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Keeping your options open: Maintenance of thermal plasticity during adaptation to a stable environment

Inês Fragata,[1,2] Miguel Lopes-Cunha,[1] Margarida Bárbaro,[1] Bárbara Kellen,[1] Margarida Lima,[1] Gonçalo S. Faria,[1] Sofia G. Seabra,[1] Mauro Santos,[3] Pedro Simões,[1,*] and Margarida Matos[1,*]

[1] *cE3c—Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal*

[2] *E-mail: irfragata@fc.ul.pt*

[3] *Departament de Genètica i de Microbiologia, Grup de Genòmica, Bioinformàtica i Biologia Evolutiva (GGBE), Universitat Autonòma de Barcelona, 08193 Bellaterra, Barcelona, Spain*

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Phenotypic plasticity may allow species to cope with environmental variation. The study of thermal plasticity and its evolution helps understanding how populations respond to variation in temperature. In the context of climate change, it is essential to realize the impact of historical differences in the ability of populations to exhibit a plastic response to thermal variation and how it evolves during colonization of new environments. We have analyzed the real-time evolution of thermal reaction norms of adult and juvenile traits in *Drosophila subobscura* populations from three locations of Europe in the laboratory. These populations were kept at a constant temperature of 18ºC, and were periodically assayed at three experimental temperatures (13ºC, 18ºC, and 23ºC). We found initial differentiation between populations in thermal plasticity as well as evolutionary convergence in the shape of reaction norms for some adult traits, but not for any of the juvenile traits. Contrary to theoretical expectations, an overall better performance of high latitude populations across temperatures in early generations was observed. Our study shows that the evolution of thermal plasticity is trait specific, and that a new stable environment did not limit the ability of populations to cope with environmental challenges.

KEY WORDS: **Clinal variation, *Drosophila*, experimental evolution, reaction norms, thermal plasticity.**

In face of both spatial and temporal changes in environment, populations will have to respond quickly or risk extinction (Hoffmann and Rieseberg 2008; Alberto et al. 2013). Phenotypic plasticity is the property of a genotype that allows it to produce different phenotypes according to the surrounding environment (Via et al. 1995; Agrawal 2001; de Jong 2005; Pigliucci 2005; Garland and Kelly 2006). This plasticity may be adaptive, maladaptive, or neutral, depending on whether the environmental-induced phenotypes are closer or further away from the evolutionary optimum in each environment (Ghalambor et al. 2007, 2015; Crispo 2008). If adaptive, plasticity for traits more loosely related to fitness may allow for high performance across environments, thus conferring homeostasis in terms of fitness (Richards et al. 2006; Liefting and Ellers 2008).

Much theoretical work has been developed with the aim of understanding how adaptive plasticity evolves and is maintained in natural environments (Huey and Kingsolver 1989; Agrawal 2001; de Jong 2005; Angilletta 2009; Auld et al. 2010; Murren et al. 2015). In general, models suggest that adaptive plasticity will evolve if (1) the environment varies spatially or temporally; (2) environmental cues related to heterogeneity are reliable; (3) plasticity leads to an increase in fitness; and (4) populations have additive genetic variance for plasticity (Kassen 2002; Garland and Kelly 2006; Ghalambor et al. 2007; Auld et al. 2010; Murren et al. 2015). The evolution of plasticity is associated with the evolution of generalists and specialists (Van Tienderen 1997). Generalists

*These authors are the joint last authors.

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are expected to evolve in heterogeneous environments, having a similar performance across environments in terms of fitness. In contrast, specialists will present higher fitness in the specific environment where they evolve (Kassen 2002). Thus, in terms of fitness, generalists will be less plastic than specialists. Nevertheless, the opposite may occur for other traits more loosely related to fitness (see Van Tienderen 1997). Expectations of how plasticity evolves or is maintained in a homogeneous environment vary depending on the existence of plasticity costs (Garland and Kelly 2006; Auld et al. 2010; Murren et al. 2015).

Fostered by the increased awareness of anthropogenic effects on climate, the mechanisms through which phenotypic plasticity emerges and/or evolves have been increasingly studied in the thermal context (Berger et al. 2013; Ketola et al. 2013, 2014; Phillips et al. 2014; Ketola and Saarinen 2015). Temperature is a major factor affecting species distributions, particularly of ectotherms (Angilletta et al. 2003; Angilletta 2009; Phillips et al. 2014), because it affects the final size of individuals through changes in development and growth, which ultimately influence organisms' performance and survival (Huey and Kingsolver 1989; Angilletta et al. 2003; Kingsolver et al. 2004; Angilletta 2009). Predictions on how temperature affects organismal performance can be extracted from empirical and theoretical work on biochemistry and thermodynamics (Huey and Kingsolver 1989; Angilletta et al. 2003; Somero 2004; Angilletta 2009). For example, enzymatic configuration affects how enzymes function at different temperatures (Hochachka and Somero 2002; Angilletta et al. 2003; Somero 2004), with more stable configurations allowing for a better performance at higher temperatures but poorer performance at low temperatures (Angilletta et al. 2003; Angilletta 2009). Therefore, specialist versus generalist or warm versus cold trade-offs should govern the evolution of thermal reaction norms (Huey and Kingsolver 1989; Gilchrist 1995; Angilletta et al. 2003; Angilletta 2009; Berger et al. 2013). The specialists versus generalists model is directly linked to the "Jack-of-all-temperatures is a master of none" hypothesis (Huey and Hertz 1984; Huey and Kingsolver 1989, 1993; Angilletta et al. 2003; Angilletta 2009). In this case a trade-off is predicted to occur between the thermal optimum and the breadth of thermal performance (Huey and Kingsolver 1989; Angilletta 2009; Kingsolver 2009). Evolution of thermal reaction norms can occur by changes in height (vertical shifts) or shape (Angilletta 2009). The first may relate to up- or downregulation of enzymes (Berger et al. 2013), affecting overall performance across temperatures without changes in plasticity. Changes in shape of the reaction norms, and thus of plasticity, are more frequently associated with structural differences of enzymes conferring temperature sensitivity (see Somero 2004; Berger et al. 2013).

Empirical work on the evolution and maintenance of thermal plasticity has mainly focused on two distinct approaches: the study of thermal performance after evolution in homogeneous versus heterogeneous environments by means of experimental evolution (Kassen 2002; Ketola et al. 2013; Berger et al. 2014; Condon et al. 2014; Ketola and Saarinen 2015), and the characterization of reaction norms in several populations from different latitudes or climates (Trotta et al. 2006; Yamahira et al. 2007; Liefting et al. 2009; Berger et al. 2013; Klepsatel et al. 2013; Phillips et al. 2014). Evolution in stable versus fluctuating thermal environments predicts the appearance of, respectively, specialists in each specific thermal environment and generalists in heterogeneous environments (Berger et al. 2014; Condon et al. 2014; Ketola and Saarinen 2015). However, few recent studies have obtained clear-cut results (Berger et al. 2013, 2014; Ketola et al. 2013; Condon et al. 2014; Ketola and Saarinen 2015; Manenti et al. 2015). On the other hand, studies comparing organisms derived from different geographical locations assume that populations (1) are subjected to different temperatures (although this assumption has been rarely quantified; see Huey and Pascual 2009), and (2) are locally adapted to those environments (Trotta et al. 2006; Liefting et al. 2009; Berger et al. 2013; Klepsatel et al. 2013). It is, therefore, expected that maximum performance occurs near the temperature for which adaptation occurred (Angilletta et al. 2003; Angilletta 2009), although again not all results fully support these predictions (Yamahira et al. 2007; Berger et al. 2013; Klepsatel et al. 2013). An approach that can help clarify this issue and open new research possibilities is the study of the real-time evolution of thermal plasticity when initially differentiated populations in nature colonize a new common environment. Moreover, when this new environment is stable, the evolution of plasticity may provide valuable information about the underlying costs of plasticity. If plasticity involves costly mechanisms, selection in a constant environment is expected to cause a decay of the underlying function, leading to loss of performance in nonselected environments (trade-offs across environments). Alternatively, relaxed selection may lead to mutational degradation in the nonselected environments (Hall and Colegrave 2008; Ketola et al. 2013; Murren et al. 2015), again causing loss of plasticity, though at a much slower evolutionary pace. In this context it is also relevant to study whether populations of different genetic backgrounds differ in plasticity costs.

Drosophila subobscura provides an excellent example of the action of natural selection at a worldwide scale, with documented parallel clinal variation in body size and chromosomal inversion frequencies on three continents (Balanyà et al. 2003; Gilchrist et al. 2004; Rezende et al. 2010). Also, this species presents clear genetic responses to temperature (Rodríguez-Trelles et al. 1996, 2013; Rodríguez-Trelles and Rodríguez 1998; Balanyà et al. 2006; Rezende et al. 2010; Calabria et al. 2012; Castañeda et al. 2015). Moreover, in an experimental evolution study, Santos et al. (2006, Santos 2007) observed a clear response to selection at different

constant temperatures. When assaying these populations at other temperatures, a reduced performance was observed, in particular for flies adapted to the cooler environment (Santos 2007). This suggests costs of local adaptation. However, several questions were not tackled by this experiment, such as what happens to the thermal plasticity of populations highly differentiated in nature that evolve in a common, stable environment? Will plasticity differ initially between populations, due to local adaptation to the previous natural environment? Are patterns of thermal plasticity similar across different life stages (i.e., juvenile vs. adult)? Will populations lose plasticity during evolution in a homogeneous environment?

To answer these questions, we collected *D. subobscura* flies from three locations along the European latitudinal cline to found three laboratory populations, and maintained all in the same new environment at constant 18ºC. We have previously reported fast convergence for these populations in several phenotypic traits (Fragata et al. 2014b). Nevertheless, convergence was not attained at the inversion frequency level (Fragata et al. 2014a) or in behavioral traits (Bárbaro et al. 2015). In the present study, we assayed these laboratory populations in both the temperature where they evolved (18ºC) and at a lower and a higher temperature (13ºC and 23ºC), to characterize the evolution of the thermal reaction norms in several juvenile and adult traits.

One clear expectation is that populations along a cline should be adapted to different environments (Kawecki and Ebert 2004; Savolainen et al. 2013). Considering the clinal variation for *D. subobscura*, we predict that initially northern populations (from colder environments) will perform better at lower temperatures, and the opposite pattern for southern populations. This will lead to differences in the shape of the thermal reaction norm across populations. As populations adapt to the homogeneous laboratory environment, we expect an increase in performance at 18ºC, and smaller increases (due to general adaptation) or even decreases (e.g., due to costs across environments) at other temperatures. Thus, temporal changes in the shape of the reaction norm are expected. Convergence of reaction norms may be attained if similar effects of laboratory adaptation or costs of plasticity are involved. However, if performance across temperatures is strongly correlated, differences in the shape of the reaction norms may not occur. With this setting, we intend to shed light on how adaptation to new environments affects the plastic response of historically differentiated populations and how this ability evolves under a similar thermal regime.

Material and Methods

FOUNDING AND MAINTENANCE OF POPULATIONS

Drosophila subobscura samples were collected in August 2010 from three low-altitude sites along the European latitudinal cline: Adraga (Portugal), Montpellier (France), and Groningen (Netherlands), from which three populations were derived in the laboratory (see Fragata et al. 2014b). Wild females were kept in separate vials with their offspring in the following two generations to equalize family contributions. During these generations inbreeding was avoided by crossing females with males from different vials (first laboratory generation), or derived from a random sample from all vials (second generation). In the third generation an equal number of offspring of each female were randomly mixed, giving rise to the outbred populations. In the fourth generation eggs collected were divided in three equal parts to originate replicate populations (e.g., Ad_1, Ad_2, and Ad_3 from the Adraga population). Three long-established populations founded from a collection in Adraga in 2001 were used as controls (TA—formerly "TW" populations—Simões et al. 2008) and assayed synchronously with the experimental populations. These populations were in the 115th generation at the time of the founding of the new populations.

All populations were maintained under the same conditions with synchronous discrete generations of 28 days, census sizes between 500 and 1200 individuals, 12L:12D at 18°C. Flies were kept in vials with controlled density of eggs (around 70 eggs per vial) and adults (50 adults per vial). For each population flies emerging in the first four to five days from a total of 24 vials were randomized using CO_2 anesthesia. Eggs were collected when flies were seven to 10 days old (around the age of peak fecundity) to found the following generation (see also Matos et al. 2004; Simões et al. 2007, 2008; Santos et al. 2012, 2013).

ADULT LIFE-HISTORY TRAITS ASSAYS

Adults were assayed at generations 6, 14, and 28 after laboratory foundation. Initial sample sizes varied between 18 and 24 mated pairs per replicate population, temperature, and assay. On the day of adult emergence at 18ºC three sets of mating pairs were placed into incubators at one of the three temperatures 13ºC, 18ºC, and 23ºC. For the first 12 days of the assay, flies were transferred daily into fresh medium and the number of eggs laid per female was counted. On the 12^{th} day, the flies were transferred to agar medium and starvation resistance was assessed. With this assay we estimated three fecundity-related traits: age of first reproduction (number of days between emergence and the first egg laying), early fecundity (total number of eggs laid during the first week of life), and peak fecundity (total number of eggs laid between days 8 and 12). Female and male starvation resistance was estimated as the number of hours until death (registered every 6 hours after transfer to agar). We also measured body size for assayed females at generations 6 and 28, using wing size estimated by geometric morphometric analysis (Dryden and Mardia 1998). Thirteen morphological landmarks of the wing were recorded with the Fly Wing 15Lmk plug-in implemented in IMAGEJ 1.33u software

(http://rsb.info.nih.gov/ij/). Wing size was estimated as centroid size, defined as the square root of the sum of the 26 squared Euclidian distances of the 13 landmarks to the centroid (see Santos et al. 2005; Fragata et al. 2010; Simões et al. 2015).

JUVENILE LIFE-HISTORY TRAITS ASSAYS

Juvenile life-history traits were assayed at generations 5 and 19. Sample sizes varied between eight and ten vials per replicate population, generation and temperature. Sixty eggs per vial were collected over a 6 hours period at 18°C. After 12, 18, and 29 days at 23°C, 18°C, and 13°C, respectively, vials where checked for emergences three times per day (9 a.m., 2 p.m., and 7 p.m.) for 10 days. The number of emerging males and females was scored. Female and male development times (FDT and MDT, respectively) were calculated as the mean total number of hours from egg to emergence of all flies in a given vial. Viability was estimated for each vial as the ratio between the number of emerged flies and the total number of eggs (60). Female and male development time and viability of a given replicate population were estimated by the average value across vials.

ANALYSIS OF REACTION NORMS FOR ADULT AND JUVENILE LIFE-HISTORY TRAITS

Preliminary analyses were performed to find the best model describing the reaction norms in our latitudinal populations. For each generation and latitudinal population, we compared linear and linear-log models, that is, where temperature (the predictor, independent variable) was either not transformed (linear model) or log-transformed (linear-log model). In general, the best model, defined by the higher R^2 was the linear-log (see Table S1). In all analyses viability data were arcsine transformed.

For both adult and juvenile life-history traits, the following analyses of covariance (ANCOVA) were used in each generation to test for the presence of plasticity.

$$Y = \mu + Rep + Temp + Rep \times Temp + \varepsilon, \quad (1)$$

where Y refers to the trait analyzed, *Rep* is the random factor replicate population (i.e., the three replicated populations), and *Temp* is the logarithm ($\log_{10}$) of the temperature used as covariate.

Nested ANCOVAs were also performed at each generation to test for differences among latitudinal populations (i.e., Adraga, Montpellier, and Groningen) in the reaction norms of both adult and juvenile life-history traits:

$$Y = \mu + Pop + Rep\{Pop\} + Temp + Pop \times Temp + Rep\{Pop\} \times Temp + \varepsilon, \quad (2)$$

where Y refers to the trait analyzed, *Pop* refers to the fixed factor "latitudinal population" (the three categories being Adraga, Montpellier, and Groningen), and $Rep\{Pop\}$ refers to the random factor replicate nested in each latitudinal population. Whenever the $Pop \times Temp$ interaction was significant, pairwise ANCOVAs between populations were performed with the same model. Additionally, pairwise comparisons between latitudinal populations were performed at each temperature at generation 6, applying false discovery rate corrections (FDR—Benjamini and Yekutieli 2001).

To analyze the evolution of plastic response across generations, we used as datapoints differences between the latitudinal populations and the average of control TA populations assayed in synchrony. Because the latter populations are long established in the laboratory and thus assumed to be in evolutionary equilibrium, this is a common procedure allowing to remove undesired environmental effects of asynchronous assays between generations (e.g., Matos et al. 2002; Simões et al. 2008). The following nested ANCOVA model was applied for all studied traits:

$$Y = \mu + Pop + Rep\{Pop\} + Temp + Gen + Pop \times Temp + Pop \times Gen + Rep\{Pop\} \times Temp + Rep\{Pop\} \times Gen + Temp \times Gen + Pop \times Gen \times Temp + Rep\{Pop\} \times Gen \times Temp + \varepsilon, \quad (3)$$

where *Gen* refers to generation analyzed as a covariate (to estimate evolutionary rates). Whenever the $Pop \times Gen \times Temp$ interaction was significant, pairwise comparisons between latitudinal populations were performed.

To test the effect of body size on the performance of adult flies at different temperatures, we used ANCOVAs including as covariate the centroid size, plus all relevant interactions, using data of assayed females of generations 6 and 28. To be coherent with previous analyses on body size using these populations (Fragata et al. 2014b), centroid size was log transformed, but the conclusions were not affected by this transformation.

For developmental time, we additionally tested for differences between sexes in the reaction norms at each generation and across generations. To account for possible confounding effects of the covariate by factor interactions on main factors, we standardized the covariate temperature using the differences to the grand mean in all datasets used. Because these analyses produced similar results as those without standardization, we present only the results of the latter. Additionally, we performed analyses with temperature as a fixed factor, which yielded similar results as the linear-log model. All analyses were performed using Statistica 8.0 (StatSoft 2007).

Results

INITIAL VARIATION OF REACTION NORMS ACROSS POPULATIONS

Adult traits

All latitudinal populations presented a plastic response to temperature at the initial sixth generation, with higher fecundity, lower

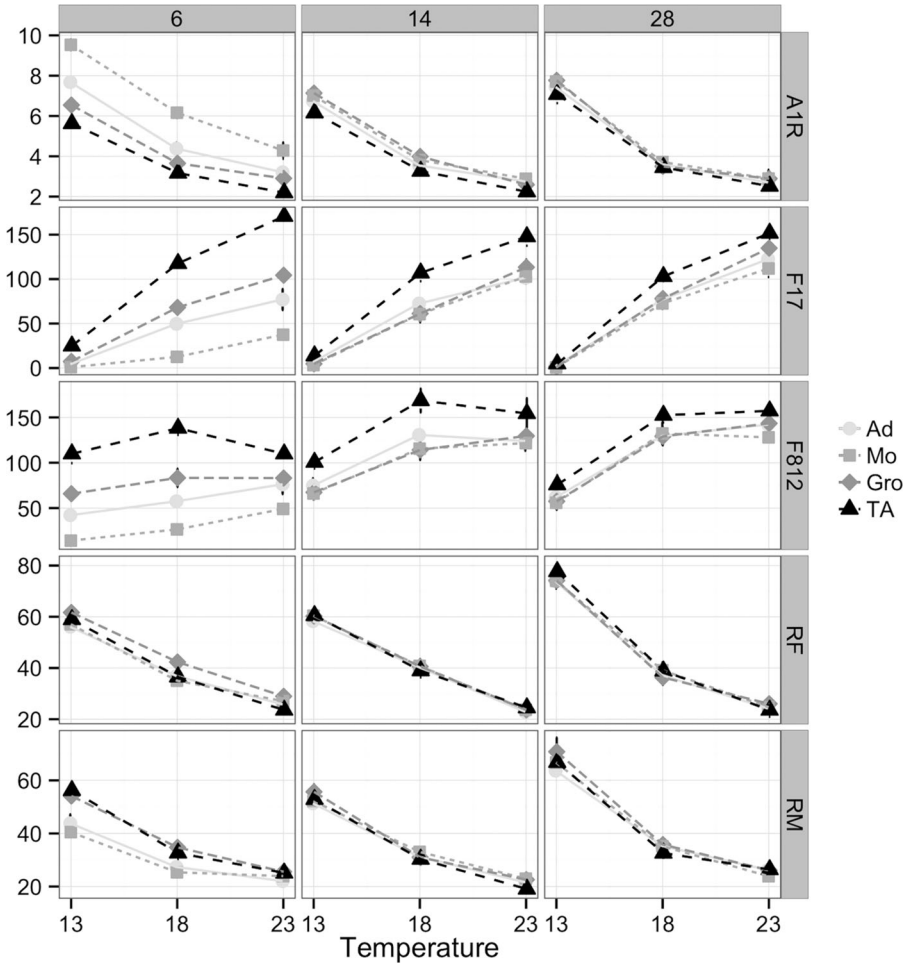

Figure 1. **Thermal reaction norms for adult traits of all latitudinal populations (Ad, Mo, Gro, and TA—controls) in the three assayed generations. A1R, age of first reproduction; F17, early fecundity; F812, peak fecundity; RF, female starvation resistance; RM, male starvation resistance. Error bars correspond to variation between replicate populations. Ad, light gray circles, solid line; Mo, gray squares, dotted line; Gro, dark gray diamonds, dashed line; TA, black triangles, dashed line.**

starvation resistance, and lower age of first reproduction at warmer temperatures (Fig. 1; Table S2—temperature $\log_{10}$-transformed). Comparisons between latitudinal populations indicated significant differences in the reaction norms for early fecundity and male starvation resistance (*Pop* × *Temp*—Table 1). Specifically, for early fecundity Montpellier showed a lower plastic response (Fig. 1, Table S3a), having also a lower mean fecundity across temperatures (Fig. 1, *Pop*—Table 1). Similarly, Montpellier males showed lower plasticity for starvation resistance, particularly compared to Groningen (Fig. 1; Table S3a), as well as lower overall starvation resistance (Fig. 1, *Pop*—Table 1). Differences between latitudinal populations in the overall performance across temperatures were also found for age of first reproduction (*Pop*—Table 1). In general, Groningen presented higher fecundity and starvation resistance across all temperatures, whereas Montpellier in general performed the worst (Fig. 1, Table S3b).

Because body size in Groningen was significantly larger than in the other latitudinal populations (Fig. S1), we performed the same analyses for female traits defining body size as covariate. A significant effect of female size on the reaction norms for early fecundity at generation 6 was found (*Size* × *Temp*—Table S4). On the other hand, overall performance and the reaction norms were no longer significantly different between populations (*Pop* and *Pop* × *Temp*—Table S4). However, the effect of body size on the reaction norm did not differ between latitudinal populations (*Pop* × *Size* × *Temp*—Table S4).

Juvenile traits

For all juvenile traits, a plastic response was observed across latitudinal populations at generation 5, with longer development time and lower viability at 13°C (Figs. 2, S2, and S3; Table S5). Nevertheless, for viability only Montpellier showed a significant

Table 1. Analyses of differences in thermal reaction norms in adult traits (ANCOVA) at each generation among populations (Adraga, Montpellier, and Groningen). Temperature (log transformed) is defined as a covariate in the analysis.

Generation	Model parameters	Age of first reproduction	Early fecundity	Peak fecundity	Female starvation resistance	Male starvation resistance
6	*Pop*	$F_{2,6.01} = 9.025^{*}$	$F_{2,6.00} = 14.760^{**}$	$F_{2,6.00} = 3.981$ m.s.	$F_{2,6.00} = 0.968$ n.s.	$F_{2,6.00} = 6.974^{*}$
	Temp	$F_{1,4.35} = 496.473^{***}$	$F_{1,6.00} = 246.338^{***}$	$F_{1,6.01} = 50.033^{***}$	$F_{1,6.01} = 972.78^{***}$	$F_{1,6.00} = 280.194^{***}$
	Pop × *Temp*	$F_{2,6.01} = 5.071$ m.s.	$F_{2,6.00} = 16.965^{**}$	$F_{2,6.01} = 1.687$ n.s.	$F_{2,6.00} = 0.580$ n.s.	$F_{2,6.00} = 6.150^{*}$
14	*Pop*	$F_{2,6.01} = 1.378$ n.s.	$F_{2,6.00} = 0.978$ n.s.	$F_{2,6.01} = 0.475$ n.s.	$F_{2,6.01} = 0.447$ n.s.	$F_{2,6.00} = 4.156$ m.s.
	Temp	$F_{1,6.01} = 1065.48^{***}$	$F_{1,6.00} = 663.126^{***}$	$F_{1,6.01} = 117.531^{***}$	$F_{1,6.02} = 2500.09^{***}$	$F_{1,6.00} = 2624.58^{***}$
	Pop × *Temp*	$F_{2,6.01} = 1.347$ n.s.	$F_{2,6.00} = 0.773$ n.s.	$F_{2,6.01} = 0.340$ n.s.	$F_{2,6.02} = 0.400$ n.s.	$F_{2,6.00} = 4.895$ m.s.
28	*Pop*	$F_{2,6.02} = 0.128$ n.s.	$F_{2,6.02} = 3.519$ m.s.	$F_{2,6.02} = 0.523$ n.s.	$F_{2,6.02} = 0.089$ n.s.	$F_{2,6.01} = 1.130$ n.s.
	Temp	$F_{1,6.02} = 804.011^{***}$	$F_{1,6.02} = 1302.6^{***}$	$F_{1,6.02} = 294.732^{***}$	$F_{1,6.02} = 1895.5^{***}$	$F_{1,6.01} = 429.092^{***}$
	Pop × *Temp*	$F_{2,6.02} = 0.081$ n.s.	$F_{2,6.02} = 3.366$ n.s.	$F_{2,6.02} = 0.607$ n.s.	$F_{2,6.02} = 0.116$ n.s.	$F_{2,6.01} = 1.129$ n.s.

Note: Significance levels: n.s. $P > 0.1$; m.s $0.1 > P > 0.05$; *$0.05 > P > 0.01$; **$0.01 > P > 0.001$; ***$P < 0.001$.

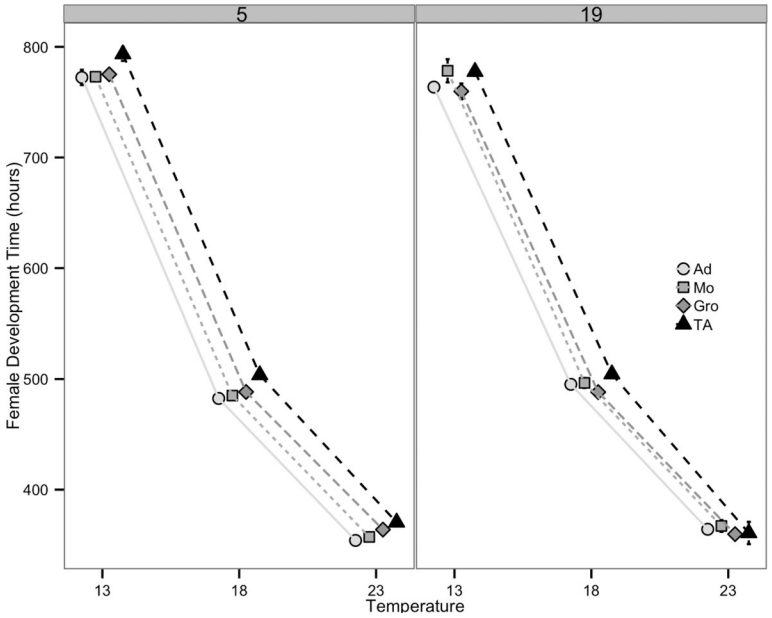

Figure 2. Thermal reaction norms for female development time of all latitudinal populations (Ad, Mo, Gro, and TA—controls) in the two assayed generations. Error bars correspond to variation between replicate populations. Ad, light gray circles, solid line; Mo, gray squares, dotted line; Gro, dark gray diamonds, dashed line; TA, black triangles, dashed line.

plastic response (Table S5b). Interestingly, Groningen presented in general longer development times both for females and males across all temperatures (Figs. 2 and S2). Pairwise comparisons at each temperature only indicated significantly longer development time in Groningen females relative to Adraga at 18ºC ($F_{(1,4.07)} = 12.856$, $P = 0.022$). At generation 5 there were significant differences in the reaction norms between sexes, with a longer development time for females at both 13°C and 18°C and no differences between males and females at 23°C (*Temp* × *Sex*—Table S6). Overall, whether including sex or not, no differences between latitudinal populations were found for the reaction norms of juvenile traits at generation 5 (*Pop* × *Temp*—Tables S5a and S6).

EVOLUTION OF REACTION NORMS

Adult traits

When analyzing each latitudinal population separately, we observed evolution of plasticity for all fecundity traits in Adraga, for early and peak fecundity and male starvation resistance in Montpellier, and for early fecundity in Groningen (Table S7). Analyzing reaction norms across populations between generations, we found significant evolutionary changes for all traits except female starvation resistance (*Temp* × *Gen*—Table 2). This corresponds to a general convergence toward the control (TA) in the shape of the reaction norm, except for early fecundity in Adraga and Montpellier (Fig. 1, Table S8). Given the absence of significant temporal changes in the reaction norm of the controls

Table 2. Analyses of differences in thermal reaction norms in adult traits (ANCOVA) across generations.

Model parameters	Age of first reproduction	Early fecundity	Peak fecundity	Female starvation resistance	Male starvation resistance
Pop	$F_{2,6.00} = 11.666^{**}$	$F_{2,6.00} = 10.456^{*}$	$F_{2,6.01} = 10.265^{*}$	$F_{2,6.00} = 1.763$ n.s.	$F_{2,6.00} = 5.218^{*}$
Gen	$F_{1,6.01} = 25.180^{**}$	$F_{1,6.01} = 71.916^{***}$	$F_{1,6.02} = 79.570^{***}$	$F_{1,6.01} = 1.696$ n.s.	$F_{1,6.00} = 17.804^{**}$
Temp	$F_{1,6.01} = 35.371^{**}$	$F_{1,6.00} = 280.639^{***}$	$F_{1,6.02} = 96.917^{***}$	$F_{1,6.01} = 6.676^{*}$	$F_{1,6.00} = 41.348^{***}$
Temp × *Gen*	$F_{1,6.01} = 11.686^{*}$	$F_{1,6.00} = 87.137^{***}$	$F_{1,6.02} = 45.014^{***}$	$F_{1,6.01} = 0.946$ n.s.	$F_{1,6.01} = 17.149^{**}$
Pop × *Gen*	$F_{2,6.01} = 8.379^{*}$	$F_{2,6.00} = 3.697$ m.s.	$F_{2,6.02} = 7.133^{*}$	$F_{2,6.01} = 1.200$ n.s.	$F_{2,6.00} = 1.916$ n.s.
Pop × *Temp* × *Gen*	$F_{2,6.01} = 4.465$ m.s.	$F_{2,6.00} = 4.391$ m.s.	$F_{2,6.02} = 4.396$ m.s.	$F_{2,6.01} = 0.877$ n.s.	$F_{2,6.00} = 2.006$ n.s.

Note: Significance levels: n.s. $P > 0.1$; m.s $0.1 > P > 0.05$; *$0.05 > P > 0.01$; **$0.01 > P > 0.001$; ***$P < 0.001$.

(Table S7), we can interpret in evolutionary terms the absolute performance changes of the experimental populations. In general, temporal changes in the shape of the reaction norms correspond to a reduction in differences between 18°C and 23°C (Fig. 1). The evolution of reaction norms did not differ significantly across latitudinal populations, although marginal effects were found for age of first reproduction, early, and peak fecundity (*Pop* × *Temp* × *Gen*—Table 2). Nevertheless, convergence in the shape of the reaction norms was attained for early fecundity and male starvation resistance, with differences found at generation 6 no longer being observed at generations 14 and 28. For the other traits, the shape of reaction norms did not differ between populations in any generation (*Pop* × *Temp*—Table 1). Also, an increase in the overall performance across all temperatures (vertical up shifts in the reaction norm) was observed for fecundity traits and male starvation resistance (*Gen*—Table 2, Fig. 1). The observed differences between latitudinal populations across temperatures at generation 6 (mostly due to Groningen flies—Table S3b) were not observed at generations 14 and 28 (*Pop*—Table 1).

Although differences in female body size were significant between populations at generation 28, their magnitude was reduced relative to generation 6, particularly the difference between Groningen and the other two populations (Fig. S1). Analyses taking into account female size using the data of generation 28 did not lead to different conclusions from analyses without this covariate (Table S4). Interestingly, and in contrast with generation 6, at generation 28 body size did not affect significantly the reaction norms for early fecundity (*Size* × *Temp*—Table S4).

Juvenile traits

In general no evolutionary changes were found for the reaction norms of juvenile traits, either across populations or for each of them (Figs. 2, S2, and S3; *Temp* × *Gen*—Table S9a). Nevertheless, contrary to generation 5, at generation 19 viability did not show plasticity across populations (Fig. S3, *Temp*—Table S5a). In particular, Montpellier presented a significant reaction norm for viability at generation 5 that was no longer observed at generation 19 (Table S5b). This may explain the significant interaction term *Pop* × *Temp* × *Gen* for this trait (Table S9a). Despite this, no comparisons between pairs of populations presented significant differences in the evolution of reaction norms (Table S9a). Additionally, at generation 19 differences in reaction norms between populations were not significant, as noted at generation 5 (*Pop* × *Temp*—Table S5a). Also, no evolutionary changes were found for any juvenile trait (*Gen*—Table S9).

When analyzing together development time of males and females, no general evolutionary changes in plasticity or between populations were found (ANCOVA $P > 0.05$). Curiously, a significant interaction term *Temp* × *Gen* × *Sex* was found ($F_{(1,7.13)} = 11.965$, $P = 0.010$). Overall, there were no clear indications of evolutionary changes either of mean performance across temperatures or reaction norms for juvenile traits.

Discussion

SIMILAR THERMAL REACTION NORMS OF ADULT TRAITS ACROSS EVOLVING POPULATIONS

Evolution in a constant, homogeneous environment led to a general convergence in the shape of the thermal reaction norms of early fecundity and male starvation resistance in the initially differentiated populations. Evolutionary convergence at 18ºC had already been found in these populations for a wide range of adult traits, erasing the initial historical differentiation between them (see Fragata et al. 2014b). Here we found that convergence between populations also occurs across other temperatures, to which they are not directly adapting.

Similar temporal changes across latitudinal populations in the shape of the reaction norm were observed for peak fecundity and age of first reproduction. These reaction norms did not differ between populations in any generation, implying parallel evolution of plasticity. In particular, peak fecundity presented the most conspicuous changes, evolving toward similarity of performance between 18ºC and 23ºC, possibly due to a weak correlation between temperatures. Because populations are evolving at 18ºC, it is expected that their performance increases at that temperature, and the lack of association between temperatures (in this

case 18ºC and 23ºC) would lead to a reduction of differences in performance between them. Alternatively, pleiotropic effects between environments might occur (as expected due to correlated responses at warmer temperatures—Huey et al. 1991; Berger et al. 2013), coupled with a limited increase in performance at 23ºC due to physiological constraints. It might be the case that the changes that we observed in the shape of the thermal reaction norms for fecundity were due to the evolution of body size. This hypothesis was discarded, as taking into account the effect of body size the plasticity changes across generations remained significant (data not shown).

Altogether, the evolution of thermal reaction norms in our study seems more associated with vertical shifts in mean trait values (i.e., overall increased performance across temperatures), rather than changes in shape (i.e., evolution of plasticity). Our findings are thus in agreement with other population comparisons from contrasting geographical origins that show vertical shifts in thermal reaction norms (Yamahira et al. 2007; Klepsatel et al. 2013). They are also in agreement with some experimental evolution studies where the evolution in different thermal regimes led to changes in trait means rather than in the shape of reaction norms (Ketola et al. 2013; Manenti et al. 2015). Vertical shifts are likely more common than changes in shape, as the latter stem from a more evolutionary conserved response related to the structural and physiological nature of enzymes (Huey and Kingsolver 1989; Somero 2004; Angilletta 2009; see also Berger et al. 2013).

HOW DOES THERMAL PLASTICITY EVOLVE IN A CONSTANT BENIGN ENVIRONMENT?

Theory predicts that in a stable environment plasticity with costs should be lost for traits more loosely related to fitness (DeWitt 1998; Hall and Colegrave 2008; Auld et al. 2010; Murren et al. 2015). On the other hand, for fitness, the expectations are quite the opposite, with evolution leading to loss of homeostatic mechanisms that promoted similar performance across environments (Richards et al. 2006). As a result, a higher performance would be expected in the selected environment, relative to other environments. These expectations can be tested by analyzing the evolution of plasticity when populations recently introduced from nature are subjected to a constant laboratory regime, as done here. The temporal patterns obtained here for the reactions norms do not support the above-mentioned expectations, as a performance peak at 18ºC did not evolve as a result of adaptation to that temperature. In fact, performance at 23ºC is always greater or similar to 18ºC for fecundity traits, in agreement with the notion that *D. subobscura* "likes it warm" (Santos 2007). Also, the long-established laboratory populations do not show a reduction in performance at 23ºC relative to 18ºC either, thus indicating no long-term costs of plasticity or local adaptation, at least at warmer temperatures. Interestingly, the evolution of a plateau between 18ºC and 23ºC for peak fecundity suggests possible limits of plasticity, rather than costs (Auld et al. 2010; Murren et al. 2015).

Several possibilities might explain the general absence of costs in our study. It is possible that these might only manifest at extreme boundaries of the thermal niche or underlimiting resources (see Auld et al. 2010; Berger et al. 2013; Condon et al. 2015). Moreover, the lack of detection of plasticity costs may be partly due to the fact that they have been looked for in the wrong place, for example, developmental instability may be the cost that more plastic genotypes pay (see Tonsor et al. 2013). It is worth noting that the detection of high costs of plasticity is infrequent (see reviews in Auld et al. 2010; Murren et al. 2015). Also, the evolution of specialized genotypes in distinct, constant thermal environments has not occurred consistently in experimental studies (see Ketola et al. 2013; Berger et al. 2014; Condon et al. 2014). Moreover, if plasticity evolved as a by-product of selection on trait values rather than through specific "plasticity genes," as our data suggest, the detection of costs of plasticity might not be straightforward (Auld et al. 2010). Nevertheless, it is important to point out that any plasticity observed, especially with regard to temperature, can result simply as a nonadaptive consequence of fundamental physiological or thermodynamic phenomena. Even one of the most clear forms of phenotypic plasticity, the relationship between body size and temperature, the so-called temperature-size rule (Atkinson 1994; Angilletta and Dunham 2003), has both adaptive and mechanistic explanations, being unclear whether this pattern is produced by natural selection or shared physiological constraints. In other words, not all observed plasticity can be straightforwardly equated to selective processes.

In contrast to adult traits, no changes in juvenile thermal plasticity were found during evolution in the new environment. This finding, coupled with the general absence of an evolutionary response in juvenile traits, suggests effects of evolutionary trade-offs with adult traits (namely between development time and fecundity), or generally low genetic variability limiting adaptive changes in juvenile traits.

HISTORICAL DIFFERENCES IN ADULT THERMAL PLASTICITY DO NOT REFLECT LOCAL ADAPTATION TO TEMPERATURE

Our study shows initial variation in thermal plasticity for early fecundity and male starvation resistance across European *D. subobscura* populations from various latitudes. Although this variation might be expected from historical evolution in likely thermally contrasting environments in nature, our results do not reflect the expected effect of local adaptation in the thermal response. In fact, Groningen from the cooler, higher latitude, in general performed best across temperatures (particularly in comparison with Montpellier) in terms of age of first reproduction, early and peak fecundity, and male starvation resistance. These results are in

contrast with the expectations of the "Hotter is better" hypothesis (Angilletta et al. 2010). Nevertheless, differences between the laboratory environment and nature may lead to inconsistencies between expectations from local adaptation and what we observe.

The better performance of Groningen in both early fecundity and starvation resistance across temperatures suggests better resource acquisition of these populations in the new (laboratory) environment (Service and Rose 1985). Another nonexclusive explanation is body size. The bigger body size of Groningen females may have contributed to their overall better performance, as differences between populations are no longer significant when taking into account its effect. Moreover, when removing the effect of body size, the reaction norms of early fecundity were no longer different between populations. This could be explained by the general positive association between body size and fecundity, increasing in magnitude between temperatures, with bigger flies (Groningen) performing better. Thus, bigger was always better across all temperatures, contrary to expectations of the adaptive evolution of the body size cline (Blanckenhorn and Demont 2004). In contrast, other studies have shown that bigger flies performed better only at lower temperatures (McCabe et al. 1997; Reeve et al. 2000; Bochdanovits and de Jong 2003).

The emergence of a better genotype across a range of temperatures has been shown in some other studies addressing thermal responses across distinct populations (e.g., Yamahira et al. 2007; Berger et al. 2013; Ketola et al. 2014). However, this is not a general finding, as studies using *D. melanogaster* have found variation in thermal plasticity for fecundity in differentiated populations but not better genotypes across environments (Trotta et al. 2006; Klepsatel et al. 2013). The fact that these latter studies involved populations from more contrasting environments (temperate vs. tropical environments) might explain their greater variation in thermal plasticity in comparison with our study. Also, the lower range of temperatures tested in our study, in comparison with Trotta et al. (2006) and Klepsatel et al. (2013), could have facilitated the occurrence of a better genotype across temperatures.

REDUCED ROLE OF HISTORICAL DIFFERENCES IN THERMAL PLASTICITY OF JUVENILE TRAITS

Contrary to the patterns observed for adult traits, development time and viability did not show any initial variation in thermal plasticity across populations. Similar to our results, Trotta et al. (2006) also found differences in thermal plasticity across differentiated populations of *D. melanogaster* for fecundity but not juvenile traits. However, studies in other species have found varying thermal reactions norms for development time across differentiated populations (Van't Land et al. 1999; Liefting et al. 2009; Berger et al. 2013). It is possible that general lack of genetic variation within populations and/or trade-offs between development time with other (namely adult) traits might be constraining the plastic response of juvenile traits (Murren et al. 2015). Although no differences between populations were found in thermal reaction norms for juvenile traits, this does not imply that development at several temperatures could not affect differently adult performance of our populations (see Gerken et al. 2015).

The overall longer development times of Groningen flies might reflect some degree of geographic differentiation, with longer development time at higher latitudes. It can be argued that this might relate to the bigger size of Groningen flies. In fact, within populations, developmental time and body size are positively correlated in many organisms including *Drosophila* (e.g., Partridge and Fowler 1993). However, the relationship between body size and developmental time along a latitudinal gradient is not straightforward (see Blanckenhorn and Demont 2004). Also, geographical patterns for development time in *Drosophila* are not clear, with some studies using *D. melanogaster* suggesting longer development times at lower latitudes (James et al. 1995; Van't Land et al. 1999), whereas others do not find this pattern (James et al. 1997; see Santos et al. 2006 for a brief review in *D. birchii* and *D. serrata*). Finding different reaction norms between our populations for adult but not juvenile traits suggests that the evolutionary history experienced in nature has shaped differently the thermal response of life stages. This highlights the importance of considering such differing responses to understand the evolutionary consequences of climate change (Kingsolver et al. 2011).

CONCLUSION

In conclusion, we found that populations from distinct geographical locations along the European *D. subobscura* cline present differential thermal plasticity for relevant adult traits. Evolution in a new, common environment led to convergence of these plastic responses for some adult traits, whereas other showed parallel evolution. In general this evolution occurred mostly through vertical shifts likely as a result of laboratory adaptation. We also found that thermal plasticity patterns are trait specific, with juvenile traits presenting no variation in plastic response between populations or across generations. Thus, our study highlights the need to analyze a wide range of traits and life stages to fully understand the genetic and physiological mechanisms that shape plastic evolutionary responses. Importantly, our study shows that evolution in a new, constant environment does not appear to have reduced the ability of populations to respond plastically to different environmental challenges, keeping their options open for tolerating rarely occurring environments.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. R^2 values for the linear and linear-log ANCOVA models for each latitudinal population and generation for adult (a) and juvenile (b) traits
Table S2. Analyses of thermal reaction norms in adult traits (ANCOVA) at each generation for each latitudinal population.
Table S3. Pairwise comparisons in adult traits at generation 6 (a) between reaction norms; (b) at each temperature.
Table S4. Analyses of thermal reaction norms in adult traits (ANCOVA) accounting for size variation, at each generation among populations (Adraga; Montpellier, and Groningen).
Table S5. Analyses of differences in thermal reaction norms in juvenile traits (ANCOVA) at each generation among populations (a) and at each generation for each population (b).
Table S6. Analyses of differences in reaction norms of development time between sexes at each generation.
Table S7. Analyses of evolution in reaction norms of adult traits, for each latitudinal population and the controls (*Temp* × *Gen*).
Table S8. Pairwise comparisons of the reaction norms for adult traits between populations and controls at each generation.
Table S9. Analyses of evolution in reaction norms of juvenile traits between populations (a) and for each population and the controls (b).
Figure S1. Female body size (log-transformed) of all populations (Ad, Mo, Gro, and TA—controls) in the two assayed generations (6 and 28).
Figure S2. Thermal reaction norms for male development time of all latitudinal populations (Ad, Mo, Gro, and TA—controls) in the two assayed generations.
Figure S3. Thermal reaction norms for viability of all latitudinal populations (Ad, Mo, Gro, and TA—controls) in the two assayed generations.