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Letter

Continent-wide differentiation of fitness traits and patterns of climate adaptation among European populations of *Drosophila melanogaster*

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Abstract

A particularly well-studied evolutionary model is the vinegar fly *Drosophila melanogaster*, a cosmopolitan insect of ancestral southern-central African origin. Recent work suggests that it expanded out of Africa ~9,000 years ago, and spread from the Middle East into Europe ~1,800 years ago. During its global expansion, this human commensal adapted to novel climate zones and habitats. Despite much work on phenotypic differentiation and adaptation on several continents (especially North America and Australia), typically in the context of latitudinal clines, little is known about phenotypic divergence among European populations. Here, we sought to provide a continent-wide study of phenotypic differentiation among European populations of *D. melanogaster*. In a consortium-wide phenomics effort, we assayed 16 fitness-related traits on a panel of 173 isofemale lines from 9 European populations, with the majority of traits measured by several groups using semi-standardized protocols. For most fitness-related traits, we found significant differentiation among populations on a continental scale. Despite inevitable differences in assay conditions among labs, the reproducibility and hence robustness of our measurements were overall remarkably good. Several fitness components (e.g., viability, development time) exhibited significant latitudinal or longitudinal clines, and populations differed markedly in multivariate trait structure. Notably, populations experiencing higher humidity/rainfall and lower maximum temperature showed higher viability, fertility, starvation resistance, and lifespan at the expense of lower heat-shock survival, suggesting a pattern of local adaptation. Our results indicate that derived populations of this tropical fly have been shaped by pervasive spatially varying multivariate selection and adaptation to different climates on the European continent.

Keywords: phenotypic variation, fitness traits, population differentiation, adaptation, *D. melanogaster*, Europe

Introduction

Over the last century, the vinegar fly *Drosophila melanogaster* has emerged as a premier experimental model system for studying various aspects of evolution, especially the process of adaptation and its genetic and phenotypic basis (Casillas & Barbadilla, 2017; David & Capy, 1988; David et al., 2004; Flatt, 2020; Hedrick & Murray, 1983; Hoffmann & Weeks, 2007; Keller, 2007; Lachaise & Silvain, 2004; Lachaise et al., 1988; Lewontin, 1974; Parsons, 1975; Powell, 1997; Prasad & Joshi, 2003). In particular, over the past decade, many population genomics studies have improved our understanding of the demography and adaptation in natural populations of this species (e.g., Barghi et al., 2019; Bergland et al., 2014, 2016; Chen et al., 2024; Coughlan et al., 2022; Fabian et al., 2012; Garud et al., 2021; Grenier et al., 2015; Huang et al., 2014; Kapun et al., 2020, 2021; Kolaczkowski et al., 2011; Lack et al., 2015, 2016; Langley et al., 2012; Machado et al., 2021; Mackay et al., 2012; Pool et al., 2012; Turner et al., 2008), as well as using experimentally evolved populations (e.g., Burke et al., 2010; Fabian et al., 2018; Graves et al., 2017; Hoedjes et al., 2019; Kawecki et al., 2021; Orozco-terWengel et al., 2012; Schlötterer et al., 2015; Turner et al., 2011).

This cosmopolitan insect originated in the seasonally dry Miombo and Mopane woodlands of tropical southern-central Africa before expanding across and then out of the African continent, causing a bottleneck in population size (Coughlan et al., 2022; Lachaise & Silvain, 2004; Lachaise et al., 1988; Mansourian et al., 2018; Nunes et al., 2008; Pool et al., 2012; Sprengelmeyer et al., 2020). While previous work has dated the out-of-Africa split time (bottleneck) to have occurred ~12–19k years ago (kya) (Arguello et al., 2019; Duchen et al., 2013; Li & Stephan, 2006; Thornton & Andolfatto, 2006), a new study suggests that the out-of-Africa event occurred ~9 kya (Chen et al., 2024). This out-of-Africa split was followed by an expansion into East Asia ~2.8–4.4 kya (Chen et al., 2024), and a spread from the Middle East into Europe ~1.8 kya (Sprengelmeyer et al., 2020). Australia and North America were colonized even more recently, i.e., only ~100–150 years ago (Hoffmann & Weeks, 2007; Keller, 2007; and references therein).

During this history of expansion and globalization, this ancestrally tropical insect and human commensal adapted to new climate zones and ecological niches, including equatorial tropical rainforests, savanna grasslands, deserts, temperate grasslands, and alpine areas (Fabian et al., 2015; Keller, 2007; Lachaise & Silvain, 2004; Lachaise et al., 1988; Mansourian et al., 2018; Pool et al., 2012 ; Sprengelmeyer and Pool 2021). Indeed, consistent with local adaptation (Kawecki & Ebert, 2004), a large body of work has documented spatially varying selection and clines in *D. melanogaster* populations along latitudinal (and sometimes also altitudinal) gradients on multiple continents (reviewed in Adrion et al., 2015; David & Capy, 1988; de Jong & Bochdanovits, 2003; Flatt, 2020; Hoffmann & Weeks, 2007; Paaby & Schmidt, 2009).

Previous studies of clinality in *D. melanogaster* have established multiple lines of evidence for spatially varying selection: (1) clinal differentiation among populations in fitness traits such as viability, size, pigmentation, lifespan, and reproductive diapause (e.g., Coyne & Beecham, 1987; David, 1982; David & Bocquet, 1975a, 1975b; David et al., 1977, 1985; Fabian et al., 2015; Gibert et al., 2004; Gilchrist & Partridge, 1999; Hangartner et al., 2015; Klepsatel et al., 2014; Pitchers et al., 2013; Rajpurohit & Nevded, 2013; Robinson et al., 2000; Schmidt et al., 2005a, 2005b; Schmidt & Conde, 2006; Van't Land et al., 1999, 2000; Zwaan et al., 2000); (2) clinal genetic variation for individual markers or polymorphisms or at the level of whole genomes (e.g., Agis & Schlötterer, 2001; Bergland et al., 2016; Betancourt et al., 2021; Bogaerts-Márquez et al., 2021; Božičević et al., 2016; Fabian et al., 2012; Kapun et al., 2016a, 2020, 2021, 2023; Kolaczkoswki et al., 2011; Mateo et al., 2018; Oakeshott et al., 1982; Reinhardt et al., 2014; Singh et al., 1982; Turner et al., 2008); and (3) functional relationships between specific clinally varying polymorphisms and fitness-related traits (e.g., Betancourt et al., 2021; Durmaz et al., 2018, 2019; Erickson et al., 2020; Glaser-Schmitt et al., 2021; Kapun et al., 2016b; Lee et al., 2013; Paaby et al., 2010, 2014; Schmidt et al., 2008; Yu & Bergland, 2022).

In addition, several studies have found pervasive genomic and phenotypic evidence for temporally (seasonally) varying selection acting in North American and European populations of *D. melanogaster* (e.g., Behrman et al., 2015, 2018; Bergland et al., 2014; Bitter et al., 2024; Cogni et al., 2015; Kapun et al., 2016a; Machado et al., 2021; Nunez et al., 2024; Rodrigues et al., 2021; Rudman et al., 2022). Together, these studies have greatly advanced our understanding of the mechanisms of spatially and temporally varying selection and the spatio-temporal scale of adaptation.

In contrast to North America (Schmidt et al., 2005a, 2005b), India (Rajpurohit et al., 2017), Australia (Hangartner et al., 2015; Hoffmann & Weeks, 2007), and sub-Saharan Africa (Fabian et al., 2015), however, we still have little systematic knowledge of phenotypic patterns of spatial differentiation and clinal adaptation among European populations of *D. melanogaster* (e.g., Ayrinhac et al., 2004; Draye & Lints, 1996; Draye et al., 1994; Imasheva et al., 1994). For instance, most work on European *D. melanogaster* has examined only a handful of populations and traits (reviewed in Flatt, 2020). Thus, large-scale patterns of phenotypic differentiation, such as phenotypic clines or patterns of local adaptation, remain quite poorly understood for European populations of the vinegar fly.

Here, we sought to address this major knowledge gap by leveraging the collaborative resources and workforce of the European *Drosophila* Population Genomics Consortium, DrosEU (https://droseu.net/) (Figure 1). In our previous work, we provided the first continent-wide analysis of patterns of genetic variation among European populations based on pool-sequencing data from 32 populations (Kapun et al., 2020). In that study, we found evidence for continent-wide selective sweeps and identified many candidate loci for local adaptation, as well as spatial frequency clines for inversion polymorphisms and transposable elements (Kapun et al., 2020; also see Kapun et al., 2021; Machado et al., 2021; Drosophila Evolution over Space and Time consortium [https://dest.bio/]). Yet, patterns of phenotypic differentiation among these European populations remained largely unknown.

To complement our population genomic analyses with information about fitness-relevant phenotypes, we performed a continent-wide phenotypic analysis of representative populations using an isofemale line approach (David et al., 2005; Parsons & Hosgood, 1967; see below). In summer and fall 2018, we sampled nine European populations, spanning 21° latitude and 41° longitude across the continent, and established a panel of 173 isofemale lines (~20 lines per population; Figure 1). In early 2019, lines were shipped to participating research groups, with the bulk of the phenotyping performed in 2019 and 2020. We assayed this DrosEU Phenotyping Panel (DPP) of isofemale lines for 16 traits (this study; see Table 1), most of which represent major phenotypic components of Darwinian fitness, including traits related to growth, size, survival, stress resistance, and reproduction (Flatt, 2020).

Measuring phenotypic traits on isofemale lines, i.e., full-sib families derived from single, inseminated females, is a convenient method for studying quantitative traits and their genetic architecture (David et al., 2005; Parsons & Hosgood, 1967). By "capturing" genotypes from a natural population, isofemale lines provide a useful "proxy" for genetic variation: Over time, as inbreeding becomes maximal, all phenotypic variation within lines eventually represents environmental variation, whereas all variation among lines eventually represents genetic variation (e.g., David et al., 2005, 2005; Falconer & Mackay, 1996; Geber, 1990; Parsons & Hosgood, 1967). A related point is that isofemale lines effectively preserve the ancestral genetic variation present in the outbred population, and the lines can be used to reconstitute the ancestral population (Nouhaud et al., 2016). Although freshly established isofemale lines often maintain segregating variation for several generations in the lab before becoming fully inbred (Endler et al., 2016), and while such variation could, in principle, contribute to lab adaptation, Nouhaud et al. (2016) were unable to detect any significant allele frequency changes between ancestral *Drosophila* populations and reconstituted ancestral populations even when isofemale lines were maintained for over 6 years in the lab. Further advantages of this approach include the fact that the repeatability of phenotypic measurements made on isofemale lines is typically quite high (David et al., 2005) and that panels of inbred isofemale lines can be used to perform genome-wide association studies (e.g., see Gardeux et al., 2024; Mackay et al., 2012). However, potential drawbacks of isofemale lines are that the inbreeding process can render deleterious recessive alleles homozygous and might lead to artifactual positive correlations among line means for fitness components when there was a negative correlation in the outbred population (Rose, 1984).

Our continent-wide phenotypic study of European *D. melanogaster* involved >100 researchers from 26 research groups in 17 countries (Figure 1). This effort resulted in >400,000 individual fly observations based on semi-standardized experimental protocols and followed by the analysis of >>100 statistical models. The high dimensionality and large size of our dataset make our study an example of "phenomics," i.e., the acquisition of comprehensive, high-dimensional phenotype data, an

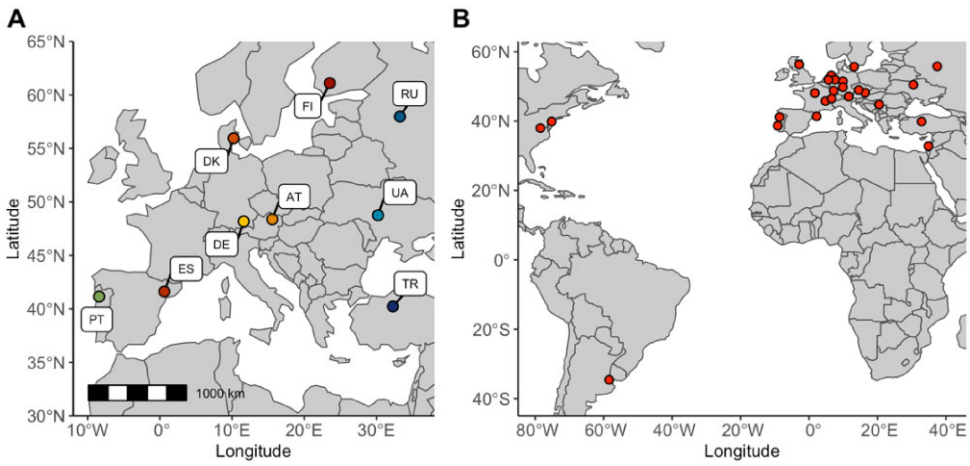

Figure 1. (A) Map of the nine locations where European populations of *Drosophila melanogaster* were sampled by the DrosEU consortium. PT, Portugal (Recarei = RE); ES, Spain (Gimenells = GI [Lleida]); TR, Turkey (Yesiloz = YE); DE, Germany (Munich = MU); AT, Austria (Mauternbach = MA); UA, Ukraine (Uman = UM); DK, Denmark (Karensminde = KA); FI, Finland (Akaa = AK); and RU, Russia (Valday = VA) (see Supplementary Table S1 and our GitHub website; also see Kapun et al., 2020, 2021). (B) Map showing the locations of the labs that contributed to phenotyping, an effort involving >100 researchers in 26 groups in 17 countries. Lines were maintained by É. Sucena (Instituto Gulbenkian de Ciência, Oeiras, Portugal) and shipped to recipient labs for phenotyping (Table 1; see also Supplementary Table S2).

important challenge and frontier in evolutionary biology (see Houle, 2010; Houle et al., 2010). As we assayed the majority of traits in multiple labs in parallel, our unprecedented phenotyping effort allowed us to examine the reproducibility of the data (see Table 1 and the *Results and discussion* section). The quantification of such repeatability has not been done on such a large scale before, yet it is important, particularly as phenotypic measurements of *Drosophila* strains are typically not replicated across labs.

Because of the massive size of our phenomic dataset, here we can only provide a summary of our most important findings. A complete description of all our methods and data, including numerous results and analyses that are not shown in the main text, can be found on our dedicated GitHub website (https://esradm.github.io/DrosEU_PhenotypingWG/).

Results and discussion

To study patterns of phenotypic variation and differentiation among the nine European populations (Figure 1), we defined 16 major traits, including several developmental, morphological, reproductive, behavioral, and stress- and survival-related traits (Table 1).

Most of these traits represent major components of fitness, including several classical life-history traits (Charlesworth, 1994; Flatt, 2020; Roff, 1992; Stearns, 1992), and might thus be phenotypic targets of selection. For several of these traits, we measured distinct "aspects" or "proxies," as shown in the second column of Table 1.

The majority of traits were measured in multiple labs (13/16 = 81%) and both males and females. All assays were carried out at 25°C, 12 hr light:12 hr dark, and a minimum relative air humidity of 60% (unless stated otherwise; for details, see the Supplementary Materials). Because strict standardization of protocols was practically challenging to implement across all 26 participating research groups, we opted to be pragmatic and to use a "semi-standardized" study design. In practice, this meant that while we strived to use standardized protocols whenever possible, we allowed for flexibility across labs in terms of implementing experimental conditions and protocols. In part, this flexibility was also necessitated by lockdowns due to the COVID-19 pandemic.

Major components of fitness vary markedly among European populations

To quantify the extent of phenotypic differentiation among populations, we used linear models (Figure 2A). Due to inevitable differences in study design, data collection, and data structure among labs, it was often not possible to fit a single global trait-specific model that could integrate all data across labs measuring the same trait. Therefore, we fitted lab-specific models instead (see the Supplementary Materials; also see Website Section 2.1).

Our analyses revealed pervasive differentiation among European populations of *D. melanogaster* for most of the phenotypic traits measured (Figure 2A). For the great majority (~70%) of the 97 linear models (i.e., combinations of traits, sexes, and labs), the factor *Population* explained a statistically significant proportion of the total phenotypic variance (Figure 2A; see Website Section 2.1 for plots of trait value estimates). The marked differences among European *D. melanogaster* in major fitness-related traits are consistent with adaptive differentiation among these populations. It is important to point out, however, that differences among populations in fitness-related traits measured in the lab do not necessarily imply that such differences are adaptive in nature (Lewontin, 2000a, 2000b).

While the range of marginal R^2 values for the factor *Population* was very broad (0.009 [fertility]–0.26 [pigmentation]; mean across all estimates = 0.081; standard error [SE] = 0.006), much of the phenotypic variance in our dataset might be genetically based. This interpretation is supported by our estimates of broad-sense heritabilities (H^2; estimated as "isofemale" heritabilities or intraclass correlation coefficients; David et al., 2005; Hoffmann & Parsons, 1988; Parsons, 1983) of the traits measured in individual labs (range across all individual estimates = 0.01 [locomotor activity]–0.67 [wing area]; mean = 0.36; SE = 0.018; for details, see Website Section 2.4). These estimates agree well with previous estimates in *D. melanogaster* (Roff & Mousseau, 1987); they are also

Table 1. Sixteen phenotypic traits assayed in our study.

Phenotypic trait	Trait aspect	Sex	Labs (PIs)
1. Viability	–	Mixed	PG, SG, KH, PS, MSR, BZ
2. Developmental time	Egg-to-pupa	Mixed	PS
	Egg-to-adult	M	PG, SG, KH, PS, MSR, BZ
		F	
3. Dry weight	–	M	HC, KH, BO
		F	
4. Thorax length	–	M	IK, NP, MR, PS
		F	
5. Wing area	Right	M	BO, NP, MR, MSR
		F	
	Left	M	
		F	
6. Fertility	–	F	JCB, CF
7. Lifespan	Line level	M	JP, EP
		F	
	Population level	M	TF
		F	
8. Cold shock mortality	–	M	JG, IK, JV
		F	
9. Chill coma recovery time	–	M	JV, JM
		F	
10. Heat shock mortality	–	M	JP, JV
		F	
11. Diapause	–	F	AB, TF, CS
12. Locomotor activity	Activity	M	ET
	Circadian phase		
	Absolute phase		
	Period		
	ND (nocturnal/diurnal ratio)		
13. Circadian eclosion timing	ZT_hours_MESA	Mixed	CW
	ZT_hours_LSPR		
	Period_MESA		
	Period_LSPR		
	Rhythmicity_LSPR_amplitude		
	Rhythmicity_JTK_p_BH_corrected		
14. Pigmentation	Total	F	JA, PG, PS
	Sixth tergite		
	Fifth tergite		
	Fourth tergite		
15. Starvation resistance	–	M	JG, BO, EP
		F	
16. Parasitoid resistance	–	Mixed	JH

Note: The following investigators and their teams contributed to the DrosEU phenotyping effort (in alphabetical order): Jessica Abbott (JA); Alan Bergland (AB); Jean-Christophe Billeter (JCB); Hervé Colinet (HC); Claudia Fricke (CF); Thomas Flatt (TF); Patricia Gibert (PG); Josefa González (JG); Sonja Grath (SG); Katja Hoedjes (KH); Jan Hrcek (JH); Iryna Kozeretska (IK); Julian Mensch (JM); Banu Onder (BO); John Parsch (JP); Elena Pasyukova (EP); Nico Posnien (NP); Michael G. Ritchie (MR); Christian Schlötterer (CS); Paul Schmidt (PS); Marina Stamenkovic-Radak (MSR); Eran Tauber (ET); Jorge Vieira (JV); Christian Wegener (CW); and Bas J. Zwaan (BZ). For more details, see Supplementary Table S2 and our GitHub website.

broadly consistent with the observation of significant genetic differentiation among these nine (as well as other) European populations in our previous genomic analyses (Kapun et al., 2020, 2021; Machado et al., 2021; also see https://dest.bio/).

Trait estimates are reproducible despite differences in assay conditions

We often observed, sometimes large, differences in trait value estimates among labs (Figure 2A; also see Website Section 2.1), potentially attributable to differences in environmental conditions among labs. Such environmental variance might include uncontrolled macro-environmental differences (e.g., diet), unknown, uncontrollable micro-environmental variance ("developmental noise"), or inadvertent differences in experimental protocols and assay conditions (Ackermann et al., 2001; Crabbe et al., 1999; Falconer & Mackay, 1996; Flatt, 2005).

For example, while several traits (e.g., thorax length, pigmentation, bristle number, ovariole number) tend to be quite repeatable and rather insensitive to small, uncontrolled variability in experimental conditions, others (behavioral, physiological, or life-history traits) can be strongly sensitive to variation in conditions (Figure 2A; cf. David et al., 2005; also see Ackermann et al., 2001; Betancourt et al., 2021; Durmaz et al., 2019; Flatt et al., 2013; Leroi et al., 1994; May et al., 2019; Min et al., 2008; Rose et al., 1996). Measurements of chill-coma recovery time, for instance, are often highly variable, sometimes even under apparently identical assay conditions within the same lab (David et al., 2005; cf. Figure 2B).

The variability in trait estimates among labs is not surprising given that we used semi-standardized experimental protocols. For example, while many labs used semi-standardized diets (i.e., diets with the same composition but not using the same brands

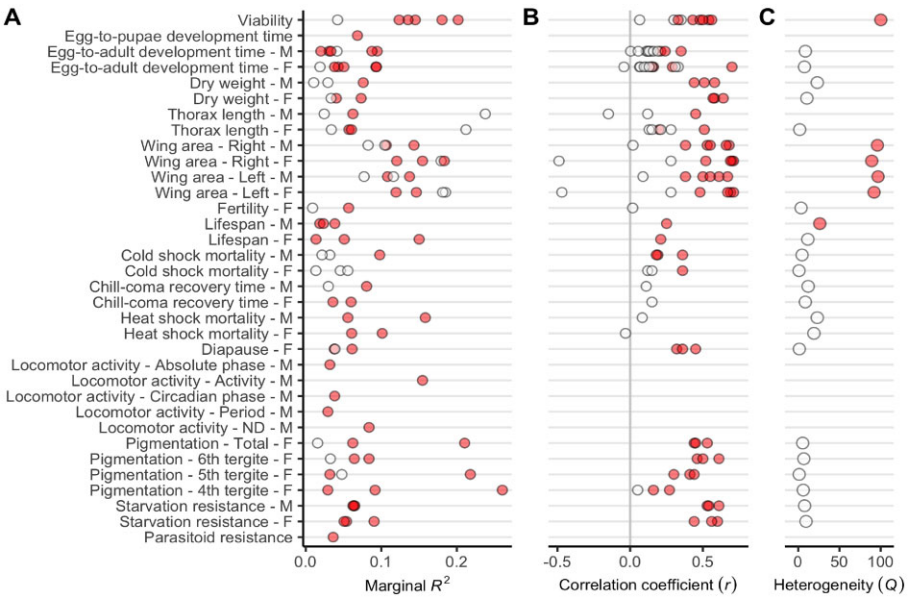

Figure 2. Phenotypic differentiation among European populations of *Drosophila melanogaster* and reproducibility of trait measurements. (A) Percentage of phenotypic variance explained by sampling location (R^2 = variance explained by the fixed-effect factor *Population*), for each trait (and sex, where applicable). Each dot represents the R^2 value extracted from each of the 97 individual linear models. (95 dots represent marginal R^2 values from linear mixed models; for two trait measurements [viability measured by the PS lab; locomotor activity — absolute phase, measured by the ET lab], we used simple linear models and extracted regular R^2 values.) Colored dots represent significant model p-values ($\alpha = 0.05$). Note that circadian eclosion timing was not analyzed using a linear modeling approach and is not shown here (see Supplementary Sections 1.6 and 2). (B) Pairwise Pearson's correlation coefficients (r) between isofemale trait values for the same phenotype estimated by different (pairs of) labs that had measured the same trait, using line coefficients extracted from linear models. Colored dots show significant correlations ($\alpha = 0.05$). Traits that were only measured by a single lab are not shown. (C) Results of the meta-analyses for the effect of "Population," showing (among-population) heterogeneity (Cochran's Q statistic), extracted from subgroup meta-analyses based on population estimates from linear models. Colored dots represent significant differences between subgroups (= populations) after Bonferroni correction ($\alpha' = \alpha/n = 0.05/26 = 0.0019$). As in (B), traits measured in single labs (as well as thorax length in males) did not enter these analyses. For further details, see the Supplementary Materials.

of ingredients), other groups used different, non-standardized diets. Indeed, as expected, differences in diet composition (protein [P]:carbohydrate [C] ratio) among labs had a significant effect on estimates (see Website Section 2.10). On the other hand, in the majority of cases, differences in the *Wolbachia* infection status of the assayed isofemale lines had no detectable impact on the studied phenotypic traits (see Website Section 2.13).

To quantify reproducibility, we calculated pairwise Pearson's correlations between trait values estimated by labs that had measured the same trait. Despite differences in assay conditions among labs, the reproducibility of estimates was overall good, with the majority of correlations between lab estimates being significantly (albeit only weakly to moderately) positive (Figure 2B; Website Section 2.3). For example, population means for dry weight, wing area, and starvation resistance were significantly positively correlated among labs, indicating robust reproducibility. Likewise, even for some complex and/or highly environmentally sensitive traits, such as lifespan and reproductive diapause, correlations were positive and significant. Conversely, for other quantitative traits such as development time, fertility, chill-coma recovery time, and heat-shock mortality, reproducibility was low, in agreement with previous findings (e.g., see David et al., 1998, 2005). Such differences between traits in their reproducibility might reflect differences in their degree of environmental sensitivity (phenotypic plasticity versus environmental canalization) (Flatt, 2005; also see Flatt, 2020; Houle, 1992, 1998; Price & Schluter, 1991; Stearns et al., 1995). From an experimental point of view, a not mutually exclusive alternative is that some traits are more "noisy" and inherently more difficult to measure than others, especially when protocols are not strictly standardized (Ackermann et al., 2001; David et al., 1998, 2005; May et al., 2019). Thus, the fact that the significant pairwise correlations in Figure 2 tend to be only weakly to modestly positive likely reflects differences in assay conditions among labs and/or genotype-by-assay environment interactions.

To obtain estimates of phenotypic differences among populations that are unlikely to be confounded by differences in assay conditions among labs, we performed meta-analyses (Balduzzi et al., 2019) of linear models across labs ("studies") and quantified heterogeneity among subgroups (= populations) by estimating Cochran's Q (see the Supplementary Materials for methodological details). These analyses confirmed significant effects of population differentiation for viability, lifespan, and wing area (Figure 2C): These three complex traits show the strongest, most consistent evidence for differentiation among European populations in our data.

Generally, however, our meta-analyses seemed to be underpowered, presumably due to the relatively small number of labs and populations involved. Indeed, meta-analyses based on Cochran's Q often tend to be underpowered (Pereira et al., 2010). Yet, despite most of our meta-analyses being nonsignificant, phenotypic differentiation among populations was significant for the majority of traits (see above: Figure 2A; ~70% of linear models showed a significant effect of the factor *Population* at $p < 0.05$). Moreover, the clear prevalence of significantly positive correlations between labs underscores the overall high degree of reproducibility of our data across labs (see above; Figure 2B).

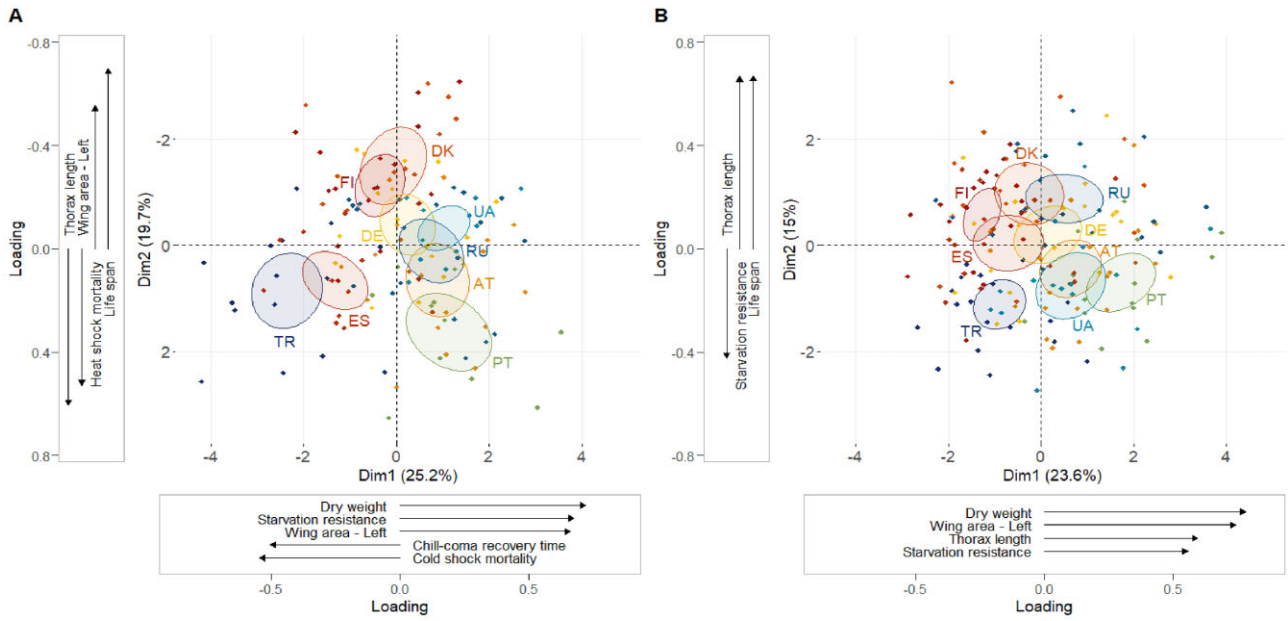

Figure 3. Principal component analysis (PCA) plots and loadings for the first principal component (PC1) and the second principal component (PC2) in (A) males and (B) females. The same nine phenotypic traits were used in both PCAs. Confidence ellipses (95%) are drawn for each of the nine populations. Phenotypic traits with greater-than-average contributions (loadings) to a given principal component are shown in the accompanying *x*- and *y*-axis vector plots. Note that in males, the *y*-axis (PC2) is inverted so that the direction of phenotypic trait correlations matches across the two sexes. For further details, see the Supplementary Materials; also see Website Section 2.8.

Our finding of high reproducibility despite variability in experimental procedures among labs is interesting given a body of work suggesting that "heterogenization" of samples or study design can improve reproducibility (Karp, 2018; Nakagawa et al., 2024; Richter et al., 2009, 2010; Usui et al., 2021; Van Der Staay et al., 2010; Voelkl & Würbel, 2016, Voelkl et al., 2018, 2020). The reason for this is the "standardization fallacy," i.e., the notion that more stringent standardization of protocols will necessarily improve the reproducibility of experimental outcomes (Voelkl & Würbel, 2016). Contrary to this idea, many environmental factors may be difficult or impossible to rigorously standardize across labs even when this is deliberately being attempted (Crabbe et al., 1999). Individuals and measurements might therefore be less variable within a single lab than among labs; together with the fact that many environmental factors might defy strict standardization, this can lead to idiosyncratic, less reproducible results among labs (Voelkl & Würbel, 2016). Thus, paradoxically, less standardized studies that, together, cover overlapping ranges of environmental conditions, and therefore explore a broader part of the underlying reaction norm, might improve reproducibility, especially when environmental heterogeneity is introduced systematically (Nakagawa et al., 2024; Richter et al., 2009; Voelkl & Würbel, 2016). This calls for improving study designs through collaborative multi-institutional studies that perform experiments in parallel and include "heterogenization" of the design (Nakagawa et al., 2024; Richter et al., 2009; Voelkl & Würbel, 2016). Such coordinated multi-lab studies are, however, rare in evolutionary biology (e.g., Ackermann et al., 2001; for a recent small-scale example involving two research groups, see Durmaz et al., 2019; Betancourt et al., 2021).

Fitness components are genetically correlated in multivariate trait space

Many components of fitness are thought to be phenotypically, physiologically, and genetically correlated with each other: As they interact to jointly determine fitness, they should be viewed from a multivariate perspective (Charlesworth, 1993; Fabian et al., 2015; Flatt, 2020; Flatt & Heyland, 2011; Houle, 2001; Lande, 1982; Lande & Arnold, 1983; Roff, 2007; Schmidt et al., 2005b; Sinervo & Svensson, 2002; Stearns, 1992; Svensson, 2023; Svensson et al., 2021).

To study multivariate phenotypes, we derived "compound" estimates across labs for each trait and line from the linear models (Supplementary Materials). Pairwise Pearson correlations of these estimates revealed many pairs of traits that were significantly genetically correlated (Website Section 2.7). We explored the main axes of variation in the ensemble of these traits (scaled to unit variance) using principal component analyses (PCAs). Initially, we included 13 traits measured on females only plus viability (see the Supplementary Materials; Website Section 2.8); we then conducted a comparison of males and females using only the nine traits that had been measured separately in both sexes (Figure 3).

In the 13-trait phenotype PCA, the first principal component (PC1) was defined by positive correlations of size, notably wing area (0.774), thorax length (0.592), and dry weight (0.753) as well as starvation resistance (0.502), and a negative correlation with lifespan (−0.453). PC2 revealed positive correlations between viability (0.782), fertility (0.633), starvation resistance (0.560), lifespan (0.417), and heat-shock mortality (0.435) (see plots and full loadings table in Website Section 2.8). Interestingly, the traits with the strongest loadings for these two axes, wing area (PC1: 0.774) and viability (PC2: 0.782), were also those with the largest Q values in the meta-analysis (see Figure 2C), reinforcing that these traits represent reliable markers of population differentiation in European *D. melanogaster*.

When comparing males and females, the overall patterns of trait correlation were similar (Figure 3). In both sexes, PC1 was defined by positive correlations between dry weight, wing area, and starvation resistance (0.783, 0.745, and 0.560 for females;

0.722, 0.664, and 0.677 for males); PC2 included a negative correlation between thorax length and lifespan (−0.424 and 0.671 for females; 0.604 and −0.699 for males). PC3 included a negative correlation between heat-shock mortality and cold-shock mortality (0.553 and −0.564 for females; −0.412 and 0.367 for males [for full loadings tables, see Website Section 2.8]). Yet, we also found several differences between the sexes. Correlations between traits were stronger in males than in females: The first four PCs explained 70.08% of the variance in males, but only 63.11% in females. Additionally, some traits showed sex-specific patterns of correlation. Interestingly, chill-coma recovery time was positively correlated with cold shock mortality in males (both PC1 and PC3), but negatively correlated with cold shock mortality in females (PC3). Furthermore, several traits (starvation resistance, wing area, and heat-shock mortality) showed sex-specific correlations along PC2 (Figure 3).

Several of the above-mentioned correlations between fitness components have been observed before, e.g., in selection experiments and/or in natural populations, such as the correlation between proxies of body size or weight and starvation resistance, or between lifespan and starvation resistance (e.g., see de Jong & Bochdanovits, 2003; Durmaz et al., 2019; Fabian et al., 2015; Flatt, 2020; Gardeux et al., 2024; Klepsatel et al., 2013; Prasad & Joshi, 2003; Stearns & Partridge, 2001). Similarly, negative correlations between size and lifespan have been found previously, yet not systematically so—this relationship is highly strain-specific and can also depend on temperature (Khazaeli et al., 2005; also see Flatt, 2020; Norry & Loeschcke, 2002).

European populations vary in multivariate trait structure

Next, we asked whether European populations of *D. melanogaster* might differ in their multivariate trait correlation structure; significant differences across space could indicate spatially varying selection on multivariate suites of fitness components.

Confidence ellipses for populations showed considerable separation along PC1 and PC2, particularly in males, as seen in Figure 3. To quantify the degree of multivariate differentiation among populations, we carried out population reallocation procedures and calculated Mahalanobis distances following discriminant function analysis (DFA) for both sexes separately (in females using the same set of traits as the PCA, and in males using the nine measured traits plus viability; for details, see the Supplementary Materials and Website Section 2.9).

Multivariate discrimination resulted in quite high levels of identifiability of populations (see Website Section 2.9). We found that 76.9% of male and 72.7% of female line estimates could be successfully reclassified according to their population of origin. The highest reclassification rates were for Turkey, with 100% of males and 95% of females reclassified correctly. Furthermore, among male flies, the highest intergroup Mahalanobis distances separated Turkey from Ukraine (24.05) and Finland (23.16), while low Mahalanobis values were observed separating Finland from Germany (3.25) and Austria from Russia (3.77). Among females, the highest Mahalanobis distances separated Ukraine from Spain (25.78) and Turkey from Finland (22.48), whereas low Mahalanobis values separated Russia from Germany (3.39) and Denmark (3.41) (for details, see Website Section 2.9). These results suggest that the Turkish population is the most distinct among the populations sampled, but that others, such as Ukraine, Finland, and Spain, also show strong differences in their multivariate trait structure.

Our results are thus consistent with ample scope for spatially varying, multivariate (including correlational) selection operating on European populations of *D. melanogaster*, similar to previous findings for North American and African populations, which differ markedly in multivariate trait structure (e.g., Fabian et al., 2015; Schmidt et al., 2005b). Notably, many of the populations that exhibit strong differences in their multivariate trait structure (i.e., Turkey, Finland, and Spain) are also among the geographically most distant populations in our dataset.

Climatic factors explain spatial patterns of trait differentiation

Many studies have documented spatially (clinally) varying selection among *D. melanogaster* populations on multiple continents (especially in North America and Australia), both at the genetic and phenotypic levels (see the *Introduction* section and references therein), but still little is known about clinal patterns on the European continent. A few studies have identified clines at the genetic level, e.g., for individual adaptive (e.g., indel) polymorphisms, neutrally evolving SNPs in short introns, as well as for transposable elements and inversion polymorphisms (Costa et al., 1992; David et al., 1986; Kapun et al., 2020, 2021; Sandrelli et al., 2007; Tauber et al., 2007), yet the evidence remains limited. Similarly, phenotypic clines in Europe remain understudied (see the *Introduction* section; for a recent overview, see Flatt, 2020).

We observed significant latitudinal differentiation among populations for viability, development time, wing area, thorax length, fertility, starvation resistance, heat-shock mortality, and lifespan (results depended on both lab and sex; for details, see Website Section 2.6). These results are broadly consistent with findings from other continents (see the *Introduction*section). For example, the latitudinal cline for wing area is in qualitative agreement with a similar cline in wing length among populations from Eastern Europe, the Caucasus, and Central Asia (Imasheva et al., 1994). Similarly, three labs found a consistent effect of latitude of origin on viability across populations, suggesting the existence of a positive latitudinal cline for this trait in Europe (see Website Section 2.6). To the best of our knowledge, a European cline for this major fitness trait has not been reported, but a similar cline has been found in South America (Folguera et al., 2008; but see Van't Land et al., 1999).

Similarly, we observed longitudinal clines for particular traits such as developmental time, pigmentation, chill-coma recovery time, and female reproductive diapause (results depended on lab and sex; see Website Section 2.6). These longitudinal phenotypic clines are particularly interesting because (1) we have previously identified a pattern of major east–west genetic structure that divides the European continent into a western and an eastern cluster of populations (Kapun et al., 2020, 2021) and (2) longitudinal phenotypic clines remain practically unknown for *D. melanogaster* to date, with very few exceptions (see Fabian et al., 2015).

On several continents, genetic and phenotypic patterns of clinality are affected by chromosomal inversion polymorphisms (Adrion et al., 2015; de Jong & Bochdanovits, 2003; Durmaz et al., 2018; Hoffmann & Weeks, 2007; Kapun & Flatt, 2019; Kapun et al., 2016a, 2016b, 2023; Lemeunier & Aulard, 1992). For example, we have previously observed latitudinal clines for *In(3L)P*, *In(3R)C*, and *In(3R)P*, as well as longitudinal clines for *In(2L)t* and *In(2R)NS*, across Europe (Kapun et al., 2020). Here, we found that population-specific mean frequencies of several polymorphic inversions (*In(2L)t*, *In(2R)NS*, *In(3L)P*, *In(3R)P*, and *In(3R)Mo*) were significantly correlated with population-specific estimates of fitness traits (see Website Section 2.14), yet Bon-

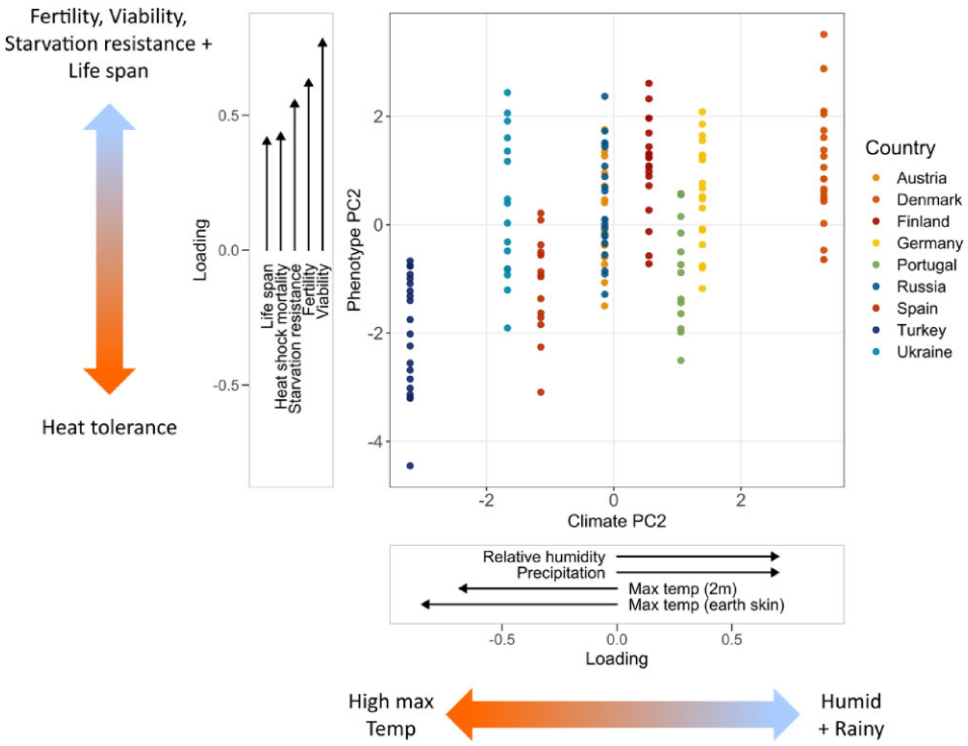

Figure 4. Correlation between phenotype principal component 2 and climate principal component 2, using all female phenotypic traits plus viability, and climatic data from the previous 30 years. Climatic and phenotypic variables with greater-than-average contributions (loadings) to a given principal component are shown in the accompanying *x*- and *y*-axis vector plots, respectively. For the corresponding results on males, which look qualitatively similar but were not significant after permutation testing, see Website Section 2.12 for details.

ferroni correction rendered these correlations nonsignificant. We also found that the presence versus the absence of inversions had significant effects on particular traits at the line level (see Website Section 2.14). Again, however, most *p*-values were nonsignificant after Bonferroni adjustment, except for a significant effect of *In(3R)P* on male heat-shock mortality. These preliminary observations suggest that inversion polymorphisms might contribute to spatial patterns of trait differentiation across the European continent.

Several major fitness components thus vary clinally across latitude and/or longitude in European populations of *D. melanogaster*. However, although latitude and longitude are often correlated with climatic variables such as temperature, they merely represent indirect proxies for causative climatic or other spatially varying factors. Moreover, clinality can also arise from demographic processes such as admixture or isolation by distance, not only spatially varying selection (Bergland et al., 2016; Flatt, 2016; Kapun et al., 2016a). Studying climatic variables might thus provide more direct and accurate evidence for the role of environmental factors in shaping population differentiation and adaptation.

To obtain summary climate variables, data for 14 climatic measures from the last 30 days and the last 30 years were retrieved from the NASA database, and their dimensionality was reduced using separate PCAs, with the resultant PC1 and PC2 explaining 94.7% (30-day data) and 89.8% (30-year data) of the total variation. Because long-term climatic trends are more likely to shape broad patterns of phenotypic evolution, we focused on the PCs obtained using 30-year data here. PC1 was driven by a negative correlation between average temperature (earth skin temperature, temperature at 2 m, and wet bulb temperature) and the number of frost days, while PC2 was driven by a negative correlation between maximum temperature (earth skin and 2 m) and relative humidity plus precipitation (for further details, see the Supplementary Materials and Website Section 2.12). We then tested whether these two climate summary variables were useful in predicting multivariate phenotypes (PCs 1, 2, and 3 of the phenotype PCAs; Website Section 2.12) using linear models, and confirmed results with a permutation procedure.

This analysis revealed a significant association between climate PC2 and phenotype PC2 for the 13-trait PCA ($F = 78.151$, $df = 1$, $p < 0.0001$; permutation $p = 0.009$): Notably, European populations experiencing higher humidity and rainfall and lower maximum temperatures (Bogaerts-Márquez et al., 2021) exhibit higher values for major fitness-related traits, including viability, fertility, starvation resistance, and lifespan (Figure 4). However, these populations are also characterized by increased heat-shock mortality; thus, local adaptation to milder, wetter climates comes at the expense of decreased heat tolerance, suggesting that there is a trade-off between viability, fertility, starvation resistance, and lifespan on the one hand and heat-shock survival on the other hand.

Thermal tolerance is mediated by the expression of heat-shock proteins (HSPs; Hoffman et al., 2013), with selection for thermal stress resistance contributing to higher constitutive levels of HSP (Sørensen et al., 2017). However, increases in HSP copy number or expression also negatively affect other phenotypic traits such as metabolic rate, fecundity, and survival (Hoekstra & Montooth, 2013; Okada et al., 2014; Roberts & Feder, 2000; Silbermann & Tatar, 2000), suggesting that pleiotropic effects of HSPs and other genes involved in thermal stress responses might be an important

factor underpinning phenotypic variation across climatic gradients (Chen et al., 2018). More generally, and consistent with our findings here, previous studies of *Drosophila* spp. have found that increased heat resistance is often associated with reduced viability, dry weight, fecundity, fertility, cold resistance, ethanol resistance, and mating frequency (see Hoffman et al., 2003).

Our finding of a trade-off between a suite of fitness components and heat-shock survival across space (i.e., between different climates) adds to a growing number of studies in *D. melanogaster* that have found evidence for patterns of local adaptation driven by spatially varying selection (Anderson et al., 2003; Betancourt et al., 2021; de Jong & Bochdanovits, 2003; Durmaz et al., 2018, 2019; Fabian et al., 2015; Kapun et al., 2016b; Paaby & Schmidt, 2009; Paaby et al., 2014; Schmidt & Paaby, 2008; Schmidt et al., 2005a, 2005b ; reviewed in Flatt, 2020).

It is also noteworthy that we failed to find a negative correlation between early fertility and lifespan (see Figure 4). This is interesting, as many studies in *Drosophila* have reported negative genetic correlations between early fecundity and lifespan, indicative of a trade-off (e.g., reviewed in Flatt, 2011, 2020; Stearns & Partridge, 2001). Whether the positive association between fertility and lifespan observed here is artifactual, e.g., due to inbreeding (Rose, 1984) or exposure to novel lab environments (Service & Rose, 1985) or whether cooler and wetter European climates are ecologically more "benign" in terms of selectively favoring higher fertility and survival remains unclear. Whatever might be the case, negative correlations between survival and reproduction are not always found, and even when they exist, multiple confounding factors can obscure them (e.g., see discussion in Flatt, 2011, 2020; Klepsatel et al., 2013; and references therein).

The association between climate PC2 and phenotype PC2 for the female and male nine-trait phenotype PCAs was, however, not significant following permutation testing ($p = 0.057$ and $p = 0.11$, respectively), perhaps due to the absence of viability from these PCAs.

Interestingly, associations between phenotype PC2 and climate PC2 had stronger support when considering 30-day data as compared to 30-year data (significant permutations for phenotypic PCAs; see Website Section 2.12), consistent with recent observations suggesting that short-term changes in the environment on the order of a few weeks or less can drive seasonal adaptation in flies (see Bitter et al., 2024; Machado et al., 2021; Nunez et al., 2024; Rudman et al., 2022; also cf. discussion in Hoffmann & Flatt, 2022).

Summary and conclusions

Here, we have undertaken a large-scale, collaborative phenomics effort to provide the first continent-wide, systematic characterization of patterns of phenotypic differentiation and clinality among European populations of the vinegar fly *D. melanogaster*, a classical model system for studying fundamental questions in evolutionary biology. Our most important findings and conclusions can be summarized as follows:

(1) European populations of *D. melanogaster* are significantly differentiated with respect to numerous phenotypic components of fitness, which might be subject to spatially varying (diversifying) selection.
(2) The majority of trait estimates were significantly positively correlated between pairs of labs that measured the same trait, suggesting a high degree of reproducibility despite differences in assay conditions among labs.
(3) PCA revealed that numerous traits were significantly correlated with each other, and DFAs showed that European populations differ markedly in their multivariate trait structure, suggesting ample scope for multivariate spatially varying selection on phenotypic components of fitness.
(4) Consistent with spatially varying selection being driven by climatic gradients, several fitness components exhibited significant latitudinal or longitudinal clinality among populations. Most notably, egg-to-adult survival (viability) and egg-to-adult development time varied latitudinally and longitudinally, respectively.
(5) Populations subject to higher humidity/rainfall and to lower maximum temperatures were characterized by higher values for viability, fertility, starvation resistance, and lifespan, yet exhibited lower heat-shock survival, suggesting a trade-off between these fitness components and revealing local climate adaptation. Together with previous and current genomic analyses of these populations (Bogaerts-Márquez et al., 2021; Kapun et al., 2020, 2021; Machado et al., 2021), it will clearly be of great interest to unravel the genetic basis underlying these phenotypic patterns of climate adaptation.

Many additional analyses and results, which we could not discuss due to space limitations, can be found on our GitHub website at https://esradm.github.io/DrosEU_PhenotypingWG/; we encourage readers to explore and make use of this rich phenomic dataset and resource.

The second resource that we wish to make available to the community is our multipopulation panel of isofemale lines, the DrosEU Phenotyping Panel (DPP). The DPP might be a useful complement to other existing *D. melanogaster* panels, such as the *Drosophila* Genetic Reference Panel (DGRP; Mackay et al., 2012), the *Drosophila* Population Genomics Project (DPGP; Pool et al., 2012), the *Drosophila* Genome Nexus (DGN; Lack et al., 2015, 2016), and the Global Diversity Lines (GDL; Grenier et al., 2015). The DPP is available upon request from Élio Sucena (jesucena@ciencias.ulisboa.pt); genomic analyses of the DPP by our consortium are currently underway.

Materials and methods

A detailed description of our materials and methods is given in the Supplementary Materials associated with the manuscript and also on our dedicated GitHub website at https://esradm.github.io/DrosEU_PhenotypingWG/.

Supplementary material

Supplementary material is available online at *Evolution Letters*.

Data and code availability

Raw data and the complete compilation of code, statistical models, analyses, and results are available on GitHub (https://esradm.github.io/DrosEU_PhenotypingWG/) and are archived on Zenodo (https://doi.org/10.5281/zenodo.15310170).

Author contributions

Contributions defined according to CRediT ontology (https://casrai.org/credit/); authors in alphabetical order: T.A.F.deA.: inves-

tigation; J.A.: investigation, methodology, writing—original draft preparation, writing—review & editing; C.A.: investigation; E.A.: investigation, methodology; T.B.: investigation; A.B.: formal analysis, investigation, methodology, software, visualization, writing—review & editing; A.B.: conceptualization, writing—review & editing; J.-C.B.: investigation; D.C.: investigation; E.C.: investigation; H.C.: investigation, methodology, data curation, supervision, writing—original draft preparation, writing—review & editing; N.C.: investigation, supervision; S.C.: investigation; S.D.: investigation; E.D.: investigation; T.D.: formal analysis, investigation, writing—review & editing; S.R.-D.: investigation; P.D.: formal analysis, investigation, writing—review & editing; E.D.M.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing—original draft preparation, writing—review & editing; M.E.: investigation; K.E.: investigation; P.E.: investigation; P.E.: investigation, methodology; F.F.: investigation; B.F.: investigation; T.F.: conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, writing—original draft preparation, writing—review & editing; C.F.: investigation, methodology; P.G.: investigation, methodology, project administration, data curation, supervision, funding acquisition; A.G.-S.: investigation, methodology, resources, writing—review & editing; A.G.: investigation; J.G.: supervision, writing—review & editing; S.G.: investigation, methodology, writing—review & editing; L.G.: investigation; E.H.: formal analysis, visualization, writing—original draft preparation; K.M.H.: investigation, methodology, data curation, supervision, writing—original draft preparation, writing—review & editing; J.H.: methodology, funding acquisition; S.J.: investigation, resources; M.J.: investigation, methodology; M.K.: resources, writing—review & editing; M.K.: formal analysis, investigation, writing—original draft preparation, writing—review & editing; E.K.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing—original draft preparation, writing—review & editing; H.K.: investigation; I.K.: methodology, supervision, data curation, resources; L.E.K.: investigation; N.K.: investigation, methodology; V.L.: investigation; M.L.: investigation, methodology; O.M.M.: data curation, formal analysis, writing—review & editing; S.-L.M.: investigation, methodology; M.M.: investigation: J.M.: investigation, supervision, writing—review & editing; M.M.: investigation; M.S.M.: investigation; V.M.: investigation, methodology; L.M.: investigation, methodology; D.V.M.: methodology, resources; S.M.: investigation; B.S.O.: investigation, supervision, resources, methodology; J.P.: funding acquisition, resources; E.G..P.: investigation, methodology, resources, supervision, writing; A.P.: investigation, methodology, data curation; T.F.P.: investigation; N.P.: investigation, validation, writing—review & editing; O.P.: investigation; F.A.P.: investigation; M.R.: investigation, validation; M.G.R.: conceptualization, formal analysis, supervision, writing—review & editing; N.V.R.: investigation, methodology; O.Y.R.: investigation, methodology; M.S.V.: investigation, methodology; data curation; C.S.: supervision, writing—review & editing; P.S.: conceptualization, investigation; M.F.S.: investigation; T.S.: investigation; S.S.S.: investigation; S.S.: investigation, methodology, data curation, writing—original draft preparation; M.S.-R.: investigation, supervision, resources; É.S.: investigation, resources; A.V.S.: investigation, methodology, formal analysis, validation, writing; M.T.: investigation, methodology, data curation, formal analysis, writing; E.T.: investigation, formal analysis, supervision, resources, writing—review & editing; V.T.: investigation; M.V.T.: investigation, methodology; E.A.T.: investigation, methodology; V.T.: formal analysis, visualization, writing—original draft preparation; J.H.: investigation; D.W.: investigation; E.R.V.: investigation, methodology; C.P.V.: formal analysis, investigation, methodology, software, validation, writing—review & editing; C.V.: investigation, methodology; J.V.: conceptualization, formal analysis, investigation, methodology, software, validation, writing—original draft preparation, writing—review & editing; X.W.: investigation; C.W.: investigation, formal analysis, resources, writing—review & editing; J.Z.: investigation; B.Z.: investigation, funding acquisition, methodology, resources, writing—original draft preparation.

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Conflict of interest

The authors declare no conflict of interest. Note: After February 24, 2022, no collaborative actions or exchanges have taken place between Ukrainian and Russian scientists within our project.

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