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MICROBIAL LOCAL ADAPTATION

Evolutionary rescue and local adaptation under different rates of temperature increase: a combined analysis of changes in phenotype expression and genotype frequency in *Paramecium* microcosms

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Abstract

Evolutionary rescue (ER) occurs when populations, which have declined due to rapid environmental change, recover through genetic adaptation. The success of this process and the evolutionary trajectory of the population strongly depend on the rate of environmental change. Here we investigated how different rates of temperature increase (from 23 to 32 °C) affect population persistence and evolutionary change in experimental microcosms of the protozoan *Paramecium caudatum*. Consistent with theory on ER, we found that those populations experiencing the slowest rate of temperature increase were the least likely to become extinct and tended to be the best adapted to the new temperature environment. All high-temperature populations were more tolerant to severe heat stress (35, 37 °C), indicating a common mechanism of heat protection. High-temperature populations also had superior growth rates at optimum temperatures, leading to the absence of a pattern of local adaptation to control (23 °C) and high-temperature (32 °C) environments. However, high-temperature populations had reduced growth at low temperatures (5–9 °C), causing a shift in the temperature niche. In part, the observed evolutionary change can be explained by selection from standing variation. Using mitochondrial markers, we found complete divergence between control and high-temperature populations in the frequencies of six initial founder genotypes. Our results confirm basic predictions of ER and illustrate how adaptation to an extreme local environment can produce positive as well as negative correlated responses to selection over the entire range of the ecological niche.

Keywords: evolutionary rescue, experimental evolution, *Paramecium*, phenotypic change, selection, temperature adaptation

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Introduction

Anthropogenic emissions of greenhouse gases are expected to result in the most rapid climate change recorded in the past 65 million years (Diffenbaugh & Field 2013). Despite many years of research on the resulting impacts of biodiversity loss on ecosystems (Bellard *et al.* 2012), predicting the impacts of climate change on individual populations, species or communities still remains a major challenge. Potential responses include shifting habitat range to track climatic change (Chen *et al.* 2011), phenotypic plasticity (Charmantier *et al.* 2008; Chevin *et al.* 2010) and rapid evolutionary change (Bell & Collins 2008; Hoffmann & Sgro 2011). Indeed, for many organisms, years or decades of climatic change may cover a large enough number of generations to produce evolutionary responses. The notion that evolutionary change can occur on the same timescale as ecological processes (known as eco-evolutionary

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dynamics, Pelletier *et al.* 2009) is increasingly recognized in the field of conservation biology (Stockwell *et al.* 2003; Alexander *et al.* 2014); it is now common sense that understanding the role of contemporary evolution is a necessity for predicting the impacts of climate change on biodiversity (Lavergne *et al.* 2010).

Basic concepts in evolutionary biology provide the framework for addressing this issue. One such concept is that of 'evolutionary rescue' (ER). It describes the capacity of populations to survive rapid environmental change by evolving adaptations that allow survival in the new environment and restore positive population growth (Bell & Collins 2008; Gonzalez *et al.* 2013). If evolutionary rescue occurs, then the population dynamics are typically defined by a *U*-shaped curve, where a strong initial population decline is followed by an increase, as surviving (and adapted) individuals rapidly reproduce (Gomulkiewicz & Holt 1995; Bell & Gonzalez 2009; Martin *et al.* 2013). The likelihood of ER depends on the effect size of the environmental stress and the rate at which environmental conditions deteriorate (Gonzalez *et al.* 2013). If an environmental change is sudden, then a large, rapid population decline is probable, increasing the chance of extinction. In this case, ER likely relies on existing genetic variation within the population or better adapted variants quickly entering the population through migration or mutation. In this scenario, it is argued that adaptation relies on a small number of mutations with large effects that fix rapidly (Bell 2013). Conversely, if environmental change is more gradual, then adaptation may proceed by the slower fixation of many mutations of small effect as the population tracks the changing environmental conditions (Lynch & Lande 1993; Bell 2013). Gradual environmental degradation increases the likelihood of successful evolutionary rescue (Bell & Gonzalez 2011). Experimental studies have demonstrated that slower rates of environmental change can result in more adapted and thus more resilient, final populations or fewer extinctions (Huey *et al.* 1991; Perron *et al.* 2008; Collins & de Meaux 2009; Bell & Gonzalez 2011). Rapidly deteriorating environments can limit both the availability of mutations due to reduced population size and can make some adapted genotypes evolutionarily inaccessible (Lindsey *et al.* 2013). Thus, the rate of environmental change affects both the likelihood of evolutionary rescue occurring, and the evolutionary trajectory and genetic architecture which results.

Predicting responses to global climate change also requires knowledge of the costs and consequences of (rapid) adaptation. Namely, this concerns the niche shape, that is the range of environmental conditions where a population can survive. One prediction is that global change requires adaptation to increasing temperature (Storey *et al.* 2012) and therefore a possible shift of the temperature niche towards a higher optimum. If adaptation to higher temperatures is costly, a shift at the warmer end of the niche may be accompanied by loss of adaptation at the cooler end (Huey & Kingsolver 1989; Huey *et al.* 1991). Such a process is known as the 'sliding niche model' (Mongold *et al.* 1999), or the 'sidestep niche model', if adaptive plasticity confers temporary expansion before the niche contracts (Gallet *et al.* 2014).

The interplay between direct and correlated responses to selection shaping the ecological niche can have important consequences for the geographic range and distribution of genetic diversity. For example, trade-offs between adaptation to higher vs. lower temperatures may lead to a pattern of locally adapted populations across a temperature gradient (e.g. Byars *et al.* 2007; Mboup *et al.* 2012; Stefansson *et al.* 2013). Conversely, positive correlated responses to selection, rather than trade-offs, may explain a niche expansion. This may occur when local adaptation to temperatures at or near the current niche boundary enhances the capacity to survive at even higher levels of stress outside of the current niche (Bell & Gonzalez 2011).

Experimental evolution is a powerful tool to address fundamental questions about adaptation to rapidly changing environments (Jessup *et al.* 2004). Here, we studied the long-term dynamics in experimental microcosms of the freshwater ciliate *Paramecium caudatum* exposed to different rates of temperature increase to ask how the rate of environmental change affects the likelihood of evolutionary rescue. By measuring growth of evolved populations over a temperature gradient (5–37 °C), we investigated how evolution in a local increasingly high-temperature environment (max. 32 °C) affected the *P. caudatum* temperature niche. In particular, we tested whether patterns of local adaptation emerged between these high-temperature populations and control populations kept at a standard permissive temperature (23 °C). Microcosm populations were initiated from a mix of several *Paramecium* founder clones; analysis of mitochondrial markers and temperature profiles of these clones were employed to test whether the observed phenotypic changes in our experimental populations could be explained by changes in the relative frequencies of the founder clones.

Materials and methods

Study organism

The ciliate *Paramecium caudatum* lives in lakes and ponds, where it feeds on bacteria and detritus (Wichterman 1986). Binary fission is the primary form of

reproduction, involving mitotic division of the germline-like micronucleus and amitotic division of the somatic and highly polyploid macronucleus. Optimum growth conditions allow 2–3 fissions per day. During the asexual stage, gene expression occurs exclusively in the macronucleus, while the micronucleus remains genetically 'silent' in terms of transcription. Thus, epigenetic de novo adaptations may arise in the macronucleus, either through frequency changes of advantageous alleles during amitotic division (phenotypic assortment, Brito *et al.* 2010) or by typical epigenetic mechanisms, such as methylation, histone modification, chromatin remodelling or noncoding RNAs (Casadesus & Low 2006; Mattick & Makunin 2006; Herrera *et al.* 2012).

Sexual reproduction occurs through conjugation and exchange of gametic nuclei, derived from the micronucleus (for a description of the sexual life cycle see Fig. S1, Supporting information). A pair of conjugating mother cells produces two sexual exconjugants, in which the old macronucleus disintegrates and a new one is built from a copy of the zygotic (micro)nucleus. However, each exconjugant retains the cytoplasmic components of its respective mother cell. Thus, a sexual event does not change the frequency of mitochondrial markers, which were chosen in this experiment to analyse the genotypic composition of evolved populations (see below). Note that we never observed any conjugating cells (during density counts) over the entire long-term experiment, suggesting that recombination was at least a rare event.

Laboratory optimum growth rates are typically around 28 °C and temperature becomes stressful at 32 °C or higher and growth stops at or below 5 °C (Krenek *et al.* 2011, 2012). Krenek *et al.* (2012) found substantial natural genetic variation in temperature responses: European clones tolerate heat stress of up to 32 °C, whereas clones from Indonesia can still grow at temperatures around 35 °C. In our laboratory, we maintain long-term cultures at 23 °C in a medium prepared from dried organic lettuce and supplemented with the food bacterium *Serratia marcescens* (Nidelet & Kaltz 2007).

Long-term selection experiment

Initially, six *P. caudatum* clones, obtained from different geographical locations (Table 1), were combined into a single mixed culture, to ensure standing genetic variation in the founder populations. Each of the original clones was maintained separately at a temperature of 23 °C for future comparison. From the mixed culture, founder populations were created in 45 mL of medium (ca. 5000 cells). These were randomly assigned either to one of three high-temperature treatments (final temperature of 32 °C) or a control treatment (23 °C; see Fig. 1). Starting from 23 °C, high-temperature populations were gradually brought to 26 °C over 2 weeks. Then, the temperature was increased to 32 °C with a 0.4 °C increase every 8 days for the 'fast' lines (14 replicates), a 0.2 °C increase for the 'intermediate' lines (12 replicates) and a 0.1 °C increase for the 'slow' lines (10 replicates). The 10 control lines were maintained at 23 °C during the entire experiment.

All 46 selection lines were maintained under the same feeding regime: After 4 days, 30 mL was removed from each population and 15 mL of fresh medium was added. After 8 days, 15 mL of fresh medium was again added, and this cycle was repeated. Thus, with at least two population doublings per 8-day period, populations from the fast treatment reached 32 °C after ca. 30 generations (120 days), from the intermediate treatment after ca. 60 generations (240 days) and from the slow treatment after ca. 120 generations (480 days). Populations then continued to grow at 32 °C for another 17 months (Fig. 1). We regularly checked density in the selection lines by removing 100 μL of culture and counting the number of *Paramecium* under a dissecting microscope.

Temperature adaptation assays

Three consecutive adaptation assays were performed, with slightly different protocols, summarized in Table S1 (Supporting information).

Assay 1. A first phenotypic adaptation assay was carried out after 16 months, when all high-temperature treatment populations had reached 32 °C. Assay temperatures were 23, 32 and 35 °C, the latter being a lethal temperature when applied over several days (Duncan *et al.* 2011). Two 10-ml samples from each of six randomly chosen selection lines per treatment (6 × 4 treatments = 24 lines) were cultured for 6 days at 23 and

Table 1 The sources and year the cultures arrived in the laboratory for each of the six clones, which were mixed to create the initial founder populations used in the study. The cytochrome *c* oxidase I (COI) haplotype was determined in this study

Clone	Source	Year	COI
VEN	Venice, Italy	2006	PcCOI_a01
K8	Japan	2001	PcCOI_b07
TUB	Tübingen, Germany	2001	PcCOI_b07
CRA	Kraków, Poland	2006	PcCOI_b07
M3	Isolated from commercial ciliate mix (USA)	2004	PcCOI_b07
GO	Stuttgart, Germany	2008	PcCOI_a18

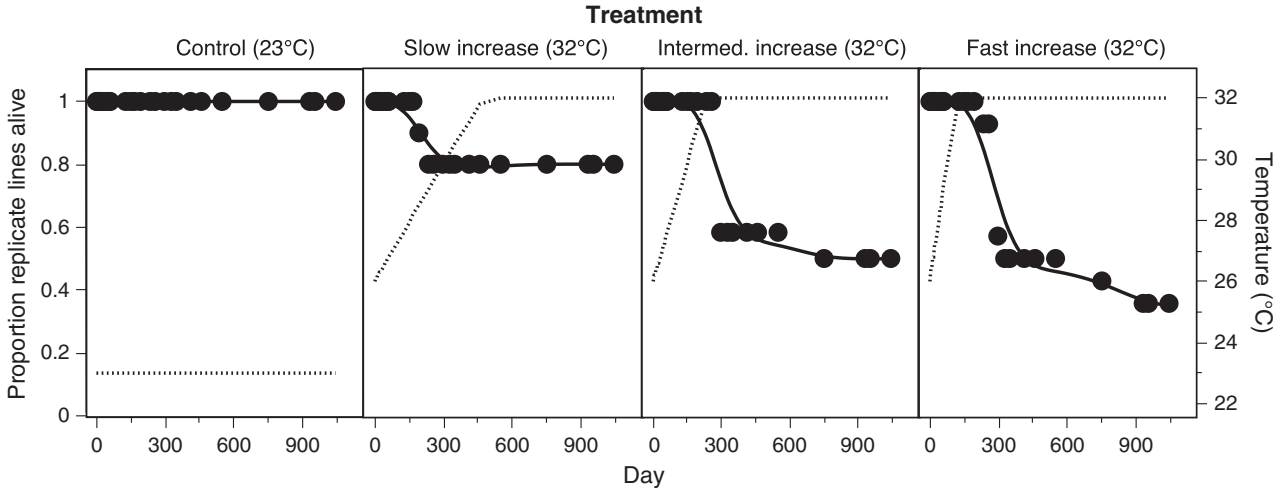

Fig. 1 Population survival through time (33 months) in the 23 °C control and in three high-temperature treatments (32 °C). Dotted lines show the change in temperature through time. Initial number of populations: 10 (control), 10 (slow increase), 12 (intermediate increase), 14 (fast increase).

32 °C, respectively, for acclimation. Cells were not conditioned at 35 °C, because this condition is lethal. Then, groups of 10 individuals were handpicked with a pipette under a dissecting microscope and transferred to a 2-ml tube containing 300 μL of medium. These assay tubes were then incubated for 6 days at 23, 32 or 35 °C; the volume was doubled every 2 days with fresh medium to promote *Paramecium* division. On days 4 and 6, density was determined from 50- or 100-μL samples (depending on treatment), using destructive sampling. We established two assay tubes per assay temperature and selection line (144 replicates in total; Table S1, Supporting information).

Assays 2 and 3. After 33 months, two additional assays were performed, using similar experimental protocols. Assay 2 comprised tests at 23, 32 and 35 °C, in two independent replicate runs of 7 days, using 12 high-temperature selection lines (four each from slow, intermediate and fast treatments) and eight control selection lines. Following 48 h of acclimation (23 °C/32 °C), each assay replicate started from 10 handpicked individuals and density was measured on day 5 and day 7. Replicates for the second run were established from day-7 populations of the first run, giving the *Paramecium* one additional week of acclimation at 23 °C or 32 °C (replicates assayed at 35 °C were established from the replicates acclimated at 32 °C). A total of 120 replicates were tested in this assay (Table S1, Supporting information).

For assay 3, we tested a range of 15 temperatures, covering the minimum, optimum and maximum growth temperature ranges (Krenek *et al.* 2012). For practical reasons, assays were separated into a 'minimum' temperature block at 5, 7, 9, 11 and 13 °C, an 'optimum' block at 19, 21, 23, 25 and 27 °C and a 'maximum' block at 29, 31, 33, 35 and 37 °C. Prior to assay, samples of each selection line were acclimated for 3 days at 23 °C for the optimum block, at 32 °C for the maximum block and gradually declining from 23 °C to 18 °C to 13 °C for the minimum block. These common garden acclimation temperatures were chosen to allow the cells to physiologically adjust to the common feature of each temperature block: permissive conditions, heat stress or cold stress.

For each assay replicate, 10 individual cells were isolated from an acclimated population and transferred twice in 100 μL of fresh medium to remove associated bacteria, before being transferred into a 2-ml tube, as described above. We tested 15 high-temperature selection lines (six fast, four intermediate and five slow) and nine control lines, with seven assay replicates per temperature. We further assayed the six founder clones, with five assay replicates per temperature. The incubators were randomly assigned to a different temperature every 48 h and the experimental samples moved between them, to ensure that samples did not remain in a single incubator for the whole duration of the experiment. For the optimum- and maximum-temperature blocks, the volume of medium was doubled on day 2 and day 4, and density measured on day 4 and day 7, as described above. For the two lethal temperatures (35 and 37 °C), assay tubes were also checked daily for the presence of living cells. For the minimum temperature range, the volume was doubled on day 4 and day 7, and density measured on day 7 and day 21. A total of 2970 replicates were tested in this assay (Table S1, Supporting information).

Molecular analysis

DNA was extracted from either 10 cells from cultures of the six founder clones or from 50 cells from selection lines (control and high-temperature treatments), using the Chelex®100 (Bio-Rad Laboratories GmbH, Germany) method (Barth *et al.* 2006). The DNA solutions were subsequently used for PCR reactions amplifying the mitochondrial cytochrome *c* oxidase I (COI) gene. The primers F199dT-B and R1143dT (Strüder-Kypke & Lynn 2010) were used for amplification following Krenek *et al.* 2015, using GoTaq® G2 Hot Start Colorless Master Mix (Promega GmbH, Germany).

Purified PCR products (NucleoSpin® Extract II Kit, Macherey-Nagel) of clonal cultures were sent to Eurofins Genomics GmbH (Germany) for direct sequencing. Sequencing in both directions was performed with Primers M13uni(-21) and M13reverse (Messing 1983). Sequence data were visualized and assembled using the programme GENEIOUS PRO v.6.1.8 (Biomatters Ltd.) and compared with the nonredundant sequence database using NCBI-BLAST and an in-house database of *Paramecium* COI sequences to infer haplotype affiliation. COI-PCR products of selection lines (amplification followed the protocol of Barth *et al.* 2006 using primers CoxL11058 and CoxH10176) were double-digested with restriction enzymes *Hind*III and *Pvu*II (New England BioLabs GmbH, Germany) producing different characteristic restriction pattern for the detected haplotypes. This simple RFLP method allowed screening the selection lines for presence or absence of the respective haplotypes within the population. This protocol can reliably detect two to three minority individuals (out of 50 in total); thus, the resolution of this method is around 5% (Fig. S2, Supporting information).

Statistical analysis

Long-term population dynamics: changes in population density over the course of the long-term experiment were compared qualitatively with corresponding changes in temperature. Population survival probability was compared among temperature treatments, using a X^2 test.

Temperature assays: for all three assays, *Paramecium* performance was estimated as the slope of the regression of the log-transformed total number of cells on the number of days elapsed. These slopes estimate the rate of *Paramecium* density change per unit time, referred to as 'growth rate' hereafter. Growth rate was used as the response variable in fully factorial mixed-model ANOVAs, with treatment origin and assay temperature as fixed factors and selection line as a nested random factor. For assay 2, we also fitted experimental run as random factor, and for assay 3, we ran separate analyses for each of the three temperature blocks. In addition, whole-tube survival at 35 and 37 °C was analysed using a Cox proportional hazards model, with time to death as the response variable. For all assays, we further conducted separate analyses for each assay temperature and compared individual treatments, using post hoc multiple comparisons.

We also analysed variation in temperature-dependent growth rate among the six founder clones. We further employed principal component analysis to examine whether multivariate phenotypic differentiation matched the mitochondrial haplotype identity of the clones. Finally, we assessed whether the temperature reaction norms of particular (groups of) clones matched with those of the selection lines, which would be indicative of selection from standing variation (i.e. among founder clones).

All data handling and statistical tests were carried out in either JMP v.12.0 (SAS Institute Inc. 2013) or in R v.3.0.2 (R Core Team 2013).

Results

Population dynamics

All 10 control lines (23 °C) survived until the end of the experiment, while almost 50% (17/36 lines) of the high-temperature treatment lines went extinct (control vs. treatment: $\chi^2_1 = 10.8$, $P = 0.001$; Fig. 1). In most cases, population extinction (14 of 17) occurred during the increase in temperature to stressful levels (≥30 °C), while three cases occurred after reaching a stable temperature of 32 °C. Furthermore, extinction probability was correlated with the rate of temperature increase in the three treatments, although this effect was formally not significant ($\chi^2_2 = 4.9$, $P = 0.086$). Extinction increased from only 20% in the slow temperature increase treatment (2/10 lines), to 50% in the intermediate treatment (6/12 lines) and to 64% in the fast treatment (9/14 lines).

Despite general month-to-month fluctuations, the impact of temperature was also reflected in the changes in population density (Fig. S3, Supporting information). Density increased when temperatures increased first to 26 °C and subsequently to temperatures within the optimum range (≈28 °C). Coinciding with the increase towards the final stressful temperature of 32 °C, we observed strong decreases in density to critically low levels (<20 cells/mL) and population extinction. Over the following months, surviving populations returned to high density, comparable to prestress levels (>200 cells/mL; Fig. S3, Supporting information).

Temperature adaptation assays

Assay 1. The first test was performed after 16 months, when all treatments had reached the final temperature

of 32 °C. Analysis of *Paramecium* growth rate revealed a significant interaction between treatment origin and assay temperature ($F_{6,37} = 9.76$, $P < 0.0001$). This effect was almost entirely explained by the higher heat tolerance of high-temperature lines to the lethal test temperature of 35 °C (Fig. 2a): While density in all lines from the 23 °C control treatment immediately declined at this temperature, high-temperature lines even showed positive growth rates over the 6-days measurement period. Slow, intermediate and fast lines did not significantly differ in heat tolerance (multiple comparisons: $P > 0.09$). However, there was no evidence of increased tolerance to 32 °C. In fact, growth rates at 32 °C were positive and did not significantly differ between control and high-temperature lines, nor did they differ at the control temperature at 23 °C (contrasts: $P > 0.2$; Fig. 2a).

Assay 2. This preliminary test after 33 months (ca. 120 generations after all treatments had reached 32 °C) confirmed the increased tolerance to extreme heat stress (35 °C) for lines from all high-temperature treatments, but also indicated better growth of fast and slow lines at 32 °C as well as 23 °C (Fig. 2b), resulting in a significant overall effect of treatment origin ($F_{3,16} = 3.98$, $P = 0.027$). Intermediate lines tended to grow less well than fast and slow lines at these two temperatures (contrast significant for 32 °C: $P < 0.05$, but not for 23 °C: $P > 0.19$), and grouped more closely with the control lines.

Assay 3. In this main assay, we found a significant treatment-origin x assay temperature interaction for growth rate across the temperature gradient (Table S2, Supporting information), as well as significant treatment-origin effects, when analysing the 15 assay temperatures separately (Table S3, Supporting information; Fig. 3).

Maximum-temperature block (31–37 °C). The two highest temperatures (35 and 37 °C) caused high and rapid mortality of assay populations. Survival analyses showed that the heat stress tolerance of the high-temperature lines at 35 °C also held at 37 °C (treatment vs. control: $P < 0.0001$), and this was equally so for slow, intermediate and fast treatment origins ($P > 0.1$ for both temperatures; Fig. S4, Supporting information). At sublethal temperatures (29–33 °C), *Paramecium* from the slow and fast treatments consistently performed the best, with the intermediate and control treatments performing the worst (Fig. 3, Table S3, Supporting information).

Optimum-temperature block (19–27 °C). As in the maximum-temperature block, the slow lines also consistently performed best at temperatures around the optimum, followed by the fast lines (Fig. 3). Furthermore, while the lines from the three high-temperature treatments show a declining trend with decreasing temperature, control lines showed a rather flat growth rate profile.

Minimum-temperature block (5–13 °C). Growth rates decreased with decreasing temperatures. However, unlike at higher temperature, the control lines performed better than the lines from the high-temperature treatments, in particular at the three lowest temperatures (5–9 °C). At the lowest temperature (5 °C), only the control lines achieved positive growth rates. The ranking of treatment lines remained very similar to that at the higher temperatures, with slow-treatment lines tending to perform best and intermediate-treatment lines performing worst (Fig. 3, Table S3, Supporting information).

Local adaptation and trade-offs

The growth patterns in the maximum and optimum blocks showed no evidence for trade-offs, with no sign of patterns of specialization (local adaptation) by selection lines to our two main selection environments (23 and 32 °C). Instead, over the time span of the three assays, high-temperature selection lines tended to become generally superior to the control lines, both at 23 and 32 °C (Fig. 4). Furthermore, we observed a positive correlation, rather than a trade-off, between growth rate at these two temperatures, such that high-temperature lines growing better at 32 °C were also growing

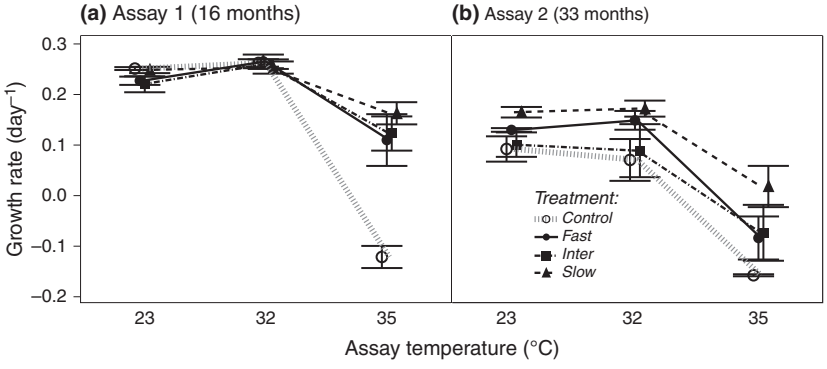

Fig. 2 Mean (±SE) growth rates at three assay temperatures, shown for selection lines from the 23 °C control treatment and from three 32 °C high-temperature treatments (slow/intermediate/fast rate of temperature increase). (a) Assay 1 was conducted when all high-temperature populations had reached 32 °C, (b) assay 2 after an additional 17 months at 32 °C. For details, see Table S1 (Supporting information).

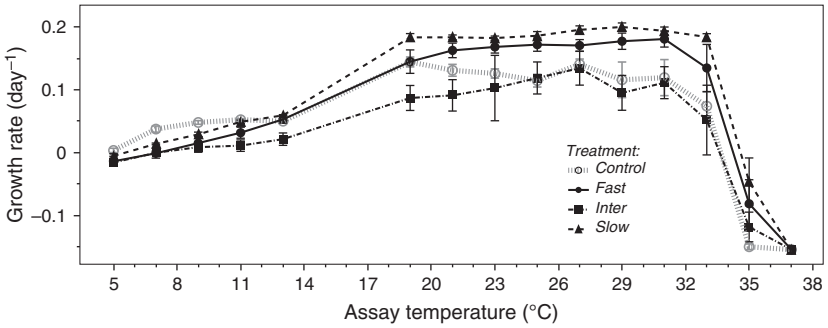

Fig. 3 Mean (±SE) growth rates in assay 3, measured at 15 assay temperatures for selection lines from the 23 °C control treatment and from three 32 °C high-temperature treatments (slow/intermediate/fast rate of temperature increase). Assay 3 was conducted after 33 months, when all high-temperature populations had spent at least 17 months at 32 °C. For details, see Table S1 (Supporting information). Note that survival analysis revealed variation at 37 °C assay temperature (Fig. S4).

better at 23 °C ($r = 0.83$, $n = 18$, $P < 0.0001$; control lines: $r = 0.91$, $n = 10$, $P = 0.0003$; Fig. S5b, Supporting information). High-temperature selection lines further showed a positive correlation between growth rate at their own temperature (32 °C) and growth rate at 35 °C ($r = 0.56$, $n = 18$, $P = 0.0152$), which was not the case for the control lines ($r = 0.13$, $n = 10$, $P > 0.5$; Fig. S5a, Supporting information).

Founder clones – single-clone comparisons

We found significant variation in growth rate among the founder clones at nearly all temperatures, including those experienced during the long-term experiment (all $P < 0.01$, except at 5 and 13 °C: $P > 0.08$). Moreover, the significant clone x assay temperature interaction ($F_{70,360} = 9.89$, $P < 0.0001$) indicated changes in the ranking of clones along the temperature gradient (Fig. S6, Supporting information). Namely, the clones VEN and CRA were worst performing at 23 °C, whereas at higher temperatures, the clones GO and VEN were the worst performing. The M3 clone showed superior growth rates at temperatures near the maximum experienced in the high-temperature treatment (29–33 °C). At the low assay temperatures, clone K8 and M3 had relatively high growth rates, whereas clone GO was performing poorly (Fig. S6, Supporting information).

Molecular analysis

Sequencing the mitochondrial cytochrome *c* oxidase I (COI) gene (767 bp after trimming) of the six founder clones (Table 1) revealed only three different haplotypes, with clone VEN representing haplotype PcCOI_a01 (GenBank Acc.No. AM072776), clone GO representing haplotype PcCOI_a18 (FN256263) and all other clones representing haplotype PcCOI_b07 (KY399443). Further attempts to distinguish these four clonal strains (CRA, K8, M3 and TUB) with molecular markers involved sequencing of four other mitochondrial loci (base pair positions 4566–6382, 8255–9280, 22 813–24 311 and 29 705–34 616 according to the *P. caudatum* mitochondrial reference genome, Acc.No. NC_014262), as well as using RAPD-10mer markers (OPA01 - OPA20, Eurofins Genomics GmbH, Germany; IT-01, IT-15, IT-17, IT-21, IT-28, Tsukii 1996; Ro270-06, Ro170-03, Ro370-01, Ro460-04, Ro470, Stoeck *et al.* 2000). However, none of these additional methods revealed any differences among the four clones in question.

Analysis of the genetic composition of the selection lines was based on the presence/absence of the above three COI haplotypes. All control lines showed the same banding pattern for only haplotype PcCOI_a01 ('a01'), meaning that VEN was the only clone remaining in these populations and the other five clones were lost. In contrast, for all high-temperature treatment lines, only haplotype PcCOI_b07 ('b07') was detected; this haplotype is shared by the strains CRA, K8, M3 and TUB, and thus, populations may consist of up to four different clones, and strains GO and VEN were lost.

Extrapolating responses to selection

Principle component analysis (PCA) indicated a match between the temperature-dependent growth profiles and the haplotype identity of our six founder clones (Fig. 5). Axis 1 of the PCA was particularly well correlated with growth at higher temperatures (29–35 °C; $r \geq 0.72$) and thus separated the four b07 clones from the two other clones (VEN and GO), which were eliminated in the high-temperature treatment. Axis 2 was best correlated with intermediate temperatures (21–25 °C; $r \geq 0.68$), and this axis further separated VEN and GO (Fig. 5). However, clone VEN, which became fixed in the 23 °C control treatment, had the lowest growth rate at this temperature in the assay.

We further assessed the possibility that evolutionary change in the high-temperature treatment was due to

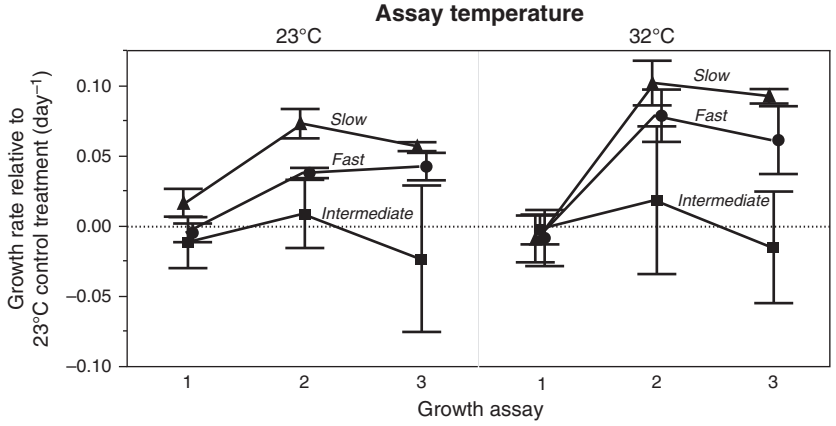

Fig. 4 Difference in mean (±SE) growth rates between 32 °C high-temperature selection lines and 23 °C control lines, obtained for the two selection environments (23C and 32 °C) in assay 1 (16 months) and assays 2 and 3 (33 months). Relative growth was taken as the difference in growth rate between high-temperature and 23 °C control lines; that is, positive values indicate a growth advantage of high-temperature lines over the 23 °C control lines. High-temperature lines originated from three treatments: slow/intermediate/fast rate of temperature increase. For assay 3, growth at 32 °C was extrapolated, by averaging over growth at 31 and 33 °C.

the fixation of one or several of the b07 founder clones (M3, K8, CRA, TUB). If selection lines were fixed for a single clone, the upper boundary of their expected growth rates is set by the best-performing clone (M3, see Fig. 6), and the lower boundary by the worst performing clone (TUB). Growth rates in-between these boundaries may reflect fixation of the other two clones or combinations of clones. Comparison of predicted growth rates (grey area in Fig. 6) and observed growth rates over the higher temperature range (25–33 °C) revealed multiple inconsistencies. Few selection lines had temperature profiles falling entirely within the area of predicted growth rates. Certain fast and slow lines showed growth rates above the predicted maximum, whereas intermediate lines grew less well than the expected minimum. Thus, growth rates of evolved lines cannot be fully explained from the standing variation among founder clones.

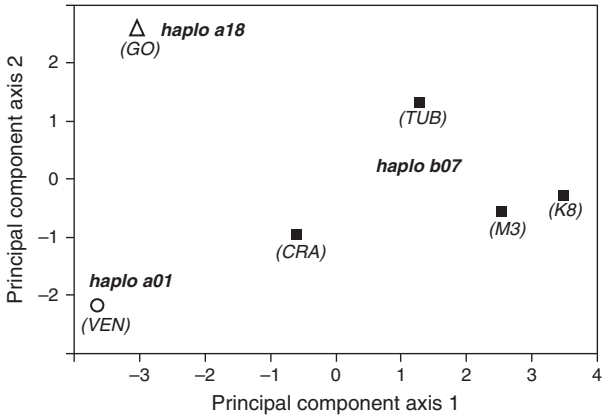

Fig. 5 Differentiation among six *Paramecium* clones, according to axis 1 and axis 2 of a principle component analysis of growth rates at 15 assay temperatures. The clones are further characterized by three different mitochondrial COI haplotypes (see Table 1).

Discussion

We observed population troughs and extinction coinciding with temperature increasing above optimum levels. Typical of evolutionary rescue (ER; Bell & Gonzalez 2009), surviving populations grew back to high density, comparable to or even higher than before the temperature increase. This was most likely due to adaptation, as high-temperature selection lines were clearly better at tolerating temperature stress than their counterparts from the permissive control treatment (23 °C).

Impact of variable rates of temperature increase

We found that those selection lines experiencing the slowest rate of temperature increase were the least likely to become extinct and tended to be the best adapted to the new temperature environment. This pattern was detectable even after all populations had spent more than a year at the same endpoint temperature of 32 °C. It conforms with results of other experimental studies, which also demonstrated that slower rates of environmental change lead to less extinctions or better adapted populations (Huey *et al.* 1991; Perron *et al.* 2008; Collins & de Meaux 2009; Bell & Gonzalez 2011). In contrast, faster environmental change is likely to decrease population size to a point at which extinction from stochastic processes is likely (Gomulkiewicz & Holt 1995), as smaller population size limits mutational availability and increases risk of extinction.

With a slower speed of change, it is also expected that more evolutionary pathways are accessible, because

there is more time for a series of beneficial mutations to arise. This has been demonstrated with *E. coli* evolving under varying speeds of increase in the concentration of an antibiotic. Slow environmental change leads to a wider range of adaptive genotypes, whereas rapid environmental change makes some genotypes inaccessible (Lindsey *et al.* 2013). Collins & de Meaux (2009) come to a similar conclusion in a study on adaptation of *Chlamydomonas* to high salt stress, where patterns of marker frequency changes were consistent with the fixation of many genes of small effect under slow environmental change, and of few genes with large effect under fast change.

We therefore expected a gradient of extinction rate and performance, from fast to intermediate to slow rates of temperature change. This was indeed the case for the extinction frequencies in each group, but not for the levels of adaptation observed in the growth assays. Mean performance of intermediate lines was often close to or below the performance of the control lines over a wide temperature range, including the 32 °C selection endpoint. The reasons for the poor performance of intermediate lines are unclear, but inspection of reaction norms of individual replicate lines from this treatment shows two prominent outliers with particularly low growth rates (see Fig. 6). We can therefore not exclude the possibility of unknown events, unrelated to the treatment. While we do know that intermediate lines are genotypically different from control lines, the limited resolution of our molecular markers did not allow us to verify whether intermediate lines also differ from the slow and fast treatment lines.

Changes in niche shape

Common to all high-temperature selection lines was the increased resistance to lethal heat stress beyond the maximum selection temperature (32 °C), indicating the potential for a strong shift of the ecological niche at the upper end of the temperature niche. Indeed, the positive across-population correlation between growth at 32 and 35 °C suggests positive correlated responses to selection at 32 °C for higher temperatures. This result is consistent with the general idea that beneficial mutations can arise in sublethal conditions, which give tolerance to lethal conditions. Such preadaptation has also been reported from selection experiments in very different organisms, such as *Drosophila* (Gilchrist *et al*. 1997), yeast (Gonzalez & Bell 2013) and *Escherichia coli* (Gallet *et al*. 2014). A straightforward explanation is that the genetic basis underlying adaptation to actual and above-actual stress is the same. In our case, adaptation may rely on differential expression of heat-shock proteins (HSP; Krenek *et al.* 2013), which would represent the common currency of responses to varying levels of heat stress (Sørensen *et al.* 2003). In a follow-up experiment, the 23 °C control lines were unable to mount a heat-stress response, even after more than 2 weeks of conditioning at high temperature (Fig. S7a,b, Supporting information); conversely, heat stress protection in the high-temperature lines was still evident after returning these cultures back to 23 °C for several months (Fig. S7c, Supporting information). These results show that the phenomenon was not a transitory plastic response that can be brought about by prolonged conditioning.

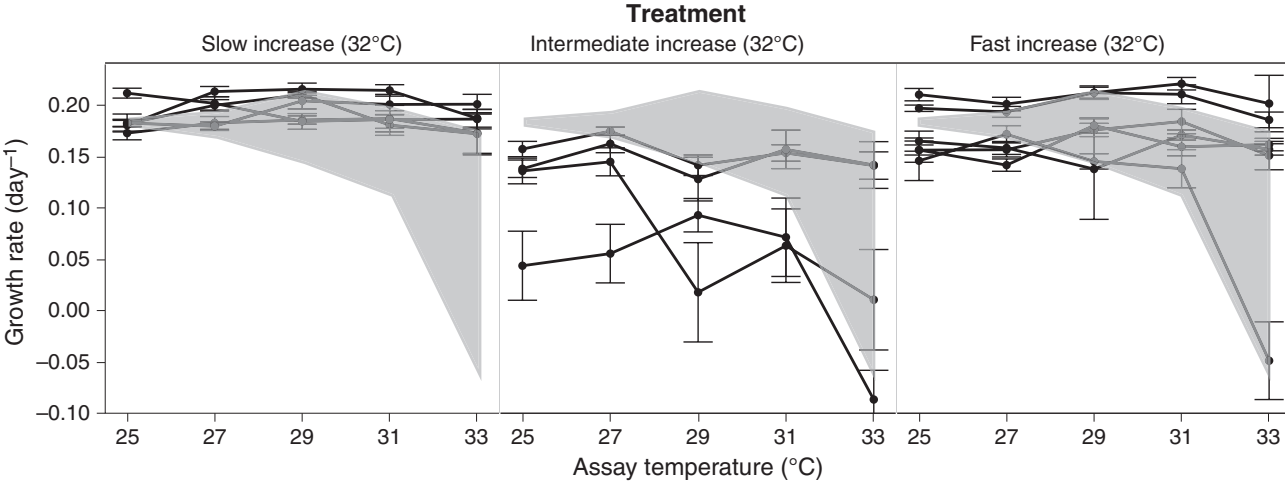

Fig. 6 Mean (±SE) growth rate of individual high-temperature selection lines over the temperature range experienced during the 33 months of the experiment. Molecular analysis indicates that selection lines are fixed for one or several of four clones (M3, TUB, CRA, K8), initially present in the founder populations (see Results). The grey area represents the range of growth rates, predicted for different combinations of the four clones. The upper grey boundary is set by the best-growing clone (M3; upper value of 95% confidence interval), the lower boundary by the worst growing clone (TUB; lower value of 95% confidence interval) at these temperatures (see Fig. S6, Supporting information). Values within the boundaries can be obtained by different combinations of the four clones.

A second finding, common to all high-temperature lines, was their reduced growth at the low-temperature end of the tested gradient (5–9 °C), when compared to control lines. This indicates a trade-off between high- and low-temperature tolerance. The evolution of trade-offs in the temperature niche has often been difficult to demonstrate or only present in some selection lines (Bennett & Lenski 1993, 2007), but here by examining the complete temperature niche, we do see evidence for trade-offs. Across-population correlations between low- and high-temperature performance were nonetheless positive, rather than negative (Fig. S5, Supporting information). This may reflect a 'big car, big house' effect, meaning that some selection lines generally grow better than others, independent of the temperature trade-off. Continued heat-stress adaptation may therefore eventually lead to super-generalists with high performance over the entire temperature niche.

Local adaptation

Local adaptation results from a process of specialization to specific habitats, in particular if a positive response to selection in one habitat produces negative correlated responses in others, for example through antagonistic pleiotropy (Kassen 2002; Kawecki & Ebert 2004). A cost of high-temperature adaptation was indeed detected at low temperatures, but not at permissive temperatures. This absence of a trade-off explains why we found no reciprocal pattern of local adaptation to our two main selection environments, 23 and 32 °C. In fact, fast and slow high-temperature lines showed globally higher growth rates over the entire optimum temperature range, including the 23 °C control temperature.

This result shows that adaptation to high temperature did not only produce a niche shift, but also a deformation of the niche, through increased performance in the optimum range. Gallet *et al.* (2014) made similar observations for *E. coli* adapting to low pH conditions, where they also showed that shifts and deformations of the niche followed different trajectories in different populations. This is all the more likely for populations experiencing different rates of environmental change (Lindsey *et al.* 2013), and it implies that knowledge of both magnitude and timing of direct and correlated responses to selection will be necessary to predict the long-term consequences. In our simple scenario, some 200 (asexual) generations at high temperature were sufficient to change the temperature profile of our populations from typical 'European/Northern hemisphere'-like to more warm-adapted 'Indonesian'-like (see Krenek *et al.* 2012). In geographical terms, we would therefore predict that *Paramecium* from high-temperature 'edge' populations can invade the 23 °C populations in the 'centre' of the range. Whether such invasion from evolving edge populations is a realistic scenario remains to be shown.

Genotypic change underlying phenotypic patterns

When population size is limited, the probability of ER critically depends on whether selection can act on standing genetic variation or whether variation must be generated de novo (Ramsayer *et al.* 2013). Our selection lines had been founded from six clones (genotypes), in an attempt to facilitate responses to selection through clone sorting or recombination. Analysis of the presence/absence of mitochondrial haplotypes revealed that the genotypic divergence between control and high-temperature selection lines was 100%, with different clones fixed in the two treatments. This observation is, at least in part, compatible with selection from standing genetic variation. Namely, in the high-temperature lines, the two clones with lowest performance at higher temperatures were found to be absent, while the haplotype with the highest growth was retained. However, the comparison of molecular marker data and phenotypic growth rate profiles strongly suggests that selection from standing (clonal) variation alone cannot explain our results. At 32 °C, even if we assume fixation of the b07 haplotype clone with the best growth rate at high temperature (M3; see Fig. 6), several selection lines still show higher growth rates than this clone. Conversely, certain selection lines performed even less well than the least-performing b07 clone (TUB). Furthermore, in the control treatment lines (constant 23 °C), only one clone (VEN) was detectable. Fixation of this clone is not expected given the low growth rate at this temperature in the assay. These deviations and unexplained outcomes may have different underlying causes. First, clones may have acquired additional adaptations through (epi)genetic change. Second, if multiple clones are still present in the evolved lines, interactions between them may decrease or increase population-level responses through clonal interference or facilitation. Such effects can be addressed in competition assays, which integrate over multiple fitness components, and not just growth rate (e.g. Fox & Morin 2001; Jiang & Morin 2004). Unfortunately, we have not yet succeeded in finding appropriate additional molecular markers that could differentiate between the four clones with the same 'b07' haplotype, fixed in the high-temperature lines. This would allow us to assess whether multiple clones still coexist, and whether clonal composition differs among selection lines, which could explain some of the observed variation among lines from the same treatment.

Finally, it is possible that sexual recombination has occurred, and this could potentially limit our

interpretations of genotype frequencies, which is based on mitochondrial, and not nuclear, markers. Recombination per se does not affect the relative frequency of the mitochondrial markers (see Fig. S1, Supporting information). It is nonetheless possible that, following recombination, a particular mitochondrial marker goes to fixation through random drift. However, this would imply that different markers become fixed in different replicate selection lines. This was not the case, suggesting that clonal selection did play an important role in our study.

Further molecular study

A first priority is a more in-depth characterization of the genotypic composition of our evolved lines. Nuclear markers are required to differentiate all of the six initial founder clones. This will be done by a whole-genome scan of each clone, concomitantly providing suitable information on how to test for recombination events. Second, we are currently investigating expression in heat-shock proteins (HSP 70) using RT-qPCR (Krenek *et al.* 2013). For example, changes in constitutive vs. induced responses could affect the balance between the costs and benefits of HSP protection (Ketola *et al.* 2004; Lopez-Maury *et al.* 2008) and thus responses to selection. If variation is detected, we envisage sequencing of the HSP genes and their promotor regions. Third, 'omics' approaches can be used to investigate de novo adaptation. Given the predominant asexual reproduction in our experiment, one possible 'fast-lane' solution to adaptation may be the epigenetic fine-tuning of gene expression in the somatic macronucleus. This might be achieved by typical epigenetic modifications, such as DNA methylation, histone modification or noncoding RNAs (Casadesus & Low 2006; Mattick & Makunin 2006; Herrera *et al.* 2012), which have stable vertical transmission in many species (Bossdorf *et al.* 2008; Yi 2012; Singh *et al.* 2014). Candidate loci responding to the experimental treatments can be identified, for example through transcriptome sequencing (RNA-Seq, Wilhelm *et al.* 2008; Glaus *et al.* 2012; Kratochwil & Meyer 2015), or by high-throughput approaches, such as Methylation Sequencing or Chromatin Immunoprecipitation Sequencing (ChIP-Seq) (Devaskar & Raychaudhuri 2007).

Conclusions

Our study corroborates basic predictions regarding evolutionary rescue under variable rates of environmental change (Gonzalez *et al.* 2013), showing that more time available for adaptation not only increases the chance of population survival, but also levels of adaptation in rescued populations. We further demonstrated that adaptation to locally extreme conditions can bring about strong divergence in the genotypic composition of populations relative to populations from permissive environments. However, adaptation at the edge of an ecological niche does not necessarily lead to specialism and patterns of local adaptation. Rather, outcomes depend on the sign and magnitude of the correlated responses to selection, which may be very different depending on the environment considered (Duputie *et al.* 2012). Knowing more about the genes and physiological mechanisms involved in adaptation will help us better understand and predict these responses.

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Data accessibility

All DNA sequences are available on GenBank (for accession numbers, see main text). Phenotypic data on selection line survival (Fig. 1) and growth rate estimates (assays 1–3) are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.f66g0.

J.K. and O.K. conceived and designed the study. J.K., C.G-B. and O.K. performed the long-term experiments and assays. J.K. and O.K. analysed the data. S.K. obtained and analysed the genetic sequences. J.K., S.K. and O.K. wrote the manuscript, and all authors contributed to revisions. All authors gave final approval for publication.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Summary of the three assay protocols used in this experiment.

Table S2 Analysis of Variance (ANOVA) of *Paramecium* growth rate, as a function of treatment origin (control and slow, intermediate or fast rate of temperature increase), assay temperature and selection line (nested within origin), carried out for three experimental blocks of assay temperature ranges (minimum, optimum, maximum).

Table S3 Analyses of Variance of *Paramecium* growth rate, as a function of treatment origin and selection line (nested within origin), carried out separately for each assay temperature.

Fig. S1 Life cycle of *Paramecium caudatum*.

Fig. S2 Gel picture showing the sensitivity PCR assay targeting the mitochondrial COI gene followed by a double-digest with restriction enzymes HindIII and PvuII.

Fig. S3 Density change through time, shown for populations surviving until the end of the experiment (bold solid line) and for populations going extinct (stippled lines), in the four selection treatments.

Fig. S4 Mean (± SE) proportion of surviving replicates at 35 and 37 °C over the 7 days of assay 3, shown for selection lines from the 23 °C control treatment and from three 32 °C high-temperature treatments (slow/intermediate/fast rate of temperature increase).

Fig. S5 Correlations between growth rates at four assay temperatures: 23, 32, 35 °C and a combined low-temperature mean (averaged over 5, 7 and 9 °C).

Fig. S6 Mean (±SE) growth rates of 6 *Paramecium* clones, measured at 15 assay temperatures.

Fig. S7 Growth performance of original 23 °C control lines and 32 °C slow lines in a follow-up experiment, investigating adaptation to new temperature environments.