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EVOLUTION OF HSP90 EXPRESSION IN *TETRAHYMENA THERMOPHILA* (PROTOZOA, CILIATA) POPULATIONS EXPOSED TO THERMALLY VARIABLE ENVIRONMENTS

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Abstract.—Evolutionary consequences of thermally varying environments were studied in the ciliated protozoan *Tetrahymena thermophila*. Replicated lines were propagated for 60 days, a maximum of 500 generations, in stable, slowly fluctuating (red spectrum), and rapidly fluctuating (blue spectrum) temperatures. The red and blue fluctuations had a dominant period length of 15 days and two hours, respectively. The mean temperature of all time series was 25°C and the fluctuating temperatures had the same minimum (10°C), maximum (40°C), and variance. During the experiment, population sizes and biomasses were monitored at three-day intervals. After the experiment, carrying capacity and maximum growth rate were measured at low (15°C), intermediate (25°C), and high (35°C) temperatures for each experimental line. Physiological changes in the lines were assessed by measuring the expression of stress-induced heat shock protein Hsp90 at 25°C, 35°C, and 39°C. Population sizes and biomasses showed no differences between stable, blue, or red temperature treatments during the experiment. Also, after the experiment, mean carrying capacities and maximum growth rates were comparable in the stable, blue, and red temperature treatments. The expression of Hsp90 was higher in lines from the blue environment than in lines from the stable environment. Lines from the red environment had an intermediate level of Hsp90 expression. This supports the hypothesis that inducible thermotolerance and expression of canalizing genes can evolve in response to rapidly varying environments. Furthermore, we found correlative evidence of benefits and disadvantages of high Hsp90 expression. Lines with high expression of Hsp90 had an increased growth rate at the highest temperature when food resources were not limiting growth. At low and intermediate temperatures the same lines had the lowest carrying capacities.

Key words.—Colored environmental noise, experimental evolution, heat shock protein, Hsp, temperature.

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The evolutionary response of ectotherm populations to variations in their thermal environment is determined by the underlying physical constraints and genetic architecture responsible for their thermotolerance (Huey and Kingsolver 1993; Angiletta et al. 2002). In addition to the distribution of temperatures, the time scale over which the temperature changes occur can be crucial for the evolutionary outcome. Natural temperature time series contain biologically significant variation that occurs in time scales both shorter and longer than the generation time of the organism. The relative dominance of short-term ''blue spectrum'' variation is predicted to have different evolutionary consequences on the thermal performance curve (Lynch and Gabriel 1987; Gilchrist 1995) and the underlying physiology than long-term ''red spectrum'' variation (Bettencourt et al. 1999).

The time scale of variations can determine the physiological reaction that organisms use to cope with suboptimal temperatures and temperature changes (Angiletta et al. 2002). Noninducible or slowly inducible mechanisms, such as the production of thermally suitable lipids (Hochachka and Somero 2002) are likely to be most beneficial in stable and slowly changing environments. In contrast, rapid or unpredictable changes may require rapidly inducible tolerance, such as the production of heat shock proteins (Morimoto 1998; Feder and Hoffmann 1999).

Heat shock protein (Hsp) expression is a ubiquitous stress tolerance mechanism that has received considerable attention. Hsps are molecular chaperones that allow proteins to maintain their correct function and prevent cytotoxic aggregation of denatured proteins (Morimoto 1998). Although Hsps are expressed constitutively in unstressed cells, expression can increase massively in response to high temperature and other physical and chemical stressors (Feder and Hofmann 1999). Expression of Hsps may also reduce the amount of resources available for growth and reproduction. Overexpression of Hsps in optimal temperatures has been found to incur fitness costs (Sanches et al. 1992; Krebs and Feder 1997, 1998). Recently, Hsps have been suggested to be primarily an adaptation to acute stress (Sørensen et al. 1999, 2001; Buchanan 2000) and, moreover, shown to be genes for canalization (e.g. Hsp90; Rutherford and Lindquist 1998; Queitsch et al. 2002). Canalization is advantageous when environments vary rapidly and unpredictably (i.e., acute stress) because a response to phenotypic selection would be maladaptive in the next generation (Kawecki 2000). In a more predictable environment the duration of directional selection is longer and the need for canalizing genes is possibly smaller.

Here we report an evolutionary experiment in which the presence and type of variability in the thermal environment is manipulated in laboratory populations of the ciliated protozoa *Tetrahymena thermophila*. In *T. thermophila* several heat inducible Hsps have previously been identified (Williams and Nelsen 1997; Williams 1999). We hypothesize that exposing *T. thermophila* populations to thermally changing environments causes an increase in population growth and Hsp90 (Hsp82 in *T. thermophila*; see Williams and Nelsen

1997) expression over an evolutionary time scale in suboptimal environments compared to populations in a stable environment. Moreover, exposing the populations to a rapidly changing (blue spectrum) environment should increase Hsp expression more than the slowly changing red spectrum environment. We also test whether the populations' growth performance or the level of Hsp90 expression at one temperature is correlated with performance at other temperatures.

Materials and Methods

Strain and Culture Medium

The *T. thermophila* strain was originally isolated from fresh water, Falmouth, MA, in 1952, after which it was cultured axenically in proteose peptone solution. The strain was obtained from the Culture Collection for Algae and Protozoa (SAMS Research Services, Argyll, U.K.; CCAP 1630/1U is equal to the strain ATCC 30008 in the American Type Culture Collection). The strain reproduces asexually, and is likely to be genetically homogeneous due to a long history of serial transfer culturing. Therefore, only the occurrence of beneficial mutations is likely to drive evolution in the populations (see also Bennett et al. 1992). To increase the number of mutations, experiments were conducted with large populations consisting of approximately 1.0×10^7 individuals. The high-density populations were produced using enhanced proteose peptone (ePP) medium containing Bacto Peptone (1% weight/volume; Becton, Dickinson and Co., Franklin Lakes, NJ) and liver concentrate (0.1% weight/volume; Sigma-Aldrich Corp., St. Louis, MO) in sterile aqueous solution. Although closely similar to proteose peptone medium, the ePP supports up to 20-fold larger population sizes (Elliott 1974).

Laboratory Experiment

The strain was first acclimated to the ePP resource with a three-day renewal cycle for three weeks (about 170 generations). Replicated populations of *T. thermophila* were then exposed to rapidly varying (blue spectrum) and slowly varying (red spectrum) temperatures and to a stable temperature of 25°C, each for a 60-day period. The rapid blue variations were scaled to occur mostly within one generation time and the slow red variations between the generations (see below). Each environment had 10 replicates. The fluctuating temperature time series had the same mean temperature (25°C), variance, minimum (10°C), and maximum (40°C). The time series were produced by summing up 1438 ($T_{max}/2 - 2$) sine waves with uniform random phase, and the amplitude of the waves was set to follow the power law such that amplitude $= 1/f^{\lambda}$, where f is frequency and λ is the spectral exponent determining the signal spectrum (Cohen et al. 1998). The three slowest frequencies (60-, 30-, and 20-day period lengths) were omitted from the 60-day time series to allow repeated low- and high-temperature periods also within the red noise series. Therefore, a period length of 15 days was the slowest possible variation studied. The spectral exponent (λ) was set to -1 or 1 to obtain blue or red noise, respectively. In the blue noise, the dominant temperature variations had a two-hour wavelength and in the red noise the dominant variations had a 15-day wavelength. Assuming that *T. thermophila* has a generation length of three hours (Elliott 1974), most of the blue noise variation occurs within one generation, whereas approximately 120 generations can occur during the 15-day period in the red noise. The time series were used to program a temperature regulator (Laakso et al. 2003; modified from Cohen et al. 1998) that maintained the desired temperature constant in the microcosms within each one-hour time step. For examples of the realized temperature time series generated in the microcosms see Figure 1.

The microcosms were modified from 250-ml polycarbonate cell culture bottles (Corning, Inc., Corning, NY) with heating resistors attached to molded aluminum plates underneath the microcosms (Laakso et al. 2003). To allow gas exchange, the bottles were closed with membrane filters (0.2 μm; Corning). To reduce water evaporation, a piece of aluminum foil with 30 holes (diameter 1 mm) was attached under the filters. A 5-ml syringe with silicon tubing was attached to the microcosm to draw aside a 10% sample of the population during the sampling for population size and the subsequent renewal of resources, which took place at three-day intervals. The population samples were taken through a 50-cm silicon tube (diameter 1.5 mm) with taps attached. After sampling for population size, the rest of the contents of the culture bottles were removed and 45 ml of fresh 1% ePP medium was pumped to the culture vessel, from a stock bottle via a silicon tube with a three-way valve and a syringe. After completing the sampling and renewal, the contents of the 5-ml syringe with the remaining population was returned to the culture bottle. To prevent microbial contamination, taps attached to sampling tubes were submerged in 75% ethanol between samplings.

Prior to starting the experiment, the microcosms, stock bottles, and other attached parts were autoclaved at 121°C, and 48 ml of fresh 1% ePP medium was added. We then aseptically added 2 ml of *T. thermophila* stock in late log-phase (cultured in 1% ePP at 25°C) to each of the microcosms. After one day incubation at 25°C, the microcosms were checked for microbial contamination by plating a fluid sample on 1% ePP agar. Microcosms were then randomized to the three temperature treatments ($n = 10$) and transferred to the temperature regulator.

Measurements during the Experiment

Biomasses and population sizes were measured from all replicates during the experiment at three-day intervals prior to the addition of resources. The 45-ml population samples were mixed well and 1-ml subsamples were fixed in Lugol's solution (20 g potassium iodide, 10 g iodide, 20 ml acetic acid, 200 ml dH_2O; 0.6% final concentration). Cell counting was performed by placing a 250-μl subsample in a glass cuvette and taking eight pictures from each sample using a binocular microscope attached to a video camera. *Tetrahymena thermophila* individuals were identified automatically from digitized images using an image recognition script written by JL (unpubl.) for Image Pro ver. 4.5 (Media Cybernetics, Silver Spring, MD). Data from objects with a morphology corresponding to *T. thermophila* individuals were included in the statistical analyses.

Possible microbial contaminations were checked weekly

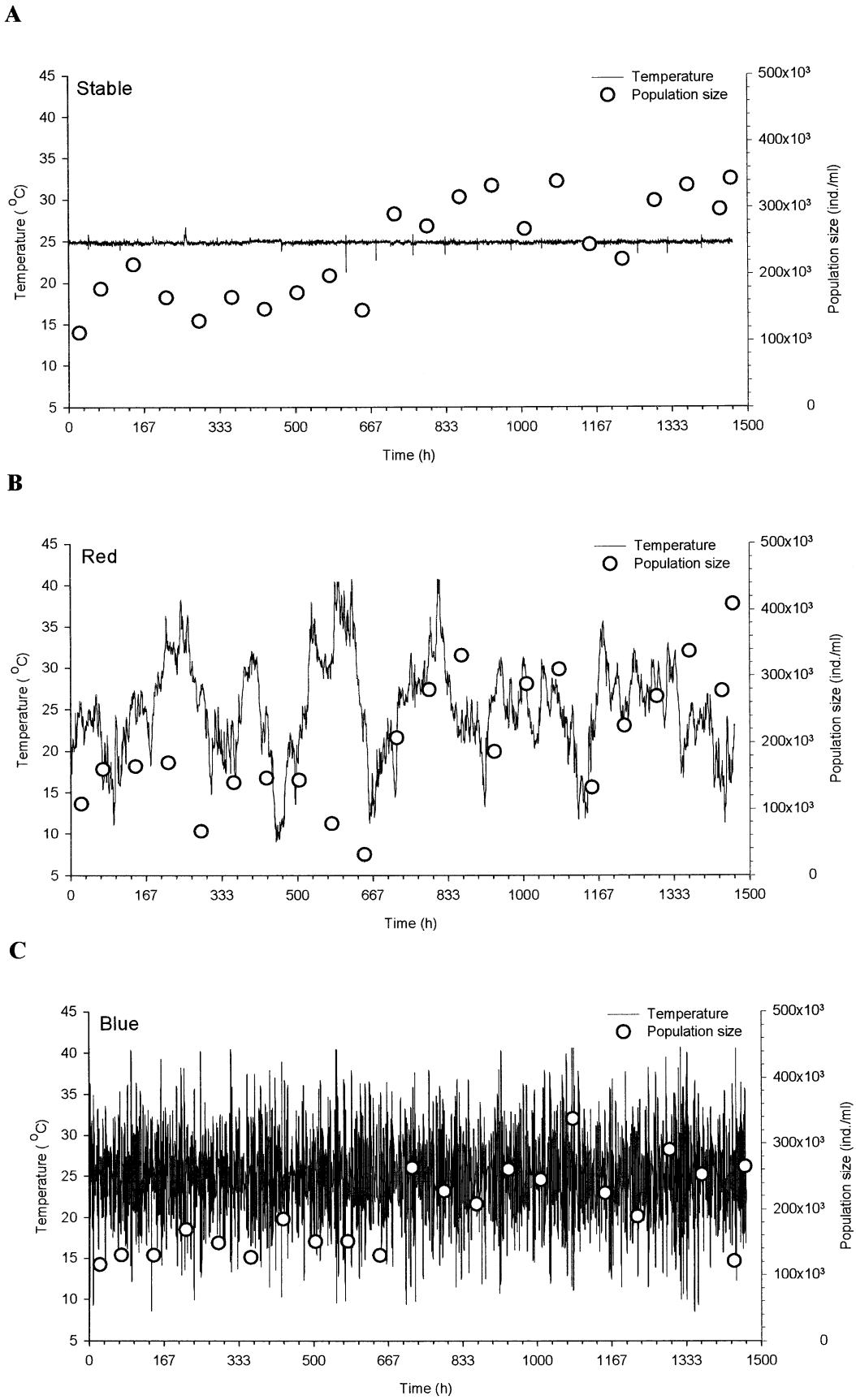

FIG. 1. Examples of different temperature time series (data presented as 10-minute moving average) and corresponding population sizes measured prior to the 90% resource renewal during experiments in stable (A), red (B), and blue (C) environments.

during the experiment by plating a fluid sample on ePP agar and monitoring the bacterial growth. Bacterial contaminations were detected in four lines, which were discarded.

Measurements at the End of the Experiment

Five uncontaminated lines from each treatment were randomly chosen to assess Hsp90 concentration as well as measurements for maximum growth rate (r_{max}) and carrying capacity (K) at the end of the experiment. r_{max} and K in batch culture experiments were measured after the main experiment at constant 15°C, 25°C, and 35°C temperatures. Each line had two replicates. One day before starting the measurements, the strains were transferred to a 25°C regime to eradicate cross-generational physiological effects of the temperature treatments (24 h equals approximately six to seven generations for *T. thermophila*; Elliott 1974). To initiate the cultures, 1 ml of each strain was transferred to 49 ml of 1% ePP medium. Sampling took place at three-hour intervals during the first 24 h, then at six-hour intervals during the next two days and at 24-h intervals thereafter. r_{max} was calculated from linear regressions of log-transformed population size (or biomass) against time, where the data showed exponential growth. Carrying capacity was measured as a maximum population size (or biomass) during the batch culture experiment.

Hsp90 Expression Analysis

Tetrahymena thermophila cells were first incubated in 250-ml cell culture bottles (Corning) containing 50 ml fresh 1% ePP at 25°C for 20 h to prevent cross-generational effects (Galego and Rodrigues-Pousada 1983). After acclimation, two 35-ml replicate samples were transferred into 50-ml centrifuge tubes (Greiner Bio-One, Kremsmuenster, Austria). After one hour incubation at 25°C the tubes were transferred to thermostatic water baths set at 35°C, 39°C, and 25°C for one additional hour. Whole cell extracts were prepared by a modification of the method described by Mosser et al. (1988). Briefly, the samples were cooled on ice and then centrifuged for 8 min at 800 g at 4°C in 15 ml of 10 mM Tris-HCl, pH 7.34 (Williams 1999). The *Tetrahymena* pellets were resuspended and the washing was repeated in eppendorf tubes. Cells were lysed in liquid nitrogen and resuspended in 1:1 volume of extraction buffer containing 25% glycerol (v/v), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM HEPES, 0.5 mM phenylmethylsulfonylfluoride (PMSF), and 0.5 mM dithiotreitol (DTT)in dH_2O. Thereafter the samples were centrifuged for 30 min (4°C, 13,500 g) and the supernatants containing the solubilized proteins were collected and stored at −70°C until the analysis. The protein concentration of the samples was determined by the Bradford (1976) method using the Bio-Rad protein assay according to manufacturer's instructions (Bio-Rad, Hercules, CA). Samples containing 20 μg of protein in sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris HCl, pH 6.8, 20% glycerol, 4% SDS, 0.1 M DTT, and 0.015% bromophenyl blue) were loaded and the proteins separated on discontinuous SDS-polyacrylamide gels (8%). The proteins were transferred from gels to nitrocellulose membranes (Schleider and Schuell, Keene, NH) using a semi-dry transfer apparatus (Bio-Rad). The membranes were blocked for one hour in 3% skimmed milk in phosphate buffered saline (PBS) with 0.3% Tween 20. Heat shock protein expression was detected indirectly using a monoclonal antibody recognizing Hsp90 (Hsp82) from *Tetrahymena thermophila* (kindly donated by N. E. Williams; see Williams and Nelsen 1997). The membranes were incubated in the primary antibody (dilution 1:750) for one hour and washed in PBS with 0.3% Tween 20 three times for 10 min. Thereafter, the membranes were incubated for one hour in horseradish peroxidase conjugated donkey anti-rabbit Ig secondary antibody (Amersham Life Sciences, Buckinghamshire, UK) using a dilution of 1:10,000 (0.3% Tween 20, 3% skimmed dry milk in PBS). Washing was repeated as described above. Enhanced chemiluminescence (ECL; Amersham) was used to develop the signal, which was captured with standard X-ray film. The films were scanned and analyzed with Image Pro (ver. 4.5). Intensities (area × mean density) were calculated as relative intensity units by normalizing samples to a control gel that contained samples from all other gels.

Data Analysis

Population data collected during the main experiment were analyzed using repeated MANOVA. Effects of the treatments on the estimates of maximum growth rate, carrying capacity and Hsp90 expression were analyzed using ANOVA and the subsequent pairwise contrasts were performed with a Bonferroni correction. When the assumptions of the parametric test were not met, a Kruskal-Wallis test was used. Correlations between Hsp90 expression and maximum growth rate and carrying capacity were calculated. To limit the number of correlation tests, only certain combinations with biological relevance to Hsp90 expression were tested. These include the correlations between Hsp90 expression and measures of population performance (r_{max} and K). Only Hsp90 expression at 35°C and 39°C was included in the analysis because no expression was detected at 25°C. A sequential Bonferroni correction was not used because our dataset is large and consequently the corrected alpha level would be very low (0.002). For discussion of the meaningfulness of the Bonferroni correction, see Moran 2003.

All statistical analyses were performed using SPSS (ver. 10.0; SPSS Inc., Chicago, IL). The following data were excluded from the analyses: in the maximum growth rate and carrying capacity measurements one line was excluded due to microbial contamination. From the Hsp90 expression experiment, six within-sample replicates were removed because background signals were too strong.

RESULTS

Development of Population Size during the Experiment

Mean population sizes (Fig. 2A) and biomasses (data not shown) almost doubled in all treatments (population size: Greenhouse-Geisser corrected $F_{2,4.503} = 15.35$, $P < 0.001$; and biomass: $F_{2,4.22} = 13.80$, $P < 0.001$). However, no differences in mean population sizes or biomasses were found between the stable, red, or blue temperature treatments (population size: treatment $F_{2,12} = 2.62$ $P = 0.11$, time × treatment $F_{2,9.01} = 1.40$, $P = 0.21$; biomass: treatment $F_{2,12} = 2.95$, $P = 0.91$, time × treatment $F_{2,8.43} = 1.42$, $P = 0.21$).

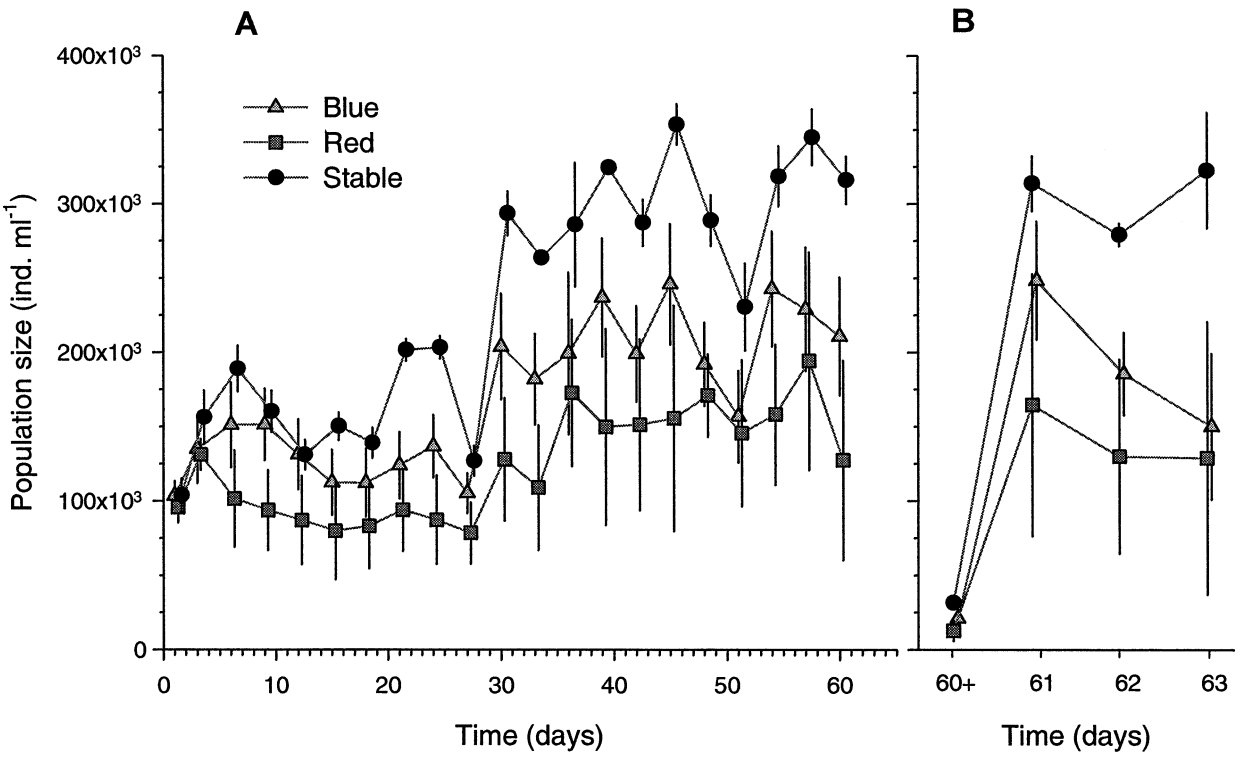

FIG. 2. (A) Average population sizes (individuals/ml, ± SE) measured prior to the 90% resource renewal occurring at three-day intervals in the stable, red, and blue environments during the 60-day experiment. The first datapoints represent the initial condition after one day of incubation at a constant 25°C. (B) Daily fluctuations in population sizes between two resource renewal episodes at the end of the experiment (days 60–63) in stable, red, and blue environments. The 90% resource renewal is used to extrapolate the population size immediately after the renewal (time 60+).

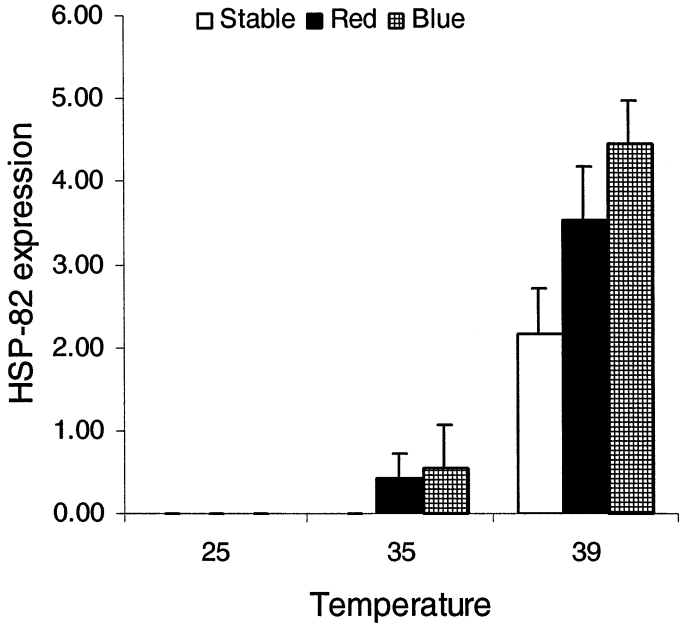

FIG. 3. Heat shock protein Hsp90 (Hsp82 in *Tetrahymena thermophila*) expression (mean ± SE) of lines evolved in stable, blue, and red environments in three constant temperatures of 25°C, 35°C, and 39°C. The amount of Hsp90 is shown relative to a standard sample. Hsp90 expression was below detection limit for all lines at 25°C and stable lines at 35°C.

Within the sampling intervals of three days, the population size grew rapidly during the first 24 h and then stabilized or slowly declined (Fig. 2B).

Measurements after the Experiment

Hsp90 expression

At 25°C no Hsp90 expression could be detected (Fig. 3). At 35°C some lines in blue and red treatments expressed Hsp90. However, expression between treatments did not differ significantly (Kruskal-Wallis ANOVA: $\chi^2 = 2.03$, $P = 0.362$). At 39°C the blue lines expressed significantly more Hsp90 than the stable lines, and red lines were intermediate ($F_{2,12} = 8.04$, $P = 0.048$; contrasts stable vs. blue: Bonferroni-corrected $P = 0.049$; stable vs. red: $P = 0.363$; red vs. blue: $P = 0.851$).

Carrying capacity and maximum growth rate

There were no differences in carrying capacity and maximum growth rates between the different environmental treatments (Tables 1 and 2). Between the different temperature regimes, growth rates were smallest at 15°C and largest at 35°C, whereas carrying capacity was smallest at 15°C and largest at 25°C (statistics not shown).

Correlations between r_{max}, K and Hsp90 expression

Hsp90 expression at 39°C, where all strains expressed Hsp90, did not correlate with maximum growth rate or car-

TABLE 1. Maximum growth rates of the population size ($r_{max}h^{-1}$ ind. ml^{-1} ± SE) and biomass ($r_{max}h^{-1}$ μm^3 ml^{-1} ± SE), and carrying capacities (K ind. ml^{-1} and K μm^3 ml^{-1} ± SE) in the three constant temperatures studied, with stable, red, and blue temperature treatments.

	Temperature history		
Temperature	Stable	Red	Blue
r_{max} count			
15°C	0.042 ± 0.001	0.036 ± 0.004	0.036 ± 0.005
25°C	0.166 ± 0.006	0.153 ± 0.014	0.161 ± 0.011
35°C	0.171 ± 0.013	0.200 ± 0.014	0.190 ± 0.011
r_{max} biomass			
15°C	0.035 ± 0.002	0.031 ± 0.0025	0.032 ± 0.003
25°C	0.159 ± 0.008	0.150 ± 0.009	0.158 ± 0.006
35°C	0.178 ± 0.014	0.205 ± 0.014	0.193 ± 0.0161
K count			
15°C	342738 ± 5529	291160 ± 11254	329003 ± 14404
25°C	405273 ± 12525	387383 ± 11226	408649 ± 12755
35°C	255614 ± 19181	297825 ± 22998	278737 ± 13567
K biomass			
15°C	$2.51 \times 10^9 \pm 0.73 \times 10^9$	$2.16 \times 10^9 \pm 0.57 \times 10^9$	$2.33 \times 10^9 \pm 1.77 \times 10^9$
25°C	$2.84 \times 10^9 \pm 1.09 \times 10^9$	$2.71 \times 10^9 \pm 0.71 \times 10^9$	$2.72 \times 10^9 \pm 1.48 \times 10^9$
35°C	$1.86 \times 10^9 \pm 1.49 \times 10^9$	$2.09 \times 10^9 \pm 1.19 \times 10^9$	$1.87 \times 10^9 \pm 0.97 \times 10^9$

rying capacity measurements. However, Hsp90 expression at 35°C correlated positively with r_{max} at 35°C (Table 3). Negative correlations were found between Hsp90 and carrying capacity at 15°C and at 25°C.

DISCUSSION

The presence of thermal variability and the time scale at which this variability occurs is predicted to have evolutionary effects on the physiology and performance of individuals (Lynch and Gabriel 1987; Parsons 1990; Huey and Kingsolver 1993; Gilchrist 1995; Kawecki 2000). In our study, we found that thermally variable environments cause an evolutionary increase in heat shock protein expression compared to a stable environment. Increases of Hsp expression induced by alternating high and low temperatures have been found in an artificial selection study on *Drosophila* by Sørensen et al. (1999). However, Cavicchi et al. (1995) and Bettencourt et al. (1999) showed that sustained high temperatures in *Drosophila* populations can result in a decrease of Hsp70 expression. It has been suggested that rapidly inducible thermotolerance mechanisms such as Hsp expression are beneficial mainly under transient thermal stress because Hsp expression is metabolically costly or has other associated trade-offs (Sørensen et al. 1999, 2001; Kawecki 2000). Our data supports this conclusion: lines from a rapidly fluctuating ''blue'' environment produced the highest amounts of Hsp90 (Hsp82 in *T. thermophila*), and the lines from a ''red'' environment were intermediate. High expression of the canalizer gene (Hsp90) has been suggested to evolve in populations that experience unpredictable environmental conditions (Kawecki 2000).

The fitness benefits of Hsps have previously been shown, for example, by Welte et al. (1993) and Feder et al. (1996). Assuming that there was little genetic variation between the individuals in the measured populations, our data gives tentative support to these findings. A small amount of variability can reasonably be expected because the populations experienced repeated and strong bottlenecks before the measurements (see Materials and Methods). Maximal growth rate was

TABLE 2. ANOVA for the effects of temperature history treatments (stable, red, and blue variations) and the stable temperatures on carrying capacity and maximum growth rate. * Because of unequal variances, the effects of the temperature history treatment were tested within temperatures for maximum growth rate measured from biomass (15°C: $F = 0.59$, $P = 0.57$; 25°C: $F = 0.13$, $P = 0.88$; 35°C: $F = 0.83$, $P = 0.46$).

	df	$F_{numbers}$/ $F_{biomass}$	$P_{numbers}$/ $P_{biomass}$
Carrying capacity K			
Temperature history	2	0.64/0.55	0.53/0.58
Temperature	2	49.8/31.7	<0.001/<0.001
History × temperature	4	2.5/1.45	0.06/0.24
Error	27		
Maximum growth rate r_{max}			
Temperature history	2	0.09/*	0.91/*
Temperature	2	215/*	<0.001/*
History × temperature	4	1.34/*	0.28/*
Error	27		

TABLE 3. Correlations between Hsp90 expression and r_{max} and K. Significant results, without correction for multiple comparisons, at $P < 0.05$ are bold.

	Spearman's $r_{numbers}/r_{biomass}$	$P_{numbers}/P_{biomass}$
Hsp90 (35°C) vs. r_{max} (15°C)	−0.401/−0.467	0.138/0.079
Hsp90 (35°C) vs. r_{max} (25°C)	−0.315/−0.333	0.273/0.244
Hsp90 (35°C) vs. r_{max} (35°C)	0.516/0.572	0.058/**0.033**
Hsp90 (35°C) vs. K (15°C)	−0.585/−0.557	**0.022**/**0.031**
Hsp90 (35°C) vs. K (25°C)	−0.547/−0.456	**0.043**/0.101
Hsp90 (35°C) vs. K (35°C)	0.297/−0.101	0.303/0.731
Hsp90 (39°C) vs. r_{max} (15°C)	−0.193/0.129	0.491/0.648
Hsp90 (39°C) vs. r_{max} (25°C)	0.081/0.262	0.782/0.366
Hsp90 (39°C) vs. r_{max} (35°C)	0.367/0.424	0.197/0.131
Hsp90 (39°C) vs. K (15°C)	−0.121/−0.311	0.666/0.260
Hsp90 (39°C) vs. K (25°C)	−0.209/−0.235	0.474/0.418
Hsp90 (39°C) vs. K (35°C)	0.011/−0.476	0.970/0.085

highest in lines with high Hsp90 expression at 35°C temperature, suggesting that Hsp expression can have fitness benefits when resources are not limiting growth. The negative correlation between Hsp expression and carrying capacity in low and intermediate temperatures suggests that increased Hsp expression, or other traits correlated with Hsp expression, was costly when resources are limiting. Overexpression of Hsps has been shown to bear costs. Feder et al. (1992) demonstrated that *Drosophila* cells overexpressing Hsp70 in normal temperatures grew slower than normal cells. Moreover, the yeast (*Saccharomyces cerevisiae*) strain lacking the ability to synthesize Hsp104 grew faster than wild-type strain (Sanches et al. 1992). The costs of Hsp expression can occur through increased energy expenditure (Krebs and Feder 1998), toxicity (Krebs and Feder 1997), or impairment of protein synthesis (Heckathorn et al. 1996). However, in our case, the expression of Hsp90 was below detection level in cold and intermediate temperatures, suggesting that other traits correlated to Hsp production may have been responsible for the reduced growth in these temperatures.

Depending on the underlying assumptions, thermal variability is predicted to increase (Lynch and Gabriel 1987) or decrease (Gilchrist 1995) the breadth of the thermal performance curve when the variability occurs within a generational time scale. Moreover, the shape of the thermal performance curve can be affected by trade-offs between performance in high and low temperatures (Huey and Hertz 1984). These changes can also be detected at population-level measurements (Huey and Kingsolver 1993; Via 1993). We found no differences between treatments in the shape or location of the thermal performance curve of populations, indicated by maximum growth rate and carrying capacity measurements in cold, intermediate, and high temperatures. However, our experiment offers only a weak test for the evolution of tolerance curves due to the limited number of assessed temperatures. Moreover, the time for accumulation of beneficial mutations was shorter in our study than in the other experimental studies (Bennett et al. 1992; Bennett and Lenski 1993).

Evolutionary improvement of fitness has been commonly demonstrated in breeding programs (Hartl and Clark 1997) and during laboratory evolution experiments (e.g., Lenski et al. 1991). During our experiment the population sizes almost doubled, suggesting an evolutionary change unrelated to the temperature treatments. The ancestral strain was allowed to adapt to 1% ePP resource for three weeks preceding the start of the experiment. However, prior to that the strain was propagated in lower quality medium (proteose peptone 2% weight/volume and yeast extract 0.25% weight/volume) with a less frequent turnover cycle of two weeks. These changes in culture conditions may for example change the efficiency of resource use or allocation of resources, with repercussions seen in the population size at the end of the three-day renewal cycle.

To summarize, our study demonstrates an evolutionary increase of Hsp90 expression in a variable environment. That the most rapidly varying environment increased Hsp90 expression most gives support to the hypothesis that inducible thermotolerance (Bettencourt et al. 1999) and expression of canalizing genes (Kawecki 2000) can evolve in response to rapidly varying environments.

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