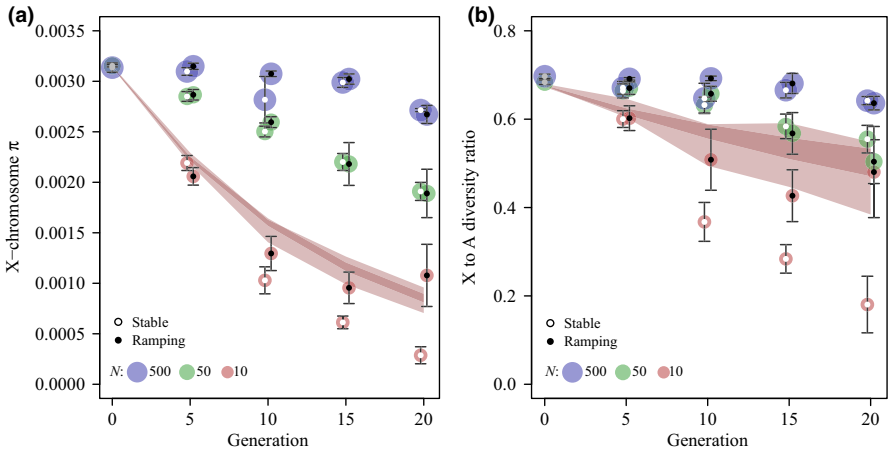

FIGURE 4 Loss of nucleotide diversity at the X-chromosome. Change in nucleotide diversity at the X-chromosome (a) and change in the X to A diversity ratio (b) across generations in the stable and ramping thermal regimes. The decreasing X to A diversity ratio for the N10 and N50 breeding regimes illustrates the effects of a lower N_e of the X-chromosome under a bottleneck (Pool & Nielsen, 2007). All values are means ± *SE*. In both regimes, the number of replicates was 11, 7 and 3 (N10, N50 and N500, respectively) in generations 5, 10 and 15. In generation 0, no N10 populations were sequenced. In generation 20, the number of replicates for the ramping regime was 3, 4 and 3, while it was 9, 7 and 3 for the stable regime (N10, N50 and N500, respectively). The coloured lines display the 95% confidence interval (strong colours: blocks of 0.1 Mb; transparent colours: blocks of 1 Mb) of the expected loss of nucleotide diversity across generations for the N10 breeding regimes under neutrality with $N_e/N = 1$ and with the N_e of X-chromosomes set to be three-fourths of that of the autosomes. Results from the statistical analysis of the X to A diversity ratio are available in Table 3 [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Analysis of X to A diversity ratio. Analyses of the effects of breeding regime (N10, N50 or N500) and thermal regime (stable or ramping) on changes in the X to A diversity ratio across generations 5, 10 and 15. Generation 20 was omitted from the analysis as only a subset of replicate populations survived to this time point. Model comparisons were done with likelihood ratio tests. As we found a significant three-way interaction in the full model, we split the data set according to breeding regime to assess possible interactions between generation and thermal regime. Model coefficients (representing the ramping regime) are given for the minimal adequate models

	Estimate (*SE*) $*10^{-1}$	$\chi^2_{(1)}$	*p*
Full model			
Intercept	4.94 (0.91)	–	–
Breeding regime	−0.05 (0.25)	–	–
Generation	−0.43 (0.08)	–	–
Thermal regime	−0.62 (1.29)	–	–
Breeding regime*Generation	0.07 (0.02)	–	–
Breeding regime*Thermal regime	0.19 (0.35)	–	–
Generation*Thermal regime	0.19 (0.11)	–	–
Breeding regime*Generation* Thermal regime	−0.04 (0.03)	24.83	<.001
N10 subset			
Intercept	4.75 (0.56)	–	–
Generation	−0.27 (0.05)	–	–
Thermal regime	−0.24 (0.80)	–	–
Generation*Thermal regime	0.12 (0.01)	23.05	<.001
N50 subset			
Intercept	5.01 (0.29)	–	–
Generation	−0.11 (0.03)	14.18	<.001
Thermal regime	–	0.14	.71
Generation*Thermal regime	–	0.13	.72
N500 subset			
Intercept	4.56	–	–
Generation	–	0.43	.51
Thermal regime	–	1.81	.18
Generation*Thermal regime	–	0.04	.84

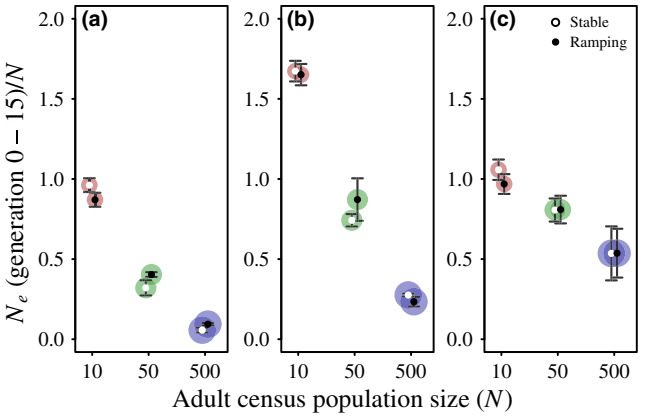

FIGURE 5 Estimates of N_e/N in the three breeding regimes. We estimated N_e of each replicate population using three different approaches: WFABC (a), NEST (b) and Wright-Fisher (c). The frequency changes in SNPs in intergenic regions were used to estimate N_e in WFABC (generations 0, 5, 10 and 15) and in Nest (generations 0 and 15). All values are means ± *SE* across replicate populations within breeding regimes. The same number of replicates was used for each of the two thermal regimes ($n_{N10} = 11$, $n_{N50} = 7$ and $n_{N500} = 3$). We found the same pattern at each separate chromosome arm (see Figs S4 and S5), and when using different subsets of generations to estimate N_e (see Figs S6 and S7) [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

The base populations had similar levels of nucleotide diversity as other *D. melanogaster* populations (Fabian et al., 2012; Kolaczkowski, Kern, Holloway, & Begun, 2011). The rate of loss of nucleotide diversity during the experiment was highly dependent on the breeding regime with higher losses in smaller populations (Figure 3). In evolutionary biology, much focus has been on the level and constancy of N_e/N in natural populations as N_e provides an estimate of evolutionary potential (Frankham, 1995; Frankham et al., 2014; Hedrick, 2005; Nunney, 1995; Palstra & Fraser, 2012). Empirical meta-analyses suggest values of N_e/N between 0.1 and 0.2 for a range of taxa, reviewed in Frankham (1995); Palstra and Fraser (2012). Theoretical estimates are as high as 0.66 for species with a lottery polygyny mating system (Nunney, 1993), but recent work based on life history data shows that the ratio may potentially be higher than 1, especially in species where age at maturity is delayed (Waples et al., 2013).

This study used three carefully controlled and replicated adult census population sizes in combination with a high number of interspaced intergenic markers (~1,500) to estimate N_e. Using different methods to estimate N_e, N_e/N was consistently higher in smaller than in larger populations. Furthermore, a N_e/N of ~1 for N10 populations was supported by comparing simulations to the observed loss of nucleotide diversity, providing evidence for a lower rate of loss of nucleotide diversity than expected for bottlenecked populations (Figure 3). In addition, results provide empirical evidence for the observation that the levels of (additive) genetic variation and evolutionary potential are sometimes similar in small and large populations (Taft & Roff, 2012; Wood, Yates, & Fraser, 2016). This finding is important for our understanding of the processes and challenges of small populations, but despite higher N_e/N in smaller populations, we still detect a substantial loss of nucleotide diversity and a decrease in egg-to-adult viability in small populations, which is exacerbated under stress. This result reemphasizes the importance of maintaining large population sizes in natural as well as domestic populations (Hoffmann, Sgrò, & Kristensen, 2017).

We propose that associative overdominance is causing the lower than expected loss of nucleotide diversity in small populations and thereby the disproportional change in N_e across N. Associative overdominance can arise by a functional overdominant locus being linked to neutral loci (Gilligan et al., 2005; Rumball et al., 1994), driving the maintenance of genetic diversity at neutral loci. It may also arise

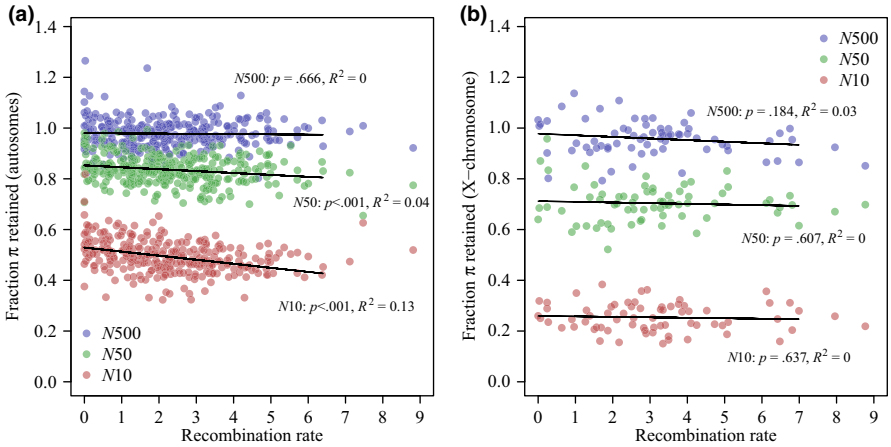

FIGURE 6 The relationship between recombination rate and fraction of nucleotide diversity retained across breeding regimes. For every genomic window of 300 kb, we calculated the average fraction of nucleotide diversity retained in generation 15 for populations reared at size 10 (N10), size 50 (N50) and size 500 (N500). The relationship between recombination rate of the genomic windows and the average fraction of retained nucleotide diversity is plotted for the autosomes (a) and the X-chromosome (b). We found a significant interaction between recombination rate and breeding regime for the autosomes (Table S2) and therefore performed separate models for each breeding regime which are represented by black lines in (a). We did not detect any effect of recombination rate on the fraction of retained nucleotide diversity on the X-chromosome (Table S2), but we performed separate models for each breeding regime, as for the autosomes, with the purpose of graphical representation and comparison to the autosomes [Colour figure can be viewed at wileyonlinelibrary.com]

from the presence of multiple recessive deleterious alleles across different haplotypes, resulting in a net advantage of heterozygote genotypes (Ohta, 1973; Pamilo & Pálsson, 1998; Zhao & Charlesworth, 2016). This mechanism has been suggested to conserve genetic variation in small populations because of strong linkage disequilibrium arising during the bottleneck coupled with a disfavoured homozygosity in the presence of recessive deleterious alleles (Ohta, 1973; Pamilo & Pálsson, 1998). The maintenance of genetic diversity may thus inflate estimates of N_e in small populations causing high N_e/N estimates. Although we cannot provide direct evidence for the presence of associative overdominance, we find this mechanism to be consistent with two separate observations in our data; the higher fraction of retained nucleotide diversity in areas of low recombination rate and a faster decrease in nucleotide diversity at the X-chromosome on which fewer recessive deleterious alleles reside due to hemizygosity in males.

Recombination rate affects the potential for directional selection to remove genetic variation through hitchhiking events. Directional selection is expected to have a higher efficacy in larger populations, which therefore could deplete genetic diversity not only at the selected site, but also in linked regions (Charlesworth, 2009). Multiple incomplete sweeps accompanied by long distance linkage may thus lead to non-neutrality of our markers (Franssen, Nolte, Tobler, & Schlötterer, 2014; Tobler et al., 2014) and reduce estimates of N_e across the genome. Conversely, different forms of balancing selection, such as associative overdominance, are likely to have a larger effect on the maintenance of genetic variation if recombination rate is low, as this may result in a higher frequency of linked recessive deleterious alleles. We found that areas of the genome, which had the lowest rates of recombination, also show the lowest loss of nucleotide diversity. This relationship was strongest in the smallest populations (N10) but also present in the intermediate-sized populations (N50). This result strongly supports the interpretation that associative overdominance is contributing to the observed loss of nucleotide diversity in the smaller populations. Large inversions may also underlie associative overdominance due to their restrictions on recombination (Frydenberg, 1963), but the consistent pattern of N_e/N in all chromosome arms (Figs S4 and S5) suggests no such effect of inversions here.

For species with heterogametic sex determination such as *D. melanogaster*, the X-chromosome diversity and N_e are expected to be three-fourths of the autosomal one, all else being equal (Wright, 1931). According to theoretical expectations and simulations, a bottleneck will initially result in a faster loss of diversity at the X-chromosome, decreasing the X to A diversity ratio (Pool & Nielsen, 2007). In the current study, the initial X to A diversity ratio of 0.69 decreased in intermediate-sized populations across generations, a pattern which is intensified in small populations (Figure 4). Nevertheless, when the expected drift on the X-chromosome is based on the autosomes and a correction factor of three-fourths, we found a significant deviation from neutral expectations for the small populations in the stable environment. The lack of recombination in *D. melanogaster* males increases the rate of recombination in the X-chromosome relative to the autosomes, which under background selection will result in an increased X to A diversity ratio (Charlesworth, 2012). However, we observed a reduced X to A diversity ratio compared to neutral expectations, again supporting the hypothesis of associative overdominance. Prior to the experiment, the founder population has likely experienced more effective purifying selection on the X-chromosome due to hemizygosity in males. Hence, the X-chromosome will have fewer recessive deleterious alleles than the autosomes, which impedes the loss of variation at small

population sizes. In natural populations, the level of X-linked variation is frequently lower than the autosomal variation, even after correcting for male hemizygosity. As such populations frequently colonize new environments, this effect has been explained by selection and increased drift during the associated bottlenecks (Gottipati, Arbiza, Siepel, Clark, & Keinan, 2011; Keinan, Mullikin, Patterson, & Reich, 2009; Veeramah, Gutenkunst, Woerner, Watkins, & Hammer, 2014). Also in *D. melanogaster*, the out of Africa habitat expansion resulted in a decreased X to A diversity ratio, when compared to the ancestral populations in Africa (Kauer, Zangerl, Dieringer, & Schlötterer, 2002). In the light of our results, we propose that associative overdominance should be considered as a mechanism generating a decreased X to A diversity ratio in natural populations.

A higher N_e/N in small populations than in larger populations has been described in beetles (Pray, Goodnight, Stevens, Schwartz, & Yan, 1996), frogs (Ficetola, Padoa-Schioppa, Wang, & Garner, 2010) and across and within salmonid fish populations (Araki, Waples, Ardren, Copper, & Blouin, 2007; Ardren & Kapuscinski, 2003; Palstra & Ruzzante, 2008). These studies have suggested several alternative explanations for this pattern, such as variance in reproductive success, which is also highly relevant in this study but notably not mutually exclusive with the presence of associative overdominance. The estimates of N_e/N ~1 in N10 populations in our study is larger than what is typically assumed for *D. melanogaster* (Nunney, 1993, 1995; Pischedda, Friberg, Stewart, Miller, & Rice, 2015), but is in accordance with the predicted effect of late age at maturity (compared to the generation time) characteristic for many insects (Waples et al., 2013). However, this prediction is dependent on an assumption of overlapping generations and random reproductive success among individuals (Waples et al., 2013). Both of these assumptions are violated in this study where we used discrete generations and a species known to show substantial higher variation in reproductive success of males than predicted from a Poisson distribution (Pischedda et al., 2015). Several studies suggested that a subtle change in the mating system may occur due to changes in census sizes, potentially affecting variation in reproductive success of males and thus N_e/N (Ardren & Kapuscinski, 2003; Palstra & Ruzzante, 2008). Such a pattern was observed in the agile frog, *Rana latastei*, where larger populations led to relatively fewer males contributing to the next generation, allegedly through more male–male competition resulting in larger harems (Ficetola et al., 2010). Conversely, in small populations, this would lead to less male–male competition and a decreased standardized variance in the family size and thus an increased N_e (Hedrick, 2005). In the current study, only five males were competing for mates in the N10 breeding regime compared to 25 males competing for mates in the N50 and N500 breeding regimes (reared under the exact same conditions, see *Materials and methods*). This could result in reduced male–male competition in N10 populations, decreasing standardized variance in the family size and increasing the N_e of small populations (Hedrick, 2005). Such a mechanism could thus contribute to the large N_e/N of N10 populations. However, adult density and horizontal surface per individual available for mating were approximately constant across all breeding regimes (10% less area in N10), whereby effects of the male–male competition dynamics on the result seem to be restricted.

In the ramping regime, flies were exposed to a small increase in average, maximum and minimum temperature every generation, and according to estimates of egg-to-adult viability, this resulted an increased environmental stress. This did not affect the rate of loss of nucleotide diversity in the autosomes; however, on the X-chromosome, we identified a slower loss of variation in the ramping regime in small populations. The fact that the difference between regimes is only observed in the X-chromosome suggests that this is not driven by specific characteristics of the environment, such as a temporal change in the selective landscape in the ramping regime, but instead relates to the hemizygosity in males. Assuming that associative overdominance is a major factor determining the loss of variation, this would imply that in the ramping regime the X-chromosome carries more deleterious alleles than it does in the stable temperature regime. We propose that the stable temperature regime matches better the natural environment of the founder population, while the ramping regime covers temperatures less frequently encountered in the wild. If deleterious alleles are more efficiently purged in commonly encountered environments, this could explain the differences between the two temperature regimes in our experiment. Interestingly, support for this idea has previously been presented in studies of gene expression intensities and alternative splicing. At benign temperatures, two *D. melanogaster* strains had similar gene expression profiles, but at more extreme temperatures their gene expression patterns diverged (Chen, Nolte, & Schlötterer, 2015; Jaksic & Schlötterer, 2016). These studies also suggested that purifying selection may have been less efficient at extreme temperatures.

The key observation in this study is that in small populations, nucleotide diversity was lost at a substantially lower rate than predicted. While several evolutionary and ecological forces could have shaped this pattern, we argue that associative overdominance is the most likely cause. We base this interpretation on the decreased loss of nucleotide diversity in regions of low recombination rate and the faster decrease in nucleotide diversity at the X-chromosome in which fewer recessive deleterious alleles reside due to hemizygosity in males. Our experimental framework provides a strong example of how and why the distribution of genetic diversity across natural populations and their chromosomes may deviate from typical neutral expectations.

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DATA ACCESSIBILITY

Sequences are deposited at the NCBI Sequence Read Archive (SRA) under the Accession no. SRP094880. Phenotypic data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.6tv76.

AUTHOR CONTRIBUTIONS

The experiments were conceived and designed by T.N.K., V.L., M.F.S. and J.B. The laboratory work was performed by M.F.S. and T.N.K. The data were analysed by M.F.S. and J.B. The data were interpreted by M.F.S., C.S., J.B., T.N.K. and V.L. The paper was written by M.F.S., C.S., T.N.K., J.B. and V.L.

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