LETTER

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Higher rates of sex evolve in spatially heterogeneous environments

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The evolution and maintenance of sexual reproduction has puzzled biologists for decades[1,2]. Although this field is rich in hypotheses[3–5], experimental evidence is scarce. Some important experiments have demonstrated differences in evolutionary rates between sexual and asexual populations[6–8]; other experiments have documented evolutionary changes in phenomena related to genetic mixing, such as recombination[9,10] and selfing[11]. However, direct experiments of the evolution of sex within populations are extremely rare (but see ref. 12). Here we use the rotifer, *Brachionus calyciflorus*, which is capable of both sexual and asexual reproduction, to test recent theory[13–15] predicting that there is more opportunity for sex to evolve in spatially heterogeneous environments. Replicated experimental populations of rotifers were maintained in homogeneous environments, composed of either high- or low-quality food habitats, or in heterogeneous environments that consisted of a mix of the two habitats. For populations maintained in either type of homogeneous environment, the rate of sex evolves rapidly towards zero. In contrast, higher rates of sex evolve in populations experiencing spatially heterogeneous environments. The data indicate that the higher level of sex observed under heterogeneity is not due to sex being less costly or selection against sex being less efficient; rather sex is sufficiently advantageous in heterogeneous environments to overwhelm its inherent costs[2]. Counter to some alternative theories[16,17] for the evolution of sex, there is no evidence that genetic drift plays any part in the evolution of sex in these populations.

Sex shuffles genotypes, changing genetic associations through recombination and segregation. Sex is thought to evolve as a byproduct of the selection on these altered genetic associations[4]. All theories for the evolution of sex invoke some mechanism that maintains genetic variation because shuffling without variation does not yield any change. Much of modern theory has focused on deleterious mutations[17–19] or host–parasite coevolution[20–22] as the key source of genetic variation. However, a more classic explanation for the maintenance of genetic variation is spatial heterogeneity in selection[23,24]. Several recent theoretical studies have shown that sex evolves more easily when there is spatial heterogeneity in selection[13–15].

If selection is the dominant evolutionary force shaping gene associations, then sex is usually disadvantageous. This is because selection leads to an excess of good allele combinations and sex destroys these combinations through recombination and segregation[3,25]. However, in spatially heterogeneous habitats, migration, not just selection, is important in determining gene associations. Maladaptive gene combinations are constantly introduced to local populations through migration. Sex is then potentially beneficial because it helps to break down maladaptive gene associations generated by differential selection and migration[13–15]. For sex to be favoured, the theory makes additional requirements about the nature of gene interactions (for example, locally adapted alleles should be dominant) but there are some reasons to expect these may be met[14]. Nonetheless, the theory is clear in predicting that the opportunity for sex to be advantageous is greater in spatially heterogeneous habitats than in homogeneous ones.

We tested this prediction in experimental populations of the monogonont rotifer *Brachionus calyciflorus* evolving in either homogeneous or heterogeneous environments. Monogonont rotifers are cyclic diploid parthenogens (that is, they normally reproduce asexually) and mixis (sexual reproduction) is induced by high rotifer densities via quorum sensing[26] (Supplementary Fig. 1). A preliminary study of our source population revealed ample genetic variation for the propensity to reproduce sexually, thus providing the necessary substrate for the evolution of sex (Supplementary Fig. 2).

In our experiments, rotifers were maintained in semi-continuous cultures at large population size ($N \approx 10,000$). We used two different food conditions to establish differentially selective environments (Methods). Each replicate population consisted of two subpopulations. In the homogeneous treatments, both subpopulations were of the same environmental type, that is, either both high-quality food or both low-quality food. In the heterogeneous treatment, each population was composed of one high-quality food subpopulation and one low-quality food subpopulation. Migration between subpopulations was performed by weekly manual transfer of individuals, for all treatments. Two migration rates were used corresponding to $m \approx 10\%$ and $m \approx 1\%$ per generation, assuming a generation time of about one day. Observed population densities (females, males, eggs and resting eggs) were similar across all food-quality environments (Supplementary Figs 3 and 4).

To confirm that our high-quality and low-quality food environments imposed different selection regimes, we measured fitness (lifetime per capita number of offspring) after 15 weeks of evolution of rotifers from the two homogeneous treatments (Fig. 1). We found that fitness was higher when measured in their evolved habitat than when fitness was measured in the alternative habitat, confirming differential adaptation.

We used two different methods to detect changes in the rate of sex in our experiment. First, we measured the fraction of isolated individual clones (84 per population with 42 from each subpopulation) that switched to sexual reproduction when exposed to a sex-inducing stimulus under standardized conditions (Methods). We performed this assay at the start of the experiment, after 6 weeks (about 40–45 generations) and again after 12 weeks (about 80–90 generations). The measured propensity for sex was significantly higher when migration took place in a heterogeneous environment (between high- and low-quality food conditions) than in a homogeneous environment (Fig. 2 and Supplementary Fig. 5) after 6 and 12 weeks (generalized linear model (GLM), $P < 0.001$) with a rapidly decreasing rate of sex in the spatially homogeneous environments. No significant differences were observed for the two migration rates or between the two homogeneous environments (Fig. 2).

To confirm that the differences between treatments observed under standardized assay conditions reflect real differences *in situ*, we used estimates of the percentage of sexually derived offspring (resting eggs) of total offspring (resting and amictic eggs) as a second measure of changes in the rate of sex over time and among treatments (Fig. 3). In the latter part of the experiment (from day 74 to day 109; Fig. 3b), the

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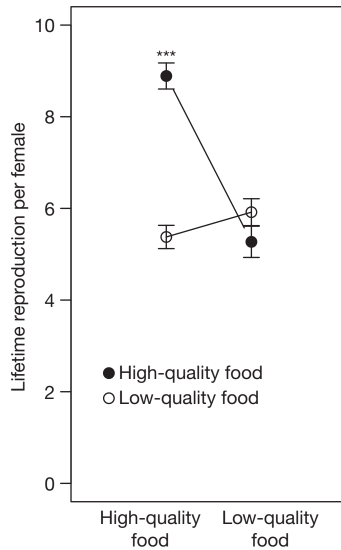

Figure 1 | Fitness in alternative environments. Lifetime reproduction (fitness) of amictic (asexual) *Brachionus calyciflorus* females was measured on individuals from populations that had evolved for 15 weeks under conditions of either high or low food quality (see text and Methods). The fitness of the same genotype of ten individual clones (third generation after isolation) was measured under high-quality food conditions (left) and low-food-quality conditions (right); $n = 18$ populations; eight populations with a migration rate of $m \approx 10\%$ and ten populations with $m \approx 1\%$ per generation. The graph shows the means ± one standard error; GLMM $^{***}P < 0.001$ for the high-quality food environment; the difference is not significant in the low-quality food environment. See Methods for fitness assay description.

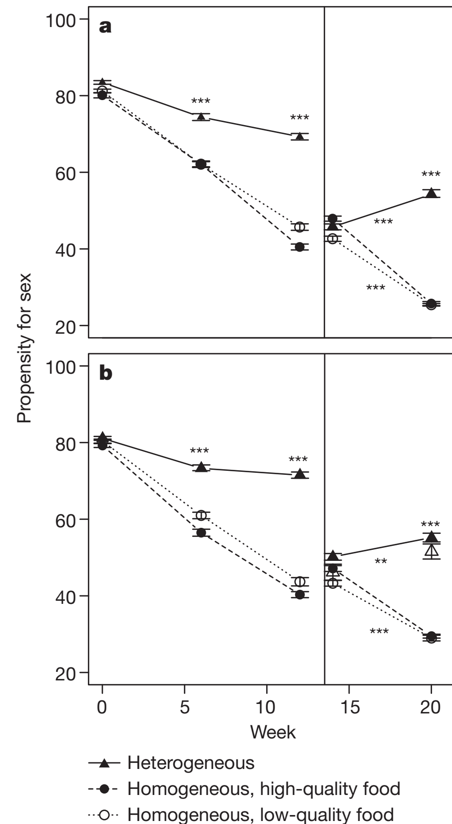

Figure 2 | Evolution of the propensity for sex in *Brachionus calyciflorus* populations from spatially heterogeneous and spatially homogeneous environments measured in a common environment. The propensity for sex was measured as the percentage of females induced into mixis (sexual reproduction) when exposed to a standardized stimulus. Vertical lines at week 14 mark the start of the second part of the experiment, when all populations were mixed and reassigned to treatments. Each data point represents the mean of 7–10 populations per treatment ± one standard error. Migration rates are shown of $m \approx 10\%$ per generation (**a**) and $m \approx 1\%$ per generation (**b**). For both migration rates, the rate of sex is significantly greater in the heterogeneous treatment than in either homogeneous treatment in comparisons at weeks 6, 12 and 20 ($^{***}P < 0.001$ for all comparisons). Between week 14 and 20, the rate of sex significantly increases in the heterogeneous treatment ($^{***}P < 0.001$ for $m \approx 10\%$; $^{**}P = 0.01$ for $m \approx 1\%$). In contrast, sex declines in the homogeneous treatments ($^{***}P < 0.001$). Open triangles (only for $m \approx 1\%$ in **b**) represent heterogeneous populations evolving at ten times the standard size of $N \approx 10{,}000$.

populations reached stable densities with no significant variation in density among treatments. This permits a reasonable comparison of rates of sex among treatments during this period. The percentage of sexually derived offspring was significantly greater in the heterogeneous treatment than in the homogeneous treatments (generalized linear mixed model (GLMM): for the comparison between the heterogeneous and homogeneous high-quality food condition, $\chi^2 = 31.458$, degrees of freedom, d.f. = 1, $P < 0.001$; for the comparison between heterogeneous and homogeneous low-quality food conditions, $\chi^2 = 24.947$, d.f. = 1, $P < 0.001$). In the heterogeneous treatment, about 15% of eggs were sexually derived whereas in both of the homogeneous treatments only about 7% of eggs were sexually derived.

Both lines of evidence above indicate that sex evolves differently in heterogeneous versus homogeneous environments. Sex declines dramatically in the homogeneous treatment but little, if at all, in the heterogeneous treatment (Fig. 2 and Supplementary Fig. 5). However, there are several interpretations of this result. First, the putative benefits of sex under heterogeneity could be sufficiently large to balance its inherent costs, resulting in a higher equilibrium rate of sex than in the homogeneous environments. Second, benefits to sex may exist under heterogeneity but these are not sufficient to fully offset its costs. Consequently, sex declines in the heterogeneous treatment but at a slower rate. Third, net selection on sex does not differ between treatments (that is, there are no benefits due to heterogeneity). Rather, selection on sex is simply less efficient in the heterogeneous environment because there is more genetic variance in fitness, that is, Hill–Robertson effects[27] impede the elimination of alleles, causing higher rates of sex.

To distinguish amongst these possibilities, we restarted the experiment at week 14. We mixed all rotifer populations from the earlier experiment, combining all replicates of all three treatments (homogeneous high-quality food, homogeneous low-quality food, and heterogeneous) to create populations with an intermediate rate of sex (Fig. 2; the vertical line marks the mixing and the start of the second part of the experiment). Rotifers were split again into 120 populations and populations grew again for 6 weeks under the same conditions as for weeks 0–13 (food conditions, migration pattern, number of replicates) and we measured the propensity for sex again after 6 weeks of evolution (week 20 in Fig. 2). To assess the possibility of less efficient selection on sex in the heterogeneous environment due to drift-related effects, we added three additional replicates to the heterogeneous treatment (lower migration rate only) in which the population size was increased tenfold.

After an additional 6 weeks (about 40–45 generations) of evolution, the propensity for sex continued to decrease in the homogeneous populations (GLMM: in Fig. 2a, $\chi^2 = 136.98$, d.f. = 3, $P < 0.001$; in Fig. 2b, $\chi^2 = 82.903$, d.f. = 3, $P < 0.001$, for both migration rates together, $\chi^2 = 220.86$, d.f. = 2, $P < 0.001$). In contrast, the propensity for sex evolved upwards in the spatially heterogeneous populations (GLMM: in Fig. 2a, $\chi^2 = 15.41$, d.f. = 2, $P < 0.001$; in Fig. 2b, $\chi^2 = 10.72$, d.f. = 1, $P = 0.01$; for both together, $\chi^2 = 20.3$, d.f. = 1, $P < 0.001$). This result indicates that the advantages to sex outweigh its costs under spatial heterogeneity. Moreover, there were no differences between the larger and the smaller population sizes (Fig. 2b, open

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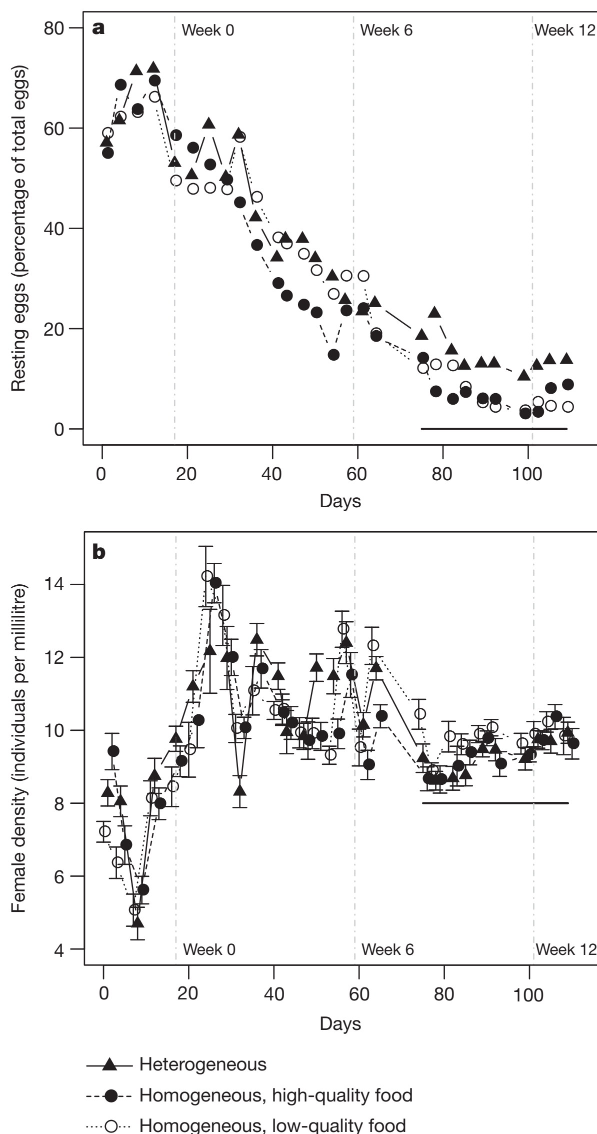

Figure 3 | *In situ* measure of the rate of sex in *Brachionus calyciflorus* populations from spatially heterogeneous and spatially homogeneous environments measured as the fraction of sexually derived offspring (resting eggs) of total offspring. Mean percentages of resting eggs out of all eggs in *Brachionus calyciflorus* populations (**a**) and mean female densities (**b**) are plotted over time. Error bars represent ± one standard error. The populations appear to reach demographic equilibrium after about 75 days. After this point (days 75–109), female densities are very similar across treatments, thereby permitting a reasonable comparison for rates of sex. During this period, the percentage of sexually produced offspring was significantly higher in the heterogeneous treatment than in the homogeneous treatments (GLMM: for the heterogeneous versus homogeneous high-quality food condition, $\chi^2 = 31.458$, d.f. = 1, $P < 0.001$; for the heterogeneous versus homogeneous low-quality food condition, $\chi^2 = 24.947$, d.f. = 1, $P < 0.001$). Vertical lines mark the time points at which the propensity for sex was measured in a common environment (Fig. 2) and horizontal lines mark the data used for the comparison of resting egg fraction between treatments.

triangles), suggesting that drift-based hypotheses for the evolution of sex[16–18] are not responsible for these results.

In this experimental system, there is the 'twofold' cost of meiosis[28] as well as a time cost because sexual reproduction takes longer than asexual reproduction. These costs tend to keep the equilibrium level of sex quite low and seem to favour complete asexuality under homogeneous conditions. Even with spatial heterogeneity, the equilibrium rate of sex is low. Nonetheless, non-zero rates of sex evolve with spatial heterogeneity despite the substantial costs. These results are therefore consistent with recent theory predicting that there is more opportunity for sex to be favoured in spatially heterogeneous environments[13–15]. However, this experiment was not designed to determine the mechanisms favouring higher levels of sex, so caution should be used in making interpretations in this regard. Below we consider alternative interpretations.

It is possible that higher levels of sex evolved here as a correlated response to selection for resting eggs. This could occur if resting eggs survive better than amictic eggs in transfers from one habitat type to another. If this were the case, then we would expect higher levels of sex when there was more movement between habitats because the strength of selection should be directly proportional to the probability of experiencing an environmental change, which is equivalent to the migration rate. However, there is little difference in the rate of sex between the two migration rates. The similarity between migration treatments is also somewhat surprising with respect to migration models of sex[13–15], though less so. Such models predict that sex is most strongly favoured at some intermediate level of migration, where the 'optimal' migration rate depends on the genetic architecture of the locally adapted traits. It is possible that the equilibrium level of sex may be fairly robust to quantitative differences in migration rate under the right genetic conditions.

Adaptation to new environments is also thought to facilitate the evolution of sex[2,10,16]. Both habitats used here were slightly different to previous culture conditions, so adaptation is likely to have occurred in all treatments. Interestingly, sex seems to increase initially (days 0 to 16 in Fig. 3a) in all treatments, despite low density during this period, then later declines. This observation is consistent with sex being advantageous when the rate of adaptation is likely to be highest but then becoming less favoured (owing to its costs) after adaptation slows. Although this pattern is interesting, it seems unlikely that 'sex for adaptation' explains the differences that develop between homogeneous and heterogeneous treatments over the longer term.

Recent theory has shown that sex may evolve as a way for genes to escape bad genetic backgrounds[3,29]; this is known as 'fitness-associated' sex. With spatial heterogeneity, maladaptive genotypes are constantly introduced through migration, providing more opportunity for fitness-associated sex to evolve. Under this hypothesis, we would expect that genotypes have more sex when tested in the alternative environment from which they were collected. However, we find no support for this prediction. Clones collected from the two different habitats in the heterogeneous environment show no difference with respect to the rate of sex when tested under the same environment (Supplementary Fig. 6).

The evolution of sex has been one of the enduring problems of evolutionary biology. Although there has been a large amount of theory, experimental tests of sex itself have been limited to comparisons of sexual and asexual populations[6,7]. Experimental evolution has been largely neglected as a means of examining the more perplexing question of how rates of sex evolve within populations (but see ref. 12). Our experiment demonstrates that rates of sex do evolve within experimental populations and that non-zero rates of sex can be favoured even in the face of real costs. This suggests that this approach can be applied to a variety of facultatively sexual organisms (able to reproduce both sexually and asexually) to test the predictions of different theoretical models. Future work should not only identify the types of conditions that favour the evolution of sex but also examine the population genetic mechanisms by which these benefits arise[4]. By doing so, we can begin bridging the sizeable gap between theory and the empirical patterns observed in nature.

It is worth noting that the rate of sex declined in all treatments compared to the initial state (Fig. 3). Because these experiments were started with field-collected organisms, this observation suggests that the equilibrium rate of sex in nature is higher than in any of the laboratory environments used here. Whether this discrepancy is due to greater environmental heterogeneity in the field or other factors (such as parasites or more targets of selection) is unknown and presents a challenge for future studies.

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METHODS SUMMARY

Creation of experimental populations. Populations were initiated from a laboratory population that had been recently hatched from a large number of field-collected resting eggs. Rotifer–algae populations were established in 500-millilitre batch cultures under two sets of conditions to create different selection regimes: high-quality food conditions and low-quality food conditions. 10% of each culture (including rotifers and algae) was replaced twice per week with the respective algae solution (2×10^6 cells ml^{-1}) taken from constant chemostat cultures. Each population was composed of two subpopulations: for the homogeneous treatment, both were either from low-quality food or both from high-quality food conditions, and for the heterogeneous treatment, the subpopulations were of opposite types. Once per week, 50% or 5% of the rotifers (females, males, eggs, resting eggs) were exchanged between the two subpopulations. Assuming a generation time of 1 to 2 days, these migration regimes are equivalent to approximately $m \approx 10\%$ and $m \approx 1\%$ per generation, respectively.

Assay of propensity to reproduce sexually. The propensity for sex was measured by isolating 42 (amictic) clones from each subpopulation (84 clones per population). Clones were individually transferred into single wells with 10 ml of high-quality food and maintained in these conditions for two amictic generations to eliminate environmental effects. For the mixis assay, two neonates of the third generation after isolation were individually transferred to a single cell of a 96-well plate with conditioned medium (sex-inducing stimulus) at two different concentrations. Conditioned medium was freshly prepared from a dense laboratory rotifer stock culture by removing rotifers from the medium and diluting it with fresh medium to a stimulus concentration representative of 22.5 females per millilitre. After the first day of the assay the initial females were removed and the offspring were scored as amictic or mictic by their produced offspring: males are produced by sexual females and females by amictic females. For the assays at the start, after 6 and 12 weeks, an additional set of tests using a lower concentration of sex-inducing stimulus (12 females per millilitre) were also performed. Results were qualitatively similar (Supplementary Fig. 5).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions L.B. and A.F.A. conceived and designed the study, L.B. performed experiments, L.B. and A.F.A. discussed and analysed the results, and shared the writing of the paper.

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METHODS

All cultures were inoculated from a *Brachionus calyciflorus* laboratory stock culture, which was started in June 2009 from field-collected resting egg hatches. Rotifer–algae cultures were kept at $25 \pm 1.5\,^{\circ}$C (12 h dark/12 h light) in tissue culture flasks (Sarsted) and moved randomly three times per week across three shelves of an incubator. Each population consisted of two subpopulations. Each subpopulation was maintained in a 500-ml batch culture at a density of about ten rotifers per millilitre, that is, about 5,000 rotifers per subpopulation giving $N \approx 10{,}000$ per population (detailed density data provided in Supplementary Fig. 3). Each subpopulation was one of two habitat types: high-quality food conditions or low-quality food conditions. 10% of each subpopulation (including rotifers and algae) was removed twice per week and an equivalent volume of the appropriate algae solution (below) was replaced. From the removed sample, rotifer densities (females, males, eggs, resting eggs) were enumerated under a stereoscope. For the replacement solution, algae were taken from long-term chemostat cultures to ensure constant food conditions over the course of experiment (high-quality food condition: nitrogen concentration in medium = 1,000 µM per litre; low-quality food condition: nitrogen concentration in medium = 160 µM with an additional 0.5 g ml^{-1} NaCl per litre); both chemostats were inoculated from the same stock four weeks before the start of the experiment (SAG 278-3, Algae Culture Collection University of Göttingen). The replacement algal solution was prepared by diluting either of the two chemostat cultures to a concentration of 2×10^6 algal cells per millilitre in nitrogen-free inorganic medium (modified after ref. 30 with 0.5 g l^{-1} NaCl added to the low-quality food environment).

Asexual rotifer females produced about one offspring per day and juveniles started reproducing a few hours after hatching (observed by L.B.). Migration of rotifers (females, males, eggs, resting eggs) between two subpopulations took place once per week by filtering out the rotifers (adults and eggs) from 50% or 5% of the volume from each subpopulation and exchanging the rotifers between subpopulation pairs. Assuming a generation time of 1 to 2 days, a 50% or 5% weekly exchange corresponds to $m \approx 10\%$ or $m \approx 1\%$ per generation, respectively. Migration started two weeks (day 18) after rotifer inoculation.

At week 15 (week 13 of migration), ten clones were isolated from each subpopulation and transferred individually to 10 ml of high-quality food. Two neonates of the third generation after isolation were used to assay lifetime reproduction (fitness) under either high-quality food or low-quality food conditions. The number of offspring of females was recorded daily and females were transferred to new wells with fresh medium at the same time until the females died.

Sex stimulus in standardized environment. Mixis in *Brachionus calyciflorus* is stimulated through a mixis-inducing protein that is excreted by *Brachionus* females and accumulates in the medium[26] (Supplementary Fig. 1). Conditioned medium (sex stimulus) was prepared freshly from a dense laboratory stock culture by removing rotifers (repeated filtration through 10 µm mesh) from the medium and diluting it with fresh medium to a desired 'equivalent rotifer density' of 22.5 or 12 females per millilitre (ref. 26). For the second part of the experiment after week 14, rotifers were only tested at 22.5 females per millilitre.

The propensity for sex was measured by isolating 42 asexual (amictic) clones from each subpopulation (84 clones per population). Clones were individually transferred into single wells with 10 ml of high-quality food and maintained under these conditions for two generations. For the mixis assay, two neonates of the third generation after isolation were individually transferred to a single cell of a 96-well plate with conditioned medium at the two different concentrations. After the first day of the assay the initial females were removed and her offspring were scored as amictic or mictic by the type of offspring that they produced. Amictic (asexual) females produce female offspring. Sexual females produce only haploid males because they are unmated in this assay (Supplementary Fig. 1).

Creation of populations with a low initial rate of sex. After week 13, experimental populations from all treatments were mixed. Because two-thirds of these populations were from the homogeneous treatment, the average rate of sex in this newly mixed population was quite low. New experimental populations were created from this mixed population and distributed amongst the same set of treatments as in the original experiment. To assess whether genetic drift was affecting evolution in the heterogeneous treatment, three larger populations were added to this treatment (for the lower migration rate only). The larger populations grew under the same conditions but each population consisted of 20 subpopulations (ten of each type) rather than two. For migration, 10% of the volume of each of the ten subpopulations of the same habitat type was removed and pooled. Half of this volume was equally distributed back to the same subpopulations (within-habitat migration) and the remainder was equally distributed amongst the ten alternative habitat subpopulations (between-habitat migration).

Data analysis. Multivariate analyses were performed in the R statistical environment using the lmer4 package[31,32]. Fitness data were analysed using generalized mixed models with environmental origin (the high- or low-quality food environment to which the populations adapted) as a fixed effect and replicated population as a random effect for the comparison of the mean fitness tested within one environment (either original or novel in Fig. 1; GLMM with quasi-Poisson error structure). Differences among environments (heterogeneous, homogeneous low-quality food, homogeneous high-quality food) in the percentages of mixis-induced females in the mixis assay (Fig. 2) were tested by using a GLM (with binomial error distribution). The effect of population size (Hill–Robertson effect) on the rate of sex within the heterogeneous treatment was tested in the same way. To test for differences in the rate of sex between the two migration rates, we compared generalized linear models with and without the migration term. For each environmental treatment, GLMM were used to test the change in the rate of sex between weeks 14 and 20, with time as a fixed effect and replicated population as a random effect (for the heterogeneous treatment, both population sizes were included). Differences in the percentage of resting eggs out of all eggs (*in situ*) were estimated from resting egg and amictic egg densities (days 75 to 109); statistical differences were determined by comparing generalized mixed models with and without treatment, treating replicated population as a random effect (quasi-binomial error correction).

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