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Phenotypic plasticity is not affected by experimental evolution in constant, predictable or unpredictable fluctuating thermal environments

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varying environments.

Abstract

The selective past of populations is presumed to affect the levels of phenotypic plasticity. Experimental evolution at constant temperatures is generally expected to lead to a decreased level of plasticity due to presumed costs associated with phenotypic plasticity when not needed. In this study, we investigated the effect of experimental evolution in constant, predictable and unpredictable daily fluctuating temperature regimes on the levels of phenotype plasticity in several life history and stress resistance traits in *Drosophila simulans*. Contrary to the expectation, evolution in the different regimes did not affect the levels of plasticity in any of the traits investigated even though the populations from the different thermal regimes had evolved different stress resistance and fitness trait means. Although costs associated with phenotypic plasticity are known, our results suggest that the maintenance of phenotypic plasticity might come at low and negligible costs, and thus, the potential of phenotypic plasticity to evolve in populations exposed to different environmental conditions might be limited.

Introduction

When the environment changes within generations, single genotypes can produce different phenotypes as a response to the environmental conditions, known as plastic responses (Bradshaw, 1965). Many examples of such phenotypic plasticity, for example antipredator behaviours (Gabriel, 2006; Auld & Relyea, 2010) and the activation of mechanisms to prevent thermal stress injuries (Sørensen *et al.*, 2003), demonstrate plasticity as an adaptive response. However, the extent of plasticity is under conflicting selective pressures, due to the costs of increased energetic demands associated with sampling the environment, activating the response, as well as to the maintenance of the plastic machinery over time (DeWitt *et al.*, 1998). It is critical to distinguish between costs associated with phenotypes or costs associated with plasticity (Callahan *et al.*, 2008). Costs of plasticity refer to a reduced fitness observed in a plastic genotype when compared to a less plastic one (DeWitt, 1998). Costs of phenotypes are the results of resource allocation from one trait to another, as well as on gathering information on the environmental conditions (Callahan *et al.*, 2008). These costs suggest that plasticity might be an efficient adaptive mechanism to respond to immediate or short-term environmental changes, whereas responses to long-term environmental changes are expected to rely more on adaptation via selection for trait means (Hoffmann & Parsons, 1991; van Heerwaarden & Sgrò, 2011).

Thermal adaption theory predicts that the ability to adapt plastically is affected by the selective past, for example by selection occurring under constant or fluctuating temperature regimes (Leroi *et al.*, 1994; Bennett & Lenski, 1996; Hallsson & Bjorklund, 2012). Selection in fluctuating temperature regimes is hypothesized to favour genotypes that can perform well under a wide range of environmental conditions (Lynch & Gabriel, 1987; Gomulkiewicz & Kirkpatrick, 1992; Scheiner, 1993; Kassen, 2002), regardless of whether the environmental fluctuations happen within or between generations (Kassen, 2002; Buckling *et al.*, 2007; Hallsson & Bjorklund, 2012). Differently, selection in a stable

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temperature regime is predicted to reduce the levels of plasticity, due to the unnecessary costs connected to the maintenance of the plastic machinery in a non-changing environment. In addition, populations with limited number of individuals maintained in a stable temperature regime were suggested to reduce the levels of plasticity through mutation degradation when the plastic response was not needed (Masel *et al.*, 2007). The benefits of a plastic response are expected to depend on the reliability of the environmental cue (Scheiner, 1993; Lande, 2009). Some authors argue that high levels of plasticity might be rare when the reliability of cues with respect to the environmental changes is low (Frank & Slatkin, 1990; Biron *et al.*, 1998; Hopper & Rosenheim, 2003). Canale & Henry (2010), instead, suggested that populations selected in an environment that changes with low predictability might show high levels of phenotypic plasticity to adapt to such varying environments. Thus, the theoretical expectation for the adaptive role of plasticity in the unpredictable fluctuating environment is not resolved.

Changes in plasticity and the existence of plasticity-associated costs were rarely confirmed by empirical studies, and the levels of plasticity have often shown a limited ability to evolve (Ketola *et al.*, 2004; Berger *et al.*, 2014; but see Gomulkiewicz & Kirkpatrick, 1992; Kassen, 2002). As most studies only assay a limited number of traits, we have an incomplete overview of how the levels of plasticity affect the fitness of populations. Furthermore, less theoretical and empirical knowledge exists on how predictable compared to unpredictable environmental changes affect the levels of plasticity.

The aim of this study was to investigate evolutionary consequences of laboratory natural selection in constant, predictable and unpredictable daily fluctuating temperature regimes on the levels of phenotypic plasticity (for more detailed information on the three thermal regimes see Manenti *et al.*, 2014). We investigated the levels of plasticity induced in eight traits, four life history traits (LHT) and four stress resistance traits (SRT), and we repeated the investigation two times, after the 10th and after the 20th generation of laboratory natural selection. These eight traits provided a diverse set of estimates of fitness of flies and of the levels of plasticity in *D. simulans*. The wild population collected in Italy showed high levels of genetic variance for plasticity in all traits except fecundity and chill coma recovery time (Manenti *et al.*, 2014), indicating a potential for the levels of plasticity to evolve.

Selection in the constant environment was predicted to decrease the levels of plasticity and optimize the performance of these flies in this constant regime. Selection in the predictable fluctuating regime was expected to maintain high levels of plasticity. It could be argued that the unpredictable regime, where the previously adaptive plastic phenotypes might be less adaptive, or even maladaptive, should lead to decreased levels of plasticity (Frank & Slatkin, 1990; Moran, 1992; Reed *et al.*, 2010). However, we suggest that high levels of plasticity might be maintained in flies as result of selection in the unpredictable fluctuating regime, as we considered plasticity as an adaptive response in this environment.

Materials and methods

Origin of population

The population of *D. simulans* used in this study was collected near to Bologna, Italy, in the first half of August 2012 (Manenti *et al.*, 2014). Around 350 flies were randomly divided among 27 plastic bottles containing 70 mL of standard medium (oatmeal–sugar–yeast–agar medium).

Experimental design

Selection procedure

Flies were divided among three different selection regimes (experimental evolution), and in each of them, three independent biological replicates were set up (bioreplicate). For each bioreplicate, 600 flies were placed in three 70-ml plastic bottles and maintained separately during the experimental evolution (Fig. 1). The three different selection regimes shared the same mean temperature (23 °C) but differed with respect to the variation and predictability of temperature changes. The constant regime (C) had constant 23 °C throughout the day. The predictable fluctuating (PF) regime repeated a regular daily fluctuation of temperatures following a sinus function, with maximum and minimum daily temperatures of 28 °C and 13 °C, respectively. The unpredictable fluctuating (UF) regime also followed a sinus function, with the difference that the maximum and minimum daily temperatures were randomly determined but not exceeding the maximum and minimum temperatures reached in the PF regime (Fig. 2). It is important to note that the temperature at the beginning and at the end of the day, at the light/dark transitions, was 23 °C, similar in all three selection regimes. The daily light intensity varied following a Gaussian model, to mimic the sun light cycle (for more details on origin of population and on the selection and test regimes, see Manenti *et al.*, 2014). The laboratory thermal selection was performed concurrently in three different thermal cabinets (internal volumes of 400 L). The cabinets consist of a thermal isolated external case, fitted with two units to heat and cool the temperature and secure the airflow. The cabinets were connected to an external computer that controlled the daily temperature and light intensity using in-house-designed software. Before running the experiment, the cabinets were calibrated for 3 months, such that differences

between average temperatures in the three cabinets were minimized to less than 0.2 °C. The small error bars associated with the temperatures scored in the C and PF regimes indicate that the temperatures in these two cabinets were controlled with high degree of repeatability and precision (see Fig. 2 and Manenti *et al.*, 2014).

Test regimes

The levels of plasticity in flies selected in the three thermal regimes were assayed by testing flies in all three thermal regimes, in a full factorial design (Fig. 1). We repeated the phenotypic assessments two times, after 10 and 20 generations of experimental evolution (20 fly generations required around 1.5 years). At each of the assay generations (after 10 and 20 generations of selection, respectively), 21 isofemale lines for each selection regime were randomly generated (7 isofemale lines for each bioreplicate). To create a line, a single female fly was allowed to lay eggs from 4 to 6 days of age in 7-mL vials with standard medium. The progeny of each isofemale line (F1) were kept in a 60-mL plastic bottle containing fresh medium for 4 days before egg laying to ensure the presence of mature females. F1 flies were allowed to lay eggs in a short time window (12–16 h), and groups of 40 eggs were transferred into 7-mL vials to achieve similar and optimal density conditions for flies (F2). From each selection regime (C, PF and UF), vials with F2 eggs were randomly divided into the three groups and placed into the three different test regimes (C, PF and UF). Thus, flies selected in different thermal regimes completed the development in all three test regimes.

Phenotypic assessments

Two- to three-day-old female flies were separated from males with the aid of light CO_2 anaesthesia. Two days thereafter, flies from all three temperature regimes were moved to a 23 °C climate chamber for the assay, at the time point when the temperature in all three regimes was 23 °C (08.00 h in the morning). The eight traits assayed in this study were divided into two groups, life history traits (LHT), represented by fecundity, body size (BS), developmental time and egg-to-adult viability, and stress resistance traits (SRT), represented by time to heat knockdown, chill coma recovery time, desiccation and starvation tolerance. In this fully crossed experimental design, each isofemale line generated from each selection regime was tested in all three thermal regimes (test regimes), resulting in 9 experimental treatments (3 × 3) for a total number of 189 isofemale lines (21 × 9). Ten *D. simulans* females from each of the 189 isofemale lines were assayed for each trait, giving a total sample size of 1890 individuals per trait, except for viability and developmental time, where we observed around 23 000 eggs until emergence.

Stress resistance-related traits (SRRT). Time to heat knockdown (KD) was the time (minutes) taken for flies

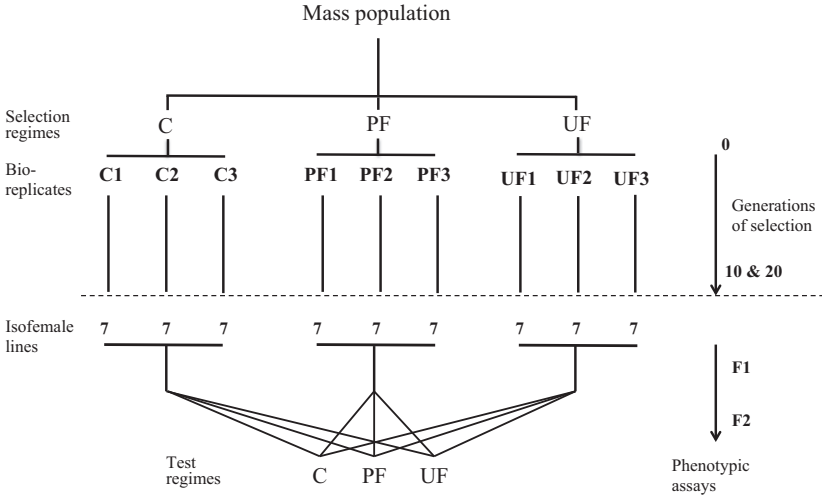

Fig. 1 Experimental design. The mass population of *D. simulans* was established based on ca. 350 flies. The mass population was randomly divided among three different selection regimes (C, PF, UF), and in each of them, three biological replicates were set up (bioreplicate). The bioreplicates were kept separately during the experimental evolution process. After 10 and 20 generations of selection, respectively, we created 7 isofemale lines for each bioreplicate. Each isofemale line was bred for two generations (F1 & F2) to obtain an adequate number of individuals for the phenotypic assays. From each selection regime (C, PF and UF), vials with F2 eggs were randomly divided into the three test regimes (C, PF and UF). Thus, flies selected in all the different thermal regimes completed the development in all three test regimes.

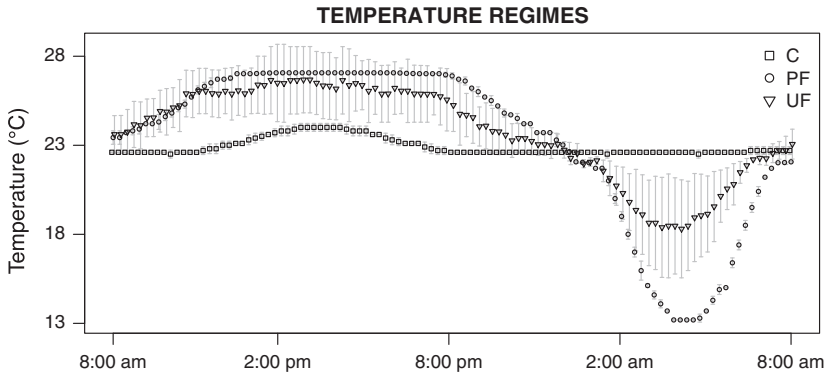

Fig. 2 Mean daily temperature ± standard deviation (SD) for the three thermal regimes recorded over 14 consecutive days. The symbols indicate the mean temperatures for each regime. Squares stand for the constant regime (C), circles correspond to the predictable fluctuating regime (PF), and triangles indicate the unpredictable fluctuating regime (UF). The grey bars (sd) indicate the consistency on daily fluctuation of temperatures. The variations in daily temperature of the C and PF regimes are minimal compared to that of the UF regime, indicating low predictability of temperature fluctuations in the latter regime. Figure modified from Manenti *et al.* (2014).

to fall into a coma where they do not react to external stimuli; chill coma recovery time (CC) was the time (minutes) necessary for the flies to spontaneously regain the ability to stand upright on their six legs; desiccation tolerance (DS) was measured as how long flies could tolerate water deprivation when the relative humidity of the air was close to 0%, before falling into coma. For DS, flies were scored every hour; starvation tolerance (ST) was scored as how long flies could tolerate food deprivation, and flies were checked every 8 hours (for more details, see Manenti *et al.*, 2014).

Life history-related traits (LHRT). Fecundity (FE) was measured as the number of adult flies produced by a single female from 3 to 6 days of age; BS of each single female was estimated by the wing centroid size calculated on the basis of 11 landmarks of the right wing (Slice, 1996). Landmark coordinates were recorded with ImageJ 1.46 software (U.S. National Institutes of Health, Bethesda, MD, USA). The centroid size was calculated as the sum of the distances between each landmark and the centre of the wing. Developmental time (DT) was the time for the complete development of flies, from egg laying of parental flies (day 0) to entering the adult stage. Egg-to-adult viability (VIA) was the percentage of adult flies that emerged from 40 eggs (for a more detailed description on how the life history traits were measured, see Manenti *et al.*, 2014).

Statistical analyses

The phenotypic assays were repeated two times, after 10 and 20 generations of selection. An additional investigation on the effect of different temperature regimes on the same life history and stress resistance traits of *D. simulans* was made before starting the selection experiment (Manenti *et al.*, 2014).

The analysis was divided into two steps, leading to two different statistical analyses. In the first step of the analysis, we investigated the levels of plasticity harboured in the different selection regimes. For each trait, except egg-to-adult viability, we applied a linear mixed model (Bates *et al.*, 2014) with selection and test regimes treated as fixed effects and with bioreplicate and isofemale line nested in bioreplicate treated as random effects. We assessed the fixed effects and their interactions by comparing the full model including all the effects to a reduced model where the fixed effects and their interaction were sequentially removed. The comparison between the two models provided a likelihood ratio (LR). The significance of the given fixed effect was assessed by chi-square-based P values obtained by comparing the two models (Schou *et al.*, 2015). Egg-to-adult viability was analysed with a generalized linear model, based on a binomial distribution.

In the second step of the analysis, we investigated the genetic variability for plasticity of each trait. We ran a liner model where test regime and isofemale line nested in the respective selection regime were both treated as fixed effects. Isofemale line was treated as fixed effect as we aimed to compare the levels of plasticity between isofemale lines. Egg-to-adult viability was analysed with a generalized linear model, based on a binomial distribution. All the analyses were performed using the Lme4 package in R (R Core Team, 2015). The data met all the assumptions for running an ANOVA.

Results

We assayed the levels of plasticity and trait means in four stress resistance (SRT) and four life history (LHT) traits after 10 and 20 generations of natural laboratory

selection in constant, predictable fluctuating and unpredictable fluctuating thermal regimes.

Generation 10

Flies from different selection regimes expressed different trait means in three of four SRT and in all LHT (Table 1), indicating an evolutionary response. The high levels of plasticity were induced by the three test regimes in all traits investigated. The interaction term 'test regime by isofemale line', nested in selection regime, was always significant, except in time to heat knockdown (Table 2), suggesting high levels of genetic variability for plasticity. Even if the selection and test regimes affected the levels of plasticity and genetic variability for plasticity in all traits investigated, ten generations of experimental evolution in different thermal regimes did not affect the levels of plasticity, as shown by the never significant interaction between selection and test regime (Table 1).

Generation 20

Flies selected in the three selection regimes showed different trait means in all SRT (Table 3, Fig. 3). For LHT, selection regimes led to statistical differences in two of four traits investigated. This indicated an evolutionary response to the thermal regimes. We found that the three selection regimes induced a strong plastic response in all traits investigated (Table 3, Fig 3). The levels of genetic variability for plasticity were highly significant in all eight LHT and SRT traits, as shown by the significant interaction between isofemale line and test regime (Table 4). However, although both test and selection regime affected the LHT and SRT, no interaction between these two terms was found in any trait investigated (Table 3, Fig. 3), meaning that flies selected in different thermal regimes showed the same levels of plasticity.

Table 1 Results from the linear mixed model on the effect of selection regime, test regime and their interaction for SRT and LHT at generation 10. For each trait, except egg-to-adult viability, we run a linear mixed model with selection and test regimes treated as fixed effects and with bioreplicate and isofemale line nested in bioreplicate treated as random effects. The table reports likelihood ratio chi-square and associated *P* values obtained by comparing the full model including all the effects to a reduced model, where fixed effects and their interactions were sequentially removed. Egg-to-adult viability was analysed with a generalized linear model, based on a binomial distribution. KD, time to heat knock down; RT, chill coma recovery time; DS, desiccation tolerance; ST, starvation tolerance; BS, body size; VIA, egg-to-adult viability; DT, developmental time; FE, fecundity. LR χ^2, likelihood ratio χ^2; *P*, probability associated with LR χ^2. The degrees of freedom of selection and test regimes are two and four in the interaction term.

	Selection regime		Test regime		Selection X test regime	
	LR χ^2	*P*	LR χ^2	*P*	LR χ^2	*P*
KD	100.4	<0.001	471.2	<0.001	0.9	0.91
RT	1.5	0.47	85.1	<0.001	1.7	0.78
DS	8.8	0.01	68.3	<0.001	9.4	0.05
ST	11.7	<0.001	51.2	<0.001	8.5	0.08
BS	7.8	0.02	31.4	<0.001	7.7	0.1
DT	29.9	<0.001	3131.6	<0.001	4.5	0.35
FE	21	<0.001	53.8	<0.001	9.2	0.06
VIA	32.9	<0.001	535.3	<0.001	4.1	0.39

Discussion

Theories on the evolution of phenotypic plasticity are strongly developed, and there are several models predicting the evolution of phenotypic plasticity under different environmental conditions (Via & Lande, 1985; Gomulkiewicz & Kirkpatrick, 1992; Gavrilets & Scheiner, 1993; de Jong, 1995; Price *et al.*, 2003; Price, 2006; Utz *et al.*, 2014). These models agree that the selective past strongly affects the levels of plasticity expressed by individuals.

We expected that the selection in different regimes would affect the levels of plasticity. In particular, we predicted the highest levels of plasticity in flies selected in the predictable fluctuating regime, followed by flies selected in the unpredictable fluctuating regime and the lowest levels of plasticity in flies selected in the constant regime.

We found that the three thermal regimes induced a marked plastic response in all traits investigated. The marked changes in the performance induced by plasticity suggest that plasticity may play a crucial role for fitness and stress resistance in *D. simulans*. Different plastic strategies might evolve under the experimental regimes in these flies in relation to the predictability of environmental changes; that is, plasticity was expected to be adaptive in the PF and UF (in a different way) regimes and not adaptive in the C regime. Even if the selection pressure in the three thermal regimes was expected to be low, given the similarity between the regimes and the supposed low selection coefficient, here we found a clear diversification in the performance of flies selected in the different thermal regimes for both stress resistance and life history traits, proving that the regimes did impose a consistent selection response.

However, after 10 and 20 generations of experimental evolution the results consistently showed no selection response for plasticity itself. This was not caused by a lack of genetic variability for plasticity. In fact, the high levels of genetic variability for plasticity were found in all traits investigated, similarly after 10 and 20 generations of experimental evolution, as revealed by the significant interaction 'isofemale line by test regime' (Tables 2 and 4). Similar results were found by James

Table 2 Results from the ANOVA and likelihood ratio (LR) χ^2 on the difference between isofemale lines (Line), thermal regime (Test regime) and their interaction (Line × test regime) for each SRT and LHT at generation 10. Test regime and isofemale line were treated as fixed effects. Egg-to-adult viability was analysed with a generalized linear model, based on a binomial distribution. The reading key for the different abbreviations used in the table is reported in the legend to Table 1. *F*, F-ratio; LR χ^2, likelihood ratio χ^2; *P*, probability associated with LR χ^2. Degrees of freedom are given within parentheses.

	Line		Test regime		Line × test regime	
	F	*P*	*F*	*P*	*F*	*P*
KD	5.1(61,1591)	<0.001	186(2,1515)	<0.001	1.1(119,1591)	0.25
RT	3.6(60,1642)	<0.001	38.6 (2,1642)	<0.001	1.3(119,1594)	<0.001
DS	3.3(60,1520)	<0.001	32.9(2,1520)	<0.001	1.9(120,1520)	<0.001
ST	5(60,1521)	<0.001	35.2(2,1521)	<0.001	3(115,1521)	<0.001
BS	6.5(60,1509)	<0.001	325(2,1509)	<0.001	3.4(112,1509)	<0.001
DT	6.7(63,14009)	<0.001	1606(2,14009)	<0.001	4(126,14009)	<0.001
FE	3.2(64,1522)	<0.001	26.1(2,1522)	<0.001	2(119,1522)	<0.001
	LR χ^2	*P*	LR χ^2	*P*	LR χ^2	*P*
VIA	901(63)	<0.001	31.4(2)	0.02	341(126)	<0.001

Table 3 Results from the linear mixed model on the effect of selection regime, test regime and their interaction for SRT and LHT at generation 20. For each trait, except egg-to-adult viability, we run a linear mixed model with selection and test regimes treated as fixed effects and with bioreplicate and isofemale line nested in bioreplicate treated as random effects. The table reported likelihood ratio chi-square and associated P values obtained by comparing the full model including all the effects to a reduced model, where fixed effects and their interaction were sequentially removed. Egg-to-adult viability was analysed with a generalized linear model, based on a binomial distribution. The reading key for the different abbreviations used in the table is reported in the legend to Table 1. The degrees of freedom of selection and test regimes are two and four in the interaction term.

	Selection regime		Test regime		Selection × test regime	
	LR χ^2	*P*	LR χ^2	*P*	LR χ^2	*P*
KD	53	<0.001	349.5	<0.001	7.7	0.1
RT	9.6	<0.001	134.7	<0.001	6.7	0.15
DS	20.9	<0.001	95.9	<0.001	1.8	0.77
ST	208.2	<0.001	14.6	<0.001	3.2	0.52
BS	29.8	<0.001	548.3	<0.001	3.7	0.44
DT	212.3	<0.001	2413.4	<0.001	2.4	0.66
FE	2.8	0.24	102.2	<0.001	3.3	0.5
VIA	2.5	0.28	7.4	0.02	1.73	0.78

et al. (1997) and van Heerwaarden & Sgrò (2011). The latter authors found high levels of plasticity induced by different developmental temperatures as well as a strong effect of latitude on morphometric traits, but they did not find any relation between latitude of origin of populations and levels of plasticity in *D. simulans*. van Heerwaarden & Sgrò (2011) suggested that gene flow might explain why the same levels of plasticity were maintained in different natural populations; however, although gene flow might be expected in nature, it is definitely excluded in our study, as flies from each selection regime were strictly kept separate.

The costs of plasticity are crucial as a key for understanding why flies showed limited levels of plasticity. The underlying assumption behind this idea is that a plastic genotype must pay a price in terms of fitness when compared to a less plastic genotype (Moran, 1992), leading to the expectation of detectable costs and limits related to plasticity. The costs associated with high levels of plasticity are assumed to be the result of maintenance of the plastic machinery over time, the sampling of the environment and, eventually, of activating the response (DeWitt, 1998). Still, this hypothesis is not supported by our results, as the same high costs suggested explaining the homogenous levels of plasticity among different populations should also have led to a decreased plasticity in flies selected in the constant regime, where maintaining plasticity would be an unnecessary use of resources. Our results indicate that if there were such costs, these were similar among selection regimes and likely limited, explaining why the selection, specifically designed to affect the level of plasticity, did fail to lead to the expected changes. In addition, twenty generations of

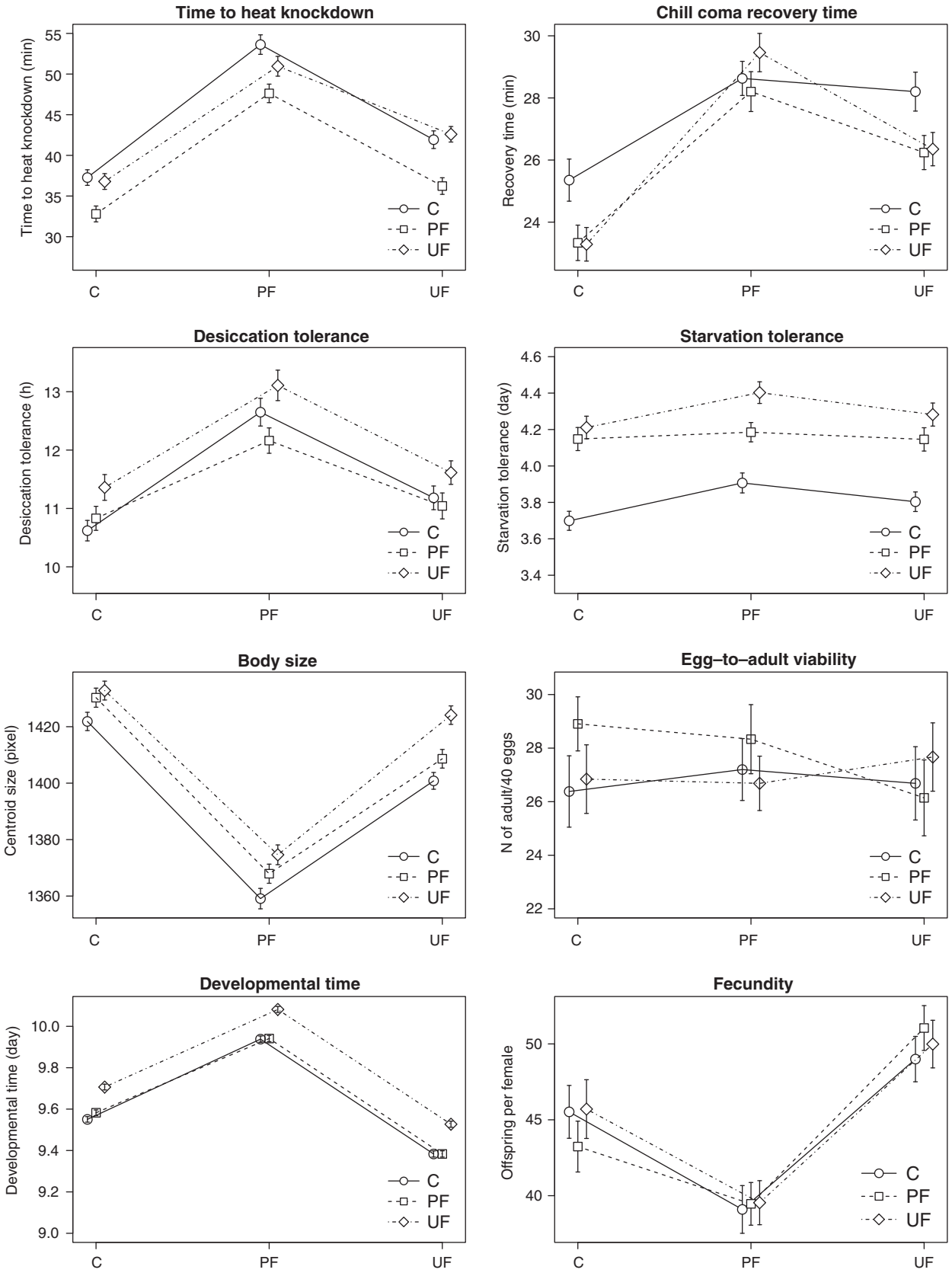
Time to heat knockdown
Time to heat knockdown (min)
55
50
45
40
35
30
C
PF
UF
Chill coma recovery time
Recovery time (min)
30
28
26
24
Desiccation tolerance
Desiccation tolerance (h)
13
12
11
10
Starvation tolerance
Starvation tolerance (day)
4.6
4.4
4.2
4.0
3.8
3.6
3.4
Body size
Centroid size (pixel)
1420
1400
1380
1360
Egg–to–adult viability
N of adult/40 eggs
30
28
26
24
22
Developmental time
Developmental time (day)
10.0
9.8
9.6
9.4
9.2
9.0
Fecundity
Offspring per female
50
45
40

Fig. 3 Phenotypic means of four stress resistance and four life history traits ± standard error of flies from different selection regimes when tested at different test regimes. The trait means are the response variable (*y*-axes), whereas the test regime (*x*-axes) and the selection regime (lines) are the explanatory variables. Open circles represent the constant selection regime (C), open triangles stand for the predictable fluctuating regime (PF), and crosses indicate the unpredictable fluctuating regime (UF).

Table 4 Results from the ANOVA and likelihood ratio (LR) χ^2 on the difference between isofemale lines (Line), thermal regime (Test regime) and their interaction (Line × test regime) for each SRT and LHT at generation 20. The reading key for the different abbreviations used in the table is reported in the legend to Table 1. *F*, *F*-ratio; LR χ^2, likelihood ratio χ^2; *P*, probability associated with LR χ^2. Degrees of freedom are given within parentheses.

	Line		Test regime		Line × test regime	
	F	*P*	*F*	*P*	*F*	*P*
KD	6.1(60,1515)	<0.001	295(2,1515)	<0.001	2.5(119,1515)	<0.001
RT	14.6(60,1594)	<0.001	11.2(2,1594)	<0.001	14.7(117,1594)	<0.001
DS	5.3(60,1540)	<0.001	52(2,1540)	<0.001	2(119,1540)	<0.001
ST	33.7(59,1633)	<0.001	9.9(2,1633)	<0.001	2.5(118,1633)	<0.001
BS	5.9(60,1539)	<0.001	319.8(2,1539)	<0.001	3.1(112,1539)	<0.001
DT	10.5(61,14917)	<0.001	20.4(2,14917)	<0.001	2.9(122,14917)	<0.001
FE	6.3(63,1681)	<0.001	30.4(2,1681)	<0.001	1.4(117,1515)	<0.001
	LR χ^2	*P*	LR χ^2	*P*	LR χ^2	*P*
VIA	384(58)	<0.001	1.59(2)	0.45	259(112)	<0.001

selection in different thermal regimes have to be considered a limited evolutionary time not enough to lead to the mutation degradation as suggested by Masel *et al.* (2007).

There is an active, yet unsolved, debate whether the same genes that regulate trait means are also responsible for the levels of plasticity in that trait (Falconer, 1952; Via & Lande, 1985, 1987) or whether they are controlled by two genetically independent mechanisms (epistasis model) regulating the levels of the plastic response and the trait mean independently (Lynch & Gabriel, 1987; Scheiner, 1993; Schlichting & Pigliucci, 1993). Our study supports the epistasis model of plasticity (Lynch & Gabriel, 1987; Scheiner & Lyman, 1989, 1991), which describes the phenotypic response to the environment as the combination of two classes of genes, regulating the trait means and the levels of a plastic response independently and interacting with each other. Even if this model does not explain why the levels of plasticity were not affected by selection in different thermal regimes, it explains the selection responses found only on trait means (T. Manenti *et al.*, *in prep.*) and not on the levels of plasticity.

Conclusion

Natural populations are exposed to environments that vary on a short or long term (Ketola *et al.*, 2004; Kingsolver *et al.*, 2009; Hallsson & Bjorklund, 2012; Manenti *et al.*, 2014). Fluctuating temperatures are considered to have a greater ecological relevance compared to constant ones, as fluctuating temperatures are a better proxy of a natural environment (Boyce *et al.*, 2006; Schreiber, 2010). In this study, we investigated the evolutionary potential of phenotypic plasticity in different ecologically relevant thermal regimes as an adaptive mechanism to cope with predictable and unpredictable thermal fluctuations. The selection in the thermal regimes was shown to affect the trait means of stress resistance, and partially of life history traits, indicating that the amplitude and predictability of daily thermal fluctuations had a marked effect on flies. Interestingly, flies selected in the different thermal regimes maintained the same levels of plasticity in all traits investigated, even if ample genetic variation was present. Thus, the ability to adapt to new environmental conditions seems to rely mainly on changes in trait means and not on the evolution of phenotypic plasticity. No changes in the levels of plasticity may be the result of low or negligible costs associated with the maintenance of the ability to respond plastically over time in *D. simulans*. We suggest that the plastic response induced by different thermal regimes seems to be crucial for fitness and stress resistance traits in *D. simulans*.

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