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An inversion supergene in *Drosophila* underpins latitudinal clines in survival traits

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Abstract

Chromosomal inversions often contribute to local adaptation across latitudinal clines, but the underlying selective mechanisms remain poorly understood. We and others have previously shown that a clinal inversion polymorphism in *Drosophila melanogaster*, *In(3R)Payne*, underpins body size clines along the North American and Australian east coasts. Here, we ask whether this polymorphism also contributes to clinal variation in other fitness-related traits, namely survival traits (lifespan, survival upon starvation and survival upon cold shock). We generated homokaryon lines, either carrying the inverted or standard chromosomal arrangement, isolated from populations approximating the endpoints of the North American cline (Florida, Maine) and phenotyped the flies at two growth temperatures (18 °C, 25 °C). Across both temperatures, high-latitude flies from Maine lived longer and were more stress resistant than low-latitude flies from Florida, as previously observed. Interestingly, we find that this latitudinal pattern is partly explained by the clinal distribution of the *In(3R)P* polymorphism, which is at ~ 50% frequency in Florida but absent in Maine: inverted karyotypes tended to be shorter-lived and less stress resistant than uninverted karyotypes. We also detected an interaction between karyotype and temperature on survival traits. As *In(3R)P* influences body size and multiple survival traits, it can be viewed as a 'supergene', a cluster of tightly linked loci affecting multiple complex phenotypes. We conjecture that the inversion cline is maintained by fitness trade-offs and balancing selection across geography; elucidating the mechanisms whereby this inversion affects alternative, locally adapted phenotypes across the cline is an important task for future work.

Introduction

Since the pioneering work of Dobzhansky, many lines of evidence suggest that chromosomal inversion polymorphisms play a major role in climatic adaptation to altitudinal and latitudinal gradients, so-called clines (Dobzhansky, 1937, 1943, 1947a, b; Wright & Dobzhansky, 1946; Lemeunier & Aulard *et al.*, 1992; Hoffmann *et al.*, 2004; Kirkpatrick & Barton, 2006; Hoffmann & Rieseberg, 2008; Schaeffer, 2008; Kirkpatrick & Kern, 2012; Kapun *et al.*, 2016a). However, while inversions have been statistically associated with many traits (Sperlich & Pfriem *et al.*, 1986; Etges, 1989; De Jong & Bochdanovits, 2003; Hoffmann *et al.*, 2004; Lowry & Willis, 2010), little is known about associations between inversions and fitness-related traits, thus limiting our understanding of how these adaptive polymorphisms are maintained by selection (e.g. Hoffmann & Rieseberg, 2008).

The latitudinal frequency clines of inversion polymorphisms in *Drosophila melanogaster*, often observed in a parallel fashion on multiple continents, provide an

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excellent opportunity to address this problem (Mettler *et al.,* 1977; Knibb *et al.,* 1981; Knibb, 1982; Lemeunier & Aulard *et al.,* 1992; De Jong & Bochdanovits, 2003; Hoffmann & Weeks, 2007). For example, a large (~ 8 Mb), cosmopolitan inversion polymorphism on the right arm of the third chromosome, *In(3R)Payne* (also called *In(3R)P*), varies clinally along latitudinal gradients on several continents, most prominently along the Australian and North American east coasts (Mettler *et al.,* 1977; Inoue & Watanabe, 1979; Stalker, 1980; Knibb *et al.,* 1981; Knibb, 1982; Das & Singh, 1991; Anderson *et al.,* 2005; Matzkin *et al.,* 2005; Fabian *et al.,* 2012; Kapun *et al.,* 2014, 2016a; Rane *et al.,* 2015). This inversion, which is of tropical African origin and approximately 130 000–150 000 years old (Corbett-Detig & Hartl, 2012), has long been thought to be an important driver of climatic adaptation in *D. melanogaster* (Weeks *et al.,* 2002; De Jong & Bochdanovits, 2003; Anderson *et al.,* 2005; Hoffmann & Weeks, 2007; Rane *et al.,* 2015; Kapun *et al.,* 2016a, b).

Potentially consistent with a role of this inversion in climate adaptation, the inverted karyotype of *In(3R)P* exhibits intermediate-to-high frequencies at low latitudes (i.e. in subtropical to tropical climates) but is rare or absent at high latitudes (i.e. in temperate, seasonal climates) on all continents or subcontinents examined so far (see references above). Along the North American east coast, for example, the inverted arrangement has a frequency of ~ 50% in Florida but its frequency decreases along the cline to ~ 0% in Maine (Mettler *et al.,* 1977; Knibb, 1982; Fabian *et al.,* 2012; Kapun *et al.,* 2014, 2016a). Recent evidence suggests that the Australian and North American clines in *In(3R)P* are adaptively maintained by spatially varying selection (Rane *et al.,* 2015; Kapun *et al.,* 2016a). Although genetic patterns of clinal variation can be severely confounded by admixture and secondary contact with ancestral populations (Bergland *et al.,* 2016; also see discussion in Flatt, 2016), the North American cline in *In(3R)P* seems to be maintained non-neutrally and independent of population structure and admixture (Kapun *et al.,* 2016a).

Interestingly, several major fitness-related traits also exhibit strong clinality across latitude (De Jong & Bochdanovits, 2003; Hoffmann & Weeks, 2007; Adrion *et al.,* 2015), and it is thus tempting to speculate that the clinal behaviour of *In(3R)P* (or potentially that of other clinal inversions) might underlie – or contribute to – these life-history clines. For instance, flies from North American high-latitude populations are characterized by increased body size, reduced wing loading, reduced fecundity, prolonged lifespan, increased resistance to starvation, cold and heat stress, and the plastic ability to undergo reproductive dormancy in response to cool temperature and short photoperiod, as compared to flies from low latitude (Coyne & Beecham, 1987; Azevedo *et al.,* 1998; de Jong & Bochdanovits, 2003; Hoffmann *et al.,* 2005; Schmidt *et al.,* 2005a, b; Schmidt & Paaby, 2008; Fabian *et al.,* 2015; Mathur & Schmidt, 2017). This suggests a pattern of climatic adaptation whereby harsh winters and seasonal stress at high latitudes favour stress resistance and overwintering ability (dormancy), along with correlated (e.g. pleiotropic) responses in terms of larger body size, increased lifespan and reduced fecundity (Schmidt & Paaby, 2008; Paaby & Schmidt, 2009; Flatt *et al.,* 2013; Paaby *et al.,* 2014). Yet, whether clinal inversion polymorphisms such as *In(3R)P* contribute to this pattern of phenotypic climatic adaptation is largely unclear (De Jong & Bochdanovits, 2003; Hoffmann *et al.,* 2004; Rako *et al.,* 2006; Hoffmann & Weeks, 2007; Hoffmann & Rieseberg, 2008; Kapun *et al.,* 2016a, b).

Consistent with a contribution of *In(3R)P* to clinal trait differentiation, we and others have previously found that the latitudinal cline in this inversion contributes to the body size cline along the Australian (Weeks *et al.,* 2002; Rako *et al.,* 2006; Kennington *et al.,* 2007) and North American (Kapun *et al.,* 2016b) east coasts. However, little is known about whether *In(3R)P* also affects other traits that covary with latitude. In Australian populations, for example, Anderson *et al.* (2003) found an association between susceptibility to cold and *In(3R)P*, but a subsequent study by Rako *et al.* (2006) failed to find a clear effect. Similarly, for the North American cline, we also failed to detect an association between *In(3R)P* and chill coma recovery (Kapun *et al.,* 2016b). Moreover, neither study found an effect of *In(3R)P* on developmental time (Rako *et al.,* 2006; Kapun *et al.,* 2016b). Thus, whether *In(3R)P* contributes to clinal variation in fitness-related traits other than body size is not clear. While it is possible that *In(3R)P* predominantly – or exclusively – affects size and not other traits, this seems rather unlikely, for two reasons: (i) the majority of significantly clinally varying SNPs in the genome reside in the region spanned by this inversion, and (ii) many of these clinal SNPs within *In(3R)P* are located in genes that are known from studies of mutants and transgenes to affect life-history traits (Fabian *et al.,* 2012; Kapun *et al.,* 2016a, b).

Here, we examine whether the clinal *In(3R)P* polymorphism affects three major survival traits in North American populations of *D. melanogaster*: adult lifespan, survival upon starvation and cold resistance (measured as survival upon cold shock). All three traits are known to vary clinally in North America as a function of latitude (or of high-latitude vs. low-latitude genotypes; e.g. Schmidt *et al.,* 2000, 2005a, b; Schmidt & Paaby, 2008; Paaby *et al.,* 2014; Mathur & Schmidt, 2017). In agreement with previous phenotypic results, we find that high-latitude flies from Maine lived longer and are more stress resistant than low-latitude flies from Florida, consistent with the idea that selection at high latitude favours genotypes and phenotypes that confer improved survival and somatic maintenance (Paaby &

Schmidt, 2009; Flatt *et al.,* 2013; Paaby *et al.,* 2014). Interestingly, we observe that the clines in these traits are partly driven by the clinal frequency gradient in *In (3R)P*: on average flies carrying the *In(3R)P* inversion from Florida live shorter and are less stress resistant than flies from Florida or Maine which possess the uninverted chromosomal segment. Our findings support the hypothesis that life-history clines are maintained by fitness trade-offs across geography and that clinal inversions, such as *In(3R)P*, make an important contribution to this phenomenon.

Materials and methods

Fly stocks and maintenance

We isolated third-chromosome isochromosomal (homokaryon) lines, either carrying two copies of the inverted *In(3R)P* arrangement or two copies of the uninverted (standard) arrangement, from two areas approximating the endpoints of the North American cline [low latitude: Florida (Homestead and Jacksonville); and high latitude: Maine (Bowdoin)], as previously described (see Kapun *et al.,* 2016b for details of sampling locations and isolation methods). Across the cline, *In(3R)P* has a frequency of ~ 50% in Florida but is absent in Maine, so that flies from high-latitude populations are fixed for the uninverted arrangement (e.g. Mettler *et al.,* 1977; Knibb, 1982; Fabian *et al.,* 2012; Kapun *et al.,* 2014, 2016a, b). Wild-type chromosomes were isogenized using a compound balancer for the second and third chromosomes [*SM6b*; *TM6B*; Bloomington *Drosophila* Stock Center (BDSC) stock #5687] in an *ebony* (e^1) mutant background (cf. Kapun *et al.,* 2016b). From Florida, where both the inverted and uninverted segments segregate, we isolated nine isochromosomal lines carrying *In(3R)P* ('Florida inverted', FI) and nine lines possessing the standard arrangement ('Florida standard', FS); from Maine, where the inverted segment is absent, we isolated nine lines with the standard arrangement only ('Maine standard', MS). Prior to phenotyping assays, which were performed at two growth temperatures, lines were kept under common garden conditions for three generations [~ 21 °C, 10 h : 14 h light : dark (LD), ~ 50% relative air humidity (RH)].

Phenotype assays

We measured three clinally varying survival traits on the homokaryon lines: lifespan, survival upon starvation and survival upon cold shock (see below). Assays were performed at two growth temperatures (18 °C, 25 °C), at 12 : 12 h LD and 60% RH, on a cornmeal/sugar/yeast/agar diet. To obtain (F1) flies for phenotypic assays, we let ~ 20–25 females and males mate and lay eggs into vials containing 8 mL of medium at room temperature [n = 46 vials for each of the 27 lines (=3 karyotypes × 9 lines); total = 1242 vials]. Depending on their fecundity, females in each vial were allowed to lay eggs for up to ~ 24 h; egg density was inspected regularly by eye and adjusted to ~ 40–50 eggs per vial. Vials were then transferred to their respective developmental temperature treatment (i.e. 18 °C vs. 25 °C; 23 replicate vials per temperature and isochromosomal line).

For lifespan assays, we collected cohorts of newly eclosed adult females and males within a 24-h period. Flies were sexed and counted under light CO_2 anaesthesia and transferred to 1-L demography cages (see Tatar *et al.,* 2001 for details of cage design) 24 h after eclosion. Each cage was initiated with 75 females and 75 males. We set up two replicate cages per line and temperature (n = 2 cages × 27 lines × 2 temperatures = 108 cages; 108 cages × 150 flies = 16 200 flies in total). Every second day at 25 °C and every third day at 18 °C, we changed food vials and removed and recorded dead flies until all flies in the experiment had died. Flies that got stuck to the food medium were censored from analysis.

For assays of survival upon starvation, we used 5- to 7-day-old mated individuals. Eclosing adults were collected in 48-h cohorts and maintained in mixed-sex groups for 4 days in their respective thermal treatments. Twenty-four hours prior to initiating the assay, flies were sexed under light CO_2 anaesthesia and transferred to fresh vials containing 10 individuals per vial and sex. On the day of the experiment, flies were transferred to food-free vials, containing 0.5% agar/water solution. For each line, temperature and sex, we used five replicate vials (n = 5 vials × 27 lines × 2 temperatures × 2 sexes = 540 vials, each with 10 flies; total = 5400 flies). Age at death was scored in 8-h intervals until all flies had died.

We used an identical experimental design for measuring survival upon cold shock; again, we used five replicate vials per group (n = 5 vials × 27 lines × 2 temperatures × 2 sexes = 540 vials, each with 10 flies; total = 5400 flies). On the day of the experiment, 5- to 7-day-old mated flies were transferred to media-free vials and the vials dipped immediately into −4 °C cold, salted ice water for 90 min (depending on acclimation, several hours of exposure to temperatures between −2 and −5 °C typically result in > 50% mortality; cf. Hoffmann, 2010). After cold shock, flies were transferred to Petri dishes (60 × 15 mm) with 2 mL fly food in one corner and left at room temperature for recovery. Survival was scored after 24 h; flies that were alive after 24 h were censored from analysis.

Note that all assays of fitness components were performed on mated flies, for two reasons. First, unmated flies live dramatically longer than mated flies – such a massive physiological effect might have masked the more subtle karyotypic differences we aimed to observe. Second, the majority of work on phenotypic

clines in *D. melanogaster*, including our work on *In(3R)P* (Kapun *et al.*, 2016b), has been done on mated flies so that – for the sake of comparison – we decided to not assay unmated flies.

All phenotypic raw data have been deposited at Dryad: https://doi.org/10.5061/dryad.3vb89dj.

Statistical analysis

The primary interest of our analysis was to determine the effects of *In(3R)P* karyotype (inverted vs. uninverted arrangement) upon survival traits; effects of clinality/geography (i.e. Florida vs. Maine) were of secondary interest. However, the biology of this system is such that the effects of karyotype vs. geography cannot be completely disentangled: as *In(3R)P* is polymorphic (with a ~ 50 : 50% frequency of inverted vs. standard arrangement) in Florida but not in Maine, where only the uninverted arrangement is present (i.e. the frequency of the inversion is ~ 0%), one cannot use a fully factorial, orthogonal design to analyse data for this inversion polymorphism when considering the cline ends. Analysing the cline ends is, however, important as this allows investigating the extent of life-history clinality, and the potential contribution of the inversion to it, across the entire latitudinal gradient. Importantly, even though the inversion is absent in Maine, this design nonetheless permits clear inferences regarding the effects of karyotype (also see Kapun *et al.*, 2016b): significant differences between FI and FS and between FI and MS, with no difference between FS and MS, imply a clear main effect of *In(3R)P* karyotype. On the other hand, a situation in which all three pairwise comparisons (FI vs. FS; FI vs. MS; FS vs. MS) are different implies that inverted vs. standard arrangements differ in their effects, yet that the two uninverted arrangement types from Florida and Maine differ too, perhaps due to an effect of geography (Florida vs. Maine). In this scenario, it is not possible to clearly separate the effects of karyotype vs. geography; nevertheless, a significant difference between FI and FS would indicate an effect of *In(3R)P* karyotype. In both cases, the inference would be that *In(3R)P* karyotype affects the trait of interest. Lastly, a scenario in which FI = FS and where both FI and FS are significantly different from MS might imply – assuming parsimony – a main effect of geography/clinal differentiation independent of *In(3R)P* karyotype.

Due to the constraint that the effects of karyotype and geography must be analysed jointly, we created a compound grouping factor K ('karyotype', partly confounded by geography) with three levels ('Florida inverted', FI; 'Florida standard', FS; and 'Maine standard', MS) and used pairwise comparisons between the levels of K in order to infer the effects of karyotype, geography or both. The second factor that entered our analyses was temperature T (18 °C vs. 25 °C). We analysed our survival (mortality) data in two ways. First, we used Cox (proportional hazards) regression to fit the fixed effects of K, T and the interaction $K \times T$. This 'global' approach gave us main effects for K (averaged across temperatures) and T (averaged across levels of K) and indicated whether – importantly – there are significant $K \times T$ (i.e. genotype by environment) interactions. Second, to dissect the source of significance of the effects of K and/or $K \times T$, we employed Kaplan–Meier survival analysis with generalized Wilcoxon (χ^2) tests (GWT) to perform pairwise comparisons (i.e. FI vs. FS; FI vs. MS; FS vs. MS) for each temperature and sex separately, followed by Bonferroni correction for multiple testing. These pairwise comparisons thus serve as post hoc tests for the Cox models. Analyses were performed in JMP v.11.2.0 (SAS, Raleigh, NC, USA).

We also provide a preliminary analysis of the potential relationship between size (wing area) and lifespan and of the effects of karyotype on the multivariate combination of life-history traits (see Appendix S1 for details).

Results

The *In(3R)P* polymorphism contributes to clinality of lifespan

We first analysed the effects of karyotype and temperature and their interaction on lifespan (Fig. 1, Fig. S1). For both females and males, Cox regression revealed effects of karyotype [likelihood ratio test (LRT); females: $\chi^2_{(2)} = 387.8$, males: $\chi^2_{(2)} = 359.0$, both $P < 0.0001$], temperature T (females: $\chi^2_{(1)} = 4301.9$, males: $\chi^2_{(1)} = 2893.4$, both $P < 0.0001$) and the $K \times T$ interaction (females: $\chi^2_{(2)} = 19.3$, males: $\chi^2_{(2)} = 31.2$, both $P < 0.0001$). This analysis, together with pairwise generalized Wilcoxon χ^2 tests (GWT), showed that Florida inverted (FI) flies lived shorter than both Florida standard (FS) and Maine standard (MS) flies (Fig. 1, Table 1, Fig. S1), implying a clear effect of *In(3R)P* karyotype on adult survival. Moreover, at 18 °C – but not at 25 °C – FS flies lived shorter than MS flies (Fig. 1, Table 1, Fig. S1). These results indicate that both inversion karyotype (inverted flies live shorter than uninverted flies from both Florida and Maine) *and* geography (at least at 18 °C, uninverted flies from Maine lived longer than both inverted and uninverted flies from Florida) affect lifespan (Fig. 1, Table 1, Fig. S1; A preliminary analysis of size data from our experiment suggests that karyotypic differences in lifespan might partly be explained by effects of *In(3R)P* on size; see Appendix S1). With regard to temperature, flies lived longer at 18 °C than at 25 °C (see significant effect of T in Cox regression above; and GWT, females: $\chi^2_{(1)} = 3490.4$, males: $\chi^2_{(1)} = 2262.5$, both $P < 0.0001$; Fig. 1, Fig. S1). At 18 °C, females lived longer than males, but we failed to find such a sex difference at 25 °C (GWT, 18 °C:

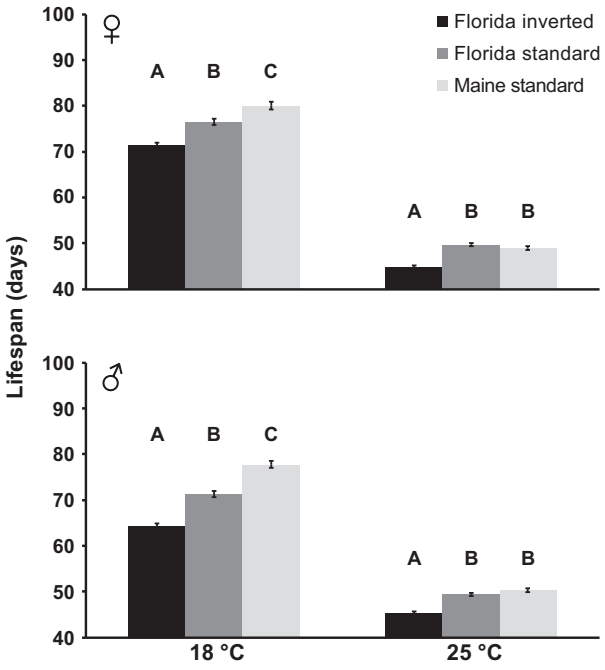

Fig. 1 *In(3R)P* shortens lifespan and lifespan varies clinally. Effects of the *In(3R)P* inversion polymorphism on adult lifespan (days) in females and males. The bar plots show means and standard errors. Black bars: Florida inverted (FI); dark grey bars: Florida standard (FS); light grey bars: Maine standard (MS). Results for pairwise comparisons among karyotypes with generalized Wilcoxon (χ^2) tests are shown in letters: groups that do not contain the same letter are significantly different from each other ($P < 0.05$). The *In(3R)P* inversion shortens lifespan as compared to uninverted karyotypes; lifespan is clinally differentiated, with high-latitude flies from Maine living longer than flies from low-latitude. See Results and Table 1 for details; for survival curves see Fig. S1.

$\chi^2_{(1)} = 115.7$, $P < 0.0001$, 25 °C: $\chi^2_{(1)} = 0.122$, $P = 0.73$; Fig. 1, Fig. S1). Thus, *In(3R)P* has a negative impact upon adult survival, and lifespan exhibits clinal differentiation, with high-latitude flies overall living longer than flies from low latitude.

The effects of *In(3R)P* on starvation survival depend on temperature

Next, we examined whether *In(3R)P* affects survival upon starvation. We found significant effects of karyotype (Cox LRT; females: $\chi^2_{(2)} = 174.9$, males: $\chi^2_{(2)} = 93.2$, both $P < 0.0001$), temperature (females: $\chi^2_{(1)} = 253.3$, males: $\chi^2_{(1)} = 660.2$, both $P < 0.0001$) and – for females – of the $K \times T$ interaction (females: $\chi^2_{(2)} = 18.2$, $P < 0.0001$; males: $\chi^2_{(2)} = 4.1$, $P = 0.13$; Fig. 2, Fig. S2). Interestingly, at 18 °C, FI flies were more starvation resistant than FS flies for both sexes, whereas at 25 °C, this pattern was reversed for females, without a significant difference in males (Fig. 2, Table 2, Fig. S2). Overall, across both temperatures, high-latitude MS flies were more starvation resistant than low-latitude FI and FS flies, indicating an effect of clinality (Fig. 2, Table 2, Fig. S2). Survival upon starvation was greater for flies reared at 18 °C than at 25 °C (see significant effect of T in Cox model above; GWT, females: $\chi^2_{(1)} = 252.4$, males: $\chi^2_{(1)} = 848.7$, both $P < 0.0001$), and females were more resistant than males at both temperatures (GWT, 18 °C: $\chi^2_{(1)} = 938.6$, 25 °C: $\chi^2_{(1)} = 1339.1$, both $P < 0.0001$; Fig. 2, Fig. S2). Together, these results show that in Florida, the effects of *In(3R)P* karyotype on starvation survival depend on temperature, and that high-latitude flies from Maine are more starvation resistant than low-latitude flies from Florida.

Table 1 Analysis of lifespan.

Sex	Temperature	Direction	χ^2	P	n
Female	18 °C	FI < FS	35.86	**< 0.0001**†	2609 (2639)
		FI < MS	137.31	**< 0.0001**†	2591 (2644)
		FS < MS	28.52	**< 0.0001**†	2520 (2571)
	25 °C	FI < FS	98.75	**< 0.0001**†	2500 (2549)
		FI < MS	71.53	**< 0.0001**†	2478 (2539)
		FS = MS	0.65	0.42	2508 (2566)
Male	18 °C	FI < FS	85.72	**< 0.0001**†	2437 (2455)
		FI < MS	247.67	**< 0.0001**†	2514 (2547)
		FS < MS	37.26	**< 0.0001**†	2453 (2484)
	25 °C	FI < FS	100.39	**< 0.0001**†	2565 (2600)
		FI < MS	109.59	**< 0.0001**†	2537 (2580)
		FS = MS	0.32	0.57	2512 (2558)

The columns show the directionality of lifespan effects for each pairwise comparison between the three karyotypes (FI = Florida inverted, FS = Florida standard, MS = Maine standard), grouped by sex and temperature. χ^2 test statistics and P-values are from generalized Wilcoxon tests. Significant effects are in bold; significance after Bonferroni correction is indicated by † ($\alpha' = 0.05/3 = 0.016$). n represents the number of dead individuals; the total cohort size is shown in parenthesis. See Results and Fig. 1 and Fig. S1 for further details.

In(3R)P confers increased mortality to cold shock

To examine whether the *In(3R)P* inversion also contributes to cold tolerance, we investigated the mortality of flies upon 24 h of exposure to cold shock at −4 °C (Fig. 3). In both females and males, karyotypes did overall not differ in cold-shock mortality (Cox LRT; females: $\chi^2_{(2)} = 2.1$, $P = 0.39$; males: $\chi^2_{(2)} \approx 0$, $P = 1.0$), but there was a significant $K \times T$ interaction for females (females: $\chi^2_{(2)} = 9.2$, $P = 0.01$; in males, the interaction term could not be fit as at 18 °C, all males survived and were censored from analysis). Temperature affected cold-shock survival in both sexes (Cox LRT; females: $\chi^2_{(1)} = 350.7$, males: $\chi^2_{(1)} = 1529.8$, both $P < 0.0001$). Pairwise comparisons between the three karyotypes with GWT showed that at 25 °C, FI inverted females survived cold shock less well than both FS and MS uninverted females; at the same time, uninverted high-latitude females from Maine survived cold shock

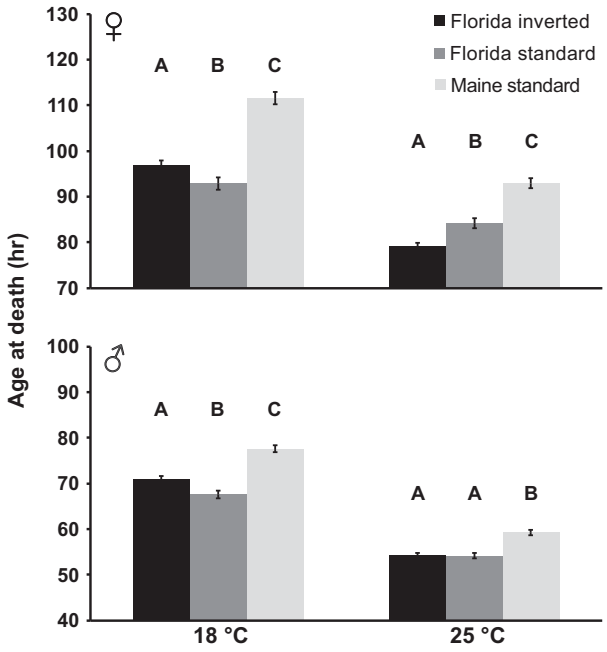

Fig. 2 *In(3R)P* affects starvation in a temperature-dependent manner. Effects of *In(3R)P* on age at death (hours) upon starvation in females and males. Shown are means and standard errors. Black bars: Florida inverted (FI), dark grey bars: Florida standard (FS), light grey bars: Maine standard (MS). Results for pairwise comparisons among karyotypes with generalized Wilcoxon (χ^2) tests are shown in letters: groups that do not contain the same letter are significantly different from each other ($P < 0.05$). At 18 °C, Florida inverted flies survive starvation better than uninverted flies, whereas this pattern is reversed for females at 25 °C. Moreover, high-latitude flies from Maine are overall more resistant than low-latitude flies from Florida. See Results and Table 2 for details; for survival curves see Fig. S2.

Table 2 Analysis of survival upon starvation.

Sex	Temperature	Direction	χ^2	*P*	*n*
Female	18 °C	FI > FS	5.35	**0.02**	881
		FI < MS	72.94	**< 0.0001**†	878
		FS < MS	90.88	**< 0.0001**†	899
	25 °C	FI < FS	10.14	**0.0014**†	899
		FI < MS	88.68	**< 0.0001**†	900
		FS < MS	25.99	**< 0.0001**†	899
Male	18 °C	FI > FS	10.44	**0.0012**†	900
		FI < MS	34.72	**< 0.0001**†	897
		FS < MS	78.31	**< 0.0001**†	897
	25 °C	FI = FS	0.15	0.70	898
		FI < MS	30.44	**< 0.0001**†	896
		FS < MS	31.60	**< 0.0001**†	894

The columns show the directionality of survival upon starvation for each pairwise comparison between the three karyotypes (FI = Florida inverted, FS = Florida standard, MS = Maine standard), grouped by sex and temperature. χ^2 test statistics and *P*-values are from generalized Wilcoxon tests. Significant effects are in bold; significance after Bonferroni correction is indicated by † ($\alpha' = 0.05/3 = 0.016$). *n* represents the total cohort size, that is the number of dead individuals (no flies were censored in this assay). See Results and Fig. 2 and Fig. S2 for further details.

better than both low-latitude karyotypes from Florida (Fig. 3; Table 3). In contrast, we found no differences among karyotypes at 18 °C and for males at both temperatures (Fig. 3, Table 3). For both females and males, flies survived cold shock better at 18 °C than at 25 °C (see significant effect of *T* in Cox regression above; GWT, females: $\chi^2_{(1)} = 652.2$, males: $\chi^2_{(1)} = 1875.1$, both $P < 0.0001$; Fig. 3). At 25 °C, females tended to survive cold shock better than males (GWT, 25 °C: $\chi^2_{(1)} = 41.0$, $P < 0.0001$; as at 18 °C, all males were censored for analysis, we did not compare the two sexes at this temperature; Fig. 3). *In(3R)P* thus confers increased mortality to cold shock, and uninverted female flies from Maine tend to survive acute cold exposure better than low-latitude flies from Florida, at least at 25°.

The *In(3R)P* inversion might represent a life-history 'supergene'

The fact that *In(3R)P* affects body size (Weeks *et al.*, 2002; Rako *et al.*, 2006; Kapun *et al.*, 2016b) and, as shown above, several survival traits indicates that this inversion might represent a 'supergene', a set of tightly linked loci that affects multiple complex phenotypes (Schwander *et al.*, 2014). Consistent with this idea, we found that karyotype has a significant upon the multivariate life-history phenotype (i.e. a linear combination of size, lifespan, starvation resistance and cold-shock survival), using multivariate analysis of variance (MANOVA; for details see Appendix S1).

Discussion

High-latitude flies are more long-lived and stress resistant

Populations of *D. melanogaster* in North America, and also on other continents, display gradients of phenotypic differentiation for fitness-related traits such as body size, fecundity, stress resistance, reproductive dormancy and longevity across latitude (Coyne & Beecham, 1987; de Jong & Bochdanovits, 2003; Hoffmann *et al.*, 2005; Schmidt *et al.*, 2005a, b; Schmidt & Paaby, 2008; Paaby *et al.*, 2014; Fabian *et al.*, 2015; Mathur & Schmidt, 2017). These patterns of clinal differentiation are hypothesized to be driven by differential selection pressures at high vs. low latitude (e.g. Paaby & Schmidt, 2009): genotypes that confer stress resistance and survival at the expense of reduced fecundity might be favoured at high latitudes, where seasonal stressors such as cold and food shortage impose strong selection on somatic maintenance, whereas at low latitude, selection might favour alternative genotypes that confer fast

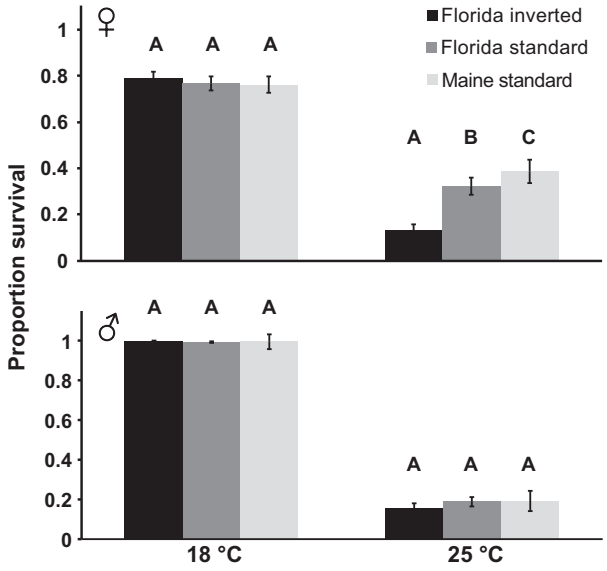

Fig. 3 At 25 °C, *In(3R)P* confers mortality upon cold shock. Effects of *In(3R)P* on the proportion of female and male flies surviving cold shock. Shown are means and standard errors. Black bars: Florida inverted (FI); dark grey bars: Florida standard (FS); light grey bars: Maine standard (MS). Results for pairwise comparisons among karyotypes with generalized Wilcoxon (χ^2) tests are shown in letters: groups that do not contain the same letter are significantly different from each other ($P < 0.05$). The *In(3R)P* inversion increases sensitivity to cold shock at 25 °C. Generally, at 25 °C, high-latitude females from Maine are more cold-shock resistant than low-latitude females from Florida. See Results and Table 3 for details.

development and high fecundity at the expense of reduced stress resistance and survival. In support of this adaptive scenario, we observed that high-latitude flies from Maine lived longer and were more resistant to starvation and cold stress than low-latitude flies from Florida, consistent with previous observations along the North American cline (Schmidt *et al.,* 2000, 2005a, b; Schmidt & Paaby, 2008; Paaby *et al.,* 2014; Mathur & Schmidt, 2017).

While the genetic basis of latitudinal clines for survival traits is poorly understood, many strongly clinally varying single nucleotide polymorphisms (SNPs) are located in genes known to be important for the determination of adult lifespan and stress resistance, for example in the insulin/insulin-like growth factor signalling (IIS) pathway (Fabian *et al.,* 2012; Kapun *et al.,* 2016a). This observation opens up an opportunity for identifying naturally segregating polymorphisms that affect lifespan and stress resistance (Flatt & Schmidt, 2009; Paaby & Schmidt, 2009).

Three examples serve to illustrate this point. A haplotype at the *methuselah* (*mth*) locus, a gene known from mutant studies to affect longevity and stress resistance, shows a 40% cline in frequency across the North American east coast that coincides with

Table 3 Analysis of survival upon cold shock.

Sex	Temperature	Direction	χ^2	*P*	*n*
Female	18 °C	FI = FS	0.64	0.43	197 (887)
		FI = MS	0.88	0.35	199 (888)
		FS = MS	0.02	0.89	210 (893)
	25 °C	FI < FS	47.39	**< 0.0001**†	694 (897)
		FI < MS	77.48	**< 0.0001**†	664 (896)
		FS < MS	4.18	**0.04**	580 (899)
Male	18 °C	–	–	–	0 (896)
		–	–	–	0 (897)
		–	–	–	0 (895)
	25 °C	FI = FS	2.01	0.16	737 (887)
		FI = MS	2.35	0.14	734 (885)
		FS = MS	0.01	0.90	719 (886)

The columns show the directionality of survival upon cold shock for each pairwise comparison between the three karyotypes (FI = Florida inverted, FS = Florida standard, MS = Maine standard), grouped by sex and temperature. χ^2 test statistics and *P*-values are from generalized Wilcoxon tests. Significant effects are in bold; significance after Bonferroni correction is indicated by † ($\alpha' = 0.05/3 = 0.016$). *n* represents the number of dead individuals; the total cohort size is shown in parenthesis. Note that at 18 °C all males survived 24 h of cold shock and were thus all censored. See Results and Fig. 3 for further details.

among-population differences in life-history traits including lifespan, even though phenotypic effects of this haplotype were not directly examined (Schmidt *et al.,* 2000; Duvernell *et al.,* 2003); subsequent work identified effects of wild-derived *mth* alleles on lifespan, fecundity and stress resistance (Paaby & Schmidt, 2008). Another example comes from a clinally varying indel polymorphism in the *insulin-like receptor* (*InR*) which affects lifespan and stress resistance in the predicted clinal direction, with the high-latitude genotype conferring improved stress resistance and survival (Paaby *et al.,* 2010, 2014). Similarly, an amino acid polymorphism in the *couch potato* (*cpo*) gene explains clinal variation in the ability of flies to undergo reproductive dormancy (Schmidt *et al.,* 2008), a plastic state associated with greatly improved stress resistance and lifespan (Schmidt & Paaby, 2008; Flatt *et al.,* 2013).

As the *In(3R)P* polymorphism is *the dominant* driver of genotypic latitudinal clines in North America (Fabian *et al.,* 2012; Kapun *et al.,* 2016a), either due to direct or indirect selection (via genetic draft/'hitchhiking'), it is interesting to ask whether the cline in *In(3R)P* might contribute to the phenotypic clines seen for survival traits. Addressing this question was the main purpose of our study.

In(3R)P contributes to latitudinal clinality of multiple survival traits

Recent evidence has shown that *In(3R)P* is adaptively maintained by spatially varying selection along the

North American cline (Kapun *et al.,* 2016a), but how this inversion polymorphism affects trait differentiation is poorly understood. In previous assays, we found that *In(3R)P* affects body size, consistent with observations from Australia (e.g. Rako *et al.,* 2006), but developmental time, egg-to-adult survival, chill coma recovery, oxidative stress resistance and lipid content were unaffected by this inversion (Kapun *et al.,* 2016b). In agreement with our findings for North America, the Australian study by Rako *et al.* (2006) also failed to find effects of *In(3R)* on developmental time and chill coma recovery. Despite these negative results for traits beyond size, here, we have found that the North American cline in *In(3R)P* underpins, at least to some extent, latitudinal clines in three survival traits, that is lifespan, starvation resistance and survival upon cold shock (Schmidt *et al.,* 2000, 2005a, b; Schmidt & Paaby, 2008; Paaby *et al.,* 2014; Mathur & Schmidt, 2017). The effects of *In(3R)P* on these traits go in the predicted clinal direction, with flies from Maine and Florida possessing the uninverted arrangement being on average longer-lived and more stress resistant than flies from Florida which carry the inverted *In(3R)P* segment. Importantly, this establishes that the *In(3R)P* polymorphism affects – and harbours genetic variance for – multiple, clinally varying components of fitness. This is in line with the observation that the genomic region spanned by *In(3R)P* contains numerous clinally varying SNPs in genes known to affect body size, lifespan, stress resistance and other fitness-related traits (Fabian *et al.,* 2012; Kapun *et al.,* 2016a, b). For example, it is noteworthy in this context that both *InR* and *cpo* (see above) are located in the region spanned by *In(3R)P*.

Although work by Rako *et al.* (2006) and us (Kapun *et al.,* 2016b) did not find an effect of *In(3R)P* on cold tolerance in terms of chill coma recovery (but see Anderson *et al.,* 2003), our experiments here show that the inverted arrangement is associated with increased mortality in response to cold shock in females. This result is in good qualitative agreement with the data of Anderson *et al.* (2003), who found that cold-shock mortality is associated with a genetic marker (*hsr-omega*) that is in linkage disequilibrium (LD) with *In(3R)P* and also with the earlier findings of Tucic (1979), who found a major effect of chromosome 3 on larval and adult cold tolerance. The fact that different measures of cold tolerance (chill coma recovery vs. cold-shock mortality) can give discordant results implies that the details of assay protocols used for measuring aspects of cold tolerance matter greatly (Macdonald *et al.,* 2004; Andersen *et al.,* 2015). Importantly, Andersen *et al.* (2015) found that measures of the time to lethality at low temperature are not correlated with chill coma recovery time.

Thus, by experimentally isolating and phenotyping *In(3R)P* karyotypes from populations approximating the endpoints of the North American cline, our results suggest that this inversion makes a major contribution to the clinality of several survival traits. An open task for future work will be to regress means of survival traits for multiple populations spanning the cline against the population frequency of *In(3R)P* as a predictor, for this would allow to estimate the amount of phenotypic variance explained by *In(3R)P*.

In a preliminary analysis using MANOVA, we also found evidence that *In(3R)P* affects the multivariate combination of life-history traits (i.e. a linear combination of a size proxy and the three survival traits; see Appendix S1), indicating that this inversion might represent a life-history 'supergene' (Schwander *et al.,* 2014).

How is the *In(3R)P* polymorphism maintained?

Although *In(3R)P* is maintained by selection along the North American cline (Kapun *et al.,* 2016a), the details of the underlying selective mechanisms remain unknown. Clearly, our results cannot fully explain how this polymorphism is maintained as the inverted segment seems to have predominantly negative effects on the measured fitness components: inverted homokaryons were on average smaller, shorter-lived and less stress resistant than uninverted homokaryons (also see Kapun *et al.,* 2016b). So, what are the fitness benefits that maintain the inverted karyotype at low latitude?

Four major considerations should be kept in mind. First, we have only phenotyped inverted vs. uninverted homokaryons but not any heterokaryons: because inversions might be maintained by overdominance or associative overdominance (Dobzhansky, 1970; Kirkpatrick & Barton, 2006), it will be critical to phenotype *In(3R)P* heterokaryons in future work (cf. Rako *et al.,* 2006). Second, inversion polymorphisms can be maintained by frequency-dependent selection. For example, Nassar *et al.* (1973) reported that *In(3R)P* might be subject to frequency-dependent selection under conditions of larval crowding. However, on theoretical grounds, it is unclear how frequency-dependent selection would be able to maintain an inversion polymorphism for a long period of time, since even small amounts of recombination or gene conversion in heterokaryons will destroy LD between the genic target(s) of balancing selection and the inversion (Kirkpatrick & Barton, 2006). Nonetheless, it would be interesting to reassess the findings of Nassar *et al.* (1973) and to directly investigate, for example, larval competitive ability as a function of *In(3R)P* karyotype. Third, there are several fitness-related traits that we have not measured on the homokaryons, including fecundity: since short-lived low-latitude flies are more fecund than long-lived high-latitude flies (Schmidt & Paaby, 2008), an important open question is whether *In(3R)P* affects fecundity and the trade-off between fecundity and lifespan. Putative effects of *In(3R)P* on fecundity and the fecundity-

lifespan trade-off (or on other traits, such as larval competitive ability) might potentially help to explain how this inversion contributes to the maintenance of stable phenotypic clines across latitude. Fourth, the fitness components through which the *In(3R)P* polymorphism is maintained might be subject to genotype by environment interactions. In our assays, we phenotyped homokaryons at two growth temperatures and indeed found several karyotype by temperature interactions. Perhaps most interestingly, we observed that at 18 °C, Florida inversion homokaryons are significantly more resistant to starvation stress than Florida uninverted homokaryons, whereas this pattern was reversed for females at 25 °C. However, this effect was very small; moreover, while the latitudinal temperature gradient is a major determinant of the cline in *In(3R)P*, other latitudinally varying environmental factors (e.g. precipitation, seasonality) seem to be important too (Kapun *et al.*, 2016a). Determining how selection maintains *In(3R)P* will ultimately depend on a more detailed understanding of the environmental factors that affect this system.

Conclusions

Here, we have asked whether a clinally varying chromosomal inversion polymorphism in *D. melanogaster*, *In (3R)P*, affects the clinal distribution of three fitness-related traits: adult lifespan, survival of starvation stress and survival upon acute cold shock. Our central finding is that the cline in *In(3R)P* contributes to the latitudinal clines observed for these survival traits, in addition to effects of geography that are independent of this inversion (Schmidt *et al.*, 2000, 2005a, b; Schmidt & Paaby, 2008; Mathur & Schmidt, 2017). Together with the fact that *In(3R)P* underpins latitudinal clines in body size (Rako *et al.*, 2006; Kapun *et al.*, 2016b), our results thus suggest that this inversion might represent a clinally varying life-history 'supergene' (On the other hand, we cannot yet rule out that the effects on multiple complex traits are due to a single pleiotropic locus within the inversion). In particular, our findings support the idea that life-history clines in *D. melanogaster* are maintained by fitness trade-offs across geography and that *In (3R)P* contributes to this maintenance. However, the precise nature of the evolutionary forces and fitness effects that keep this chromosomal inversion polymorphic in some places but not others remains to be elucidated.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Survival curves as a function of *In(3R)P* karyotype and temperature. Effects of *In(3R)P* and temperature (18 °C vs. 25 °C) on the proportion adult survival in females and males. The different curves represent Florida inverted (black), Florida standard (red), and Maine standard (blue). See Results, Fig. 1, and Table 1 for details.

Figure S2 Starvation survival curves as a function of *In (3R)P* and temperature. Effects of *In(3R)P* and temperature (18 °C vs. 25 °C) on the proportion adult survival upon starvation in females and males. The different curves show Florida inverted (black), Florida standard (red), Maine standard (blue). See Results, Fig. 2 and Table 2 for details.

Appendix S1 A preliminary analysis of trait relationships. Supporting results text describing a (i) preliminary analysis of the relationship between wing area, as a proxy of body size and lifespan and (ii) MANOVA on multivariate life-history phenotype (i.e., a linear combination of size, lifespan, starvation and cold survival).

Data deposited at Dryad: https://doi.org/10.5061/dryad.3vb89dj

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