Genetic variation in behavioral and physiological response to environmental change

Benedict Adam Lenhart

Hillsboro, Virginia

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Abstract

The ability of a population to evolutionarily adapt to environmental changes is in part mediated by the genetic variation within the population. Genetic variation allows for presence of alleles with varying fitness contributions, and researchers have sought to characterize how different evolutionary forces act to preserve genetic variation within and across different environments. By using modern genetic resources and methods of trait quantification, we can further understand how genetically diverse populations adapt to rapid changes in environment such as catastrophic natural disasters. In my dissertation, I study how elements of natural genetic variation present within the fruit fly model organism *Drosophila melanogaster* allow for variation in trait responses to rapid changes in environment. In my first chapter, I report how genetic variation in *Drosophila* results in differences in the physiological response to fasting. My second chapter tests the impacts cosmopolitan inversions have on physiological, behavioral, life-history, and morphology, and identifies association mapping strategies that improve our ability to link phenotypic variation to inversions. My third chapter describes the role of a common inversion in the regulation of different aspects of behavior, and the environmental sensitivity of this relationship. Ultimately, my dissertation pushes forward the study of how genetic variation allows a range of responses to rapid environmental changes. Through genomic analysis and experimental validation, I further characterize our understanding of the role inversions play in altering traits, and additionally offer insights into how natural genetic variation can be considered during study design, especially when investigating traits and genomic regions associated with inversions.

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Introduction

Animals must adapt in the face of new environmental stresses and challenges (Bijlsma & Loeschcke, 2005; Dobzhansky, 1942; P. C. Lee, 1991). Adaptation can take the form of phenotypic plasticity at the individual level (Ghalambor et al., 2007), or a population-level adaption through change in allele frequency (Günther & Coop, 2013). In the face of rapid, cyclically changing environments such as seasons, variation in selection can drive rapid adaptation in a wide array of phenotypes such as starvation resistance, metabolic rate, and behavior (Hoffmann & Harshman, 1999; Shaw & Couzin, 2013; Swanson, 2010). In many of these instances, the fitness of given trait value for a phenotype can fluctuate across seasons (Simons, 2009; Wittmann et al., 2017). Animals with longer generation time relative to seasons can respond to changing fitness landscapes with phenotypic plasticity, while populations with shorter generation times relative to seasonal change instead undergo evolutionary adaptation mediated by standing genetic variation (Botero et al., 2015). It is an area of ongoing study how the genetic variation necessary for rapid and repeated adaptation is maintained over time within a population.

Balancing selection and the different forces underlying it has been proposed as a mechanism of preservation of genetic variation within populations. In a deterministic model, directional selective pressure will generally lead fitness-conferring alleles to fixation (Patwa & Wahl, 2008). Strong selection can even push nearby, highly linked loci to fixation as well (Stephan, 2019). Both of these processes reduce genetic variation within a population. In contrast, the forces included within balancing selection can maintain alleles within intermediate frequencies (Turelli & Barton, 2004). Forms of balancing selection include adaptive tracking, in which allele frequences can rapidly shift to track changes in fitness landscapes (Bergland et al., 2014; Bertram & Masel, 2019), or other forms of fluctuating selection, in which the direction of selective pressures on one or more alleles changes and thus prevents total fixation (Fitzpatrick et al., 2007). Balancing selection can be traced to pleiotropy, in which a given loci impacts multiple traits in an adaptive or mal-adaptive manner, and is potentially subject to contrasting selection across environments (Barton, 1990; Bitter et al., 2024), as well as from gene by environment interactions, in which the effect of a loci on a trait is itself environment specific (Turelli & Barton, 2004). Drivers of balancing selection preserve the natural variation responsible for rapid adaptation, however the genetic architecture involved in maintaining natural variation across seasonal cycles remains incompletely understood.

Genomic inversions are a structural variant with a complex effect on the architecture of a genome and previous evidence of involvement in environment adaption. Inversions

suppress recombination (Dobzhansky & Epling, 1948), and thus can allow for balancing selection and maintenance of genetic variation within a population (Berdan et al., 2023; Charlesworth & Flatt, 2021; Wellenreuther & Bernatchez, 2018). Genomic inversions generate linkage between many genes and mutations, allowing for the maintenance of high-fitness and deleterious variants within the inversion (Kirkpatrick & Barton, 2006; Saltz et al., 2017). This complex impact on fitness has contributed to a role of inversions in organismal adaptation across the tree of life (Harringmeyer & Hoekstra, 2022; Lowry & Willis, 2010; Stefansson et al., 2005). One way inversions can provide an avenue for the preservation of gene variation is gene by environment interactions, in which the fitness and selection on loci fluctuate throughout different environments and thus prevents total fixation (Turelli & Barton, 2004). Additionally, the many genes potentially contained within inversions can result in pleiotropic effects on multiple traits (Kirkpatrick & Barton, 2006; Saltz et al., 2017), in which complex selection pressure can drive balancing selection (Barton, 1990). Inversions are a potentially powerful tool for studying adaptation to rapid environmental change, especially within models carrying them at intermediate frequencies.

The fruit fly *Drosophila melanogaster's* powerful genetic tools, wealth of past literature, and unique genomic identity make it an ideal model for studies into the mechanisms into adaptation. *D. melanogaster* populations are found worldwide, and are subject to rapidly changing environments as these populations persist through seasons (Bergland et al., 2014b; Rodrigues et al., 2021). Research into *Drosophila* lineages with significant genetic variation includes the DGRP (Mackay et al., 2012), globally-derived isofemale lines (Behrman et al., 2018; Grenier et al., 2015; Kao et al., 2015), and a publicly available database of allele frequencies across space and time (Kapun et al., 2021). Some of the considerable phenotyping work done on *Drosophila* has been consolidated for use and meta-analysis (Gardeux et al., 2023; Nunez et al., 2024). Additionally, *Drosophila* are a popular model for investigating human health and dietary outcomes due to their high homology with human genetics (Faria & Sucena, 2017; K. P. Lee et al., 2008). *D. melanogaster* are genomically unique in that they not only contain several large inversions maintained across the globe (Nunez et al., 2024; Stalker, 1980; van Delden & Kamping, 1989), but in that the demographic history indicates these insects have migrated and persist worldwide (Bergland et al., 2016; David & Capy, 1988; Stephan & Li, 2007) The adaptation of Drosophila to novel environments and seasons and rich genetic resources of the species indicates Drosophila are a strong model for studies into investigating the role inversions play in influencing fruit fly traits and mediating adaptation to new environments.

With my dissertation, I study how genetic variation within a population could allow for rapid adaptation to new environments, which traits are most impacted by these variants, and

how to better identify variant-trait associations. I first address the question of how populations could adapt to changes in nutrient availability by completing a dietary manipulation assay consisting of *Drosophila* with diverse genetic backgrounds. Fly populations collected in environments diverse in location and season were raised under different nutrient conditions to measure the impact on starvation. Next, I addressed the question of how genomic inversions could at as mediators of adaption by examining which phenotypes are significantly impacted by presence of inversion within *Drosophila melanogaster*. This study follows up with a set of comparative GWAS models to identify which method is better able to identify associations within inverted regions. Last, I address the question of how cosmopolitan inversion In(2L)t could be mediating seasonal adaptation by examining its role in aspects of behavior across different environmental conditions.

In my first chapter, I place different *Drosophila* lineages through regular fasting to understand how their genetic background influences subsequent fat storage and starvation resistance. I replicated an intermittent fasting design previously established (Zhang et al., 2018), and replicated the finding that intermittent fasting can improve starvation resistance within common laboratory backgrounds (Catterson et al., 2018; Zhang et al., 2018). However, more recently established lineages (Behrman et al., 2018; Grenier et al., 2015; Kao et al., 2015; Mackay et al., 2012) showed considerable variance in the extent to which fasting altered their energy storage and ability to survive nutrient deprivation. Some lines improved in starvation resistance post fasting, while others declined in resistance. These results suggest that improvement of starvation resistance via fasting is highly genetically variable, and supports the general conclusion that phenotypes observed within common lab settings may not be replicated in other genetic backgrounds.

In my second chapter, I identify traits impacted by common *Drosophila* inversions, and test the ability of different methods to identify association between inversion-linked loci and these traits. I utilize phenotype studies in the DGRP that have been subsequently aggregated (Gardeux et al., 2023) into a single data set with information on trait classes such as behavior, life history, and morphology. I modeled the effect of inversion presence on these traits and discovered that there are several inversions within *Drosophila* that have a significant impact on several different classes of traits. I followed up with a comparative GWAS study that tested the ability of different GWAS methods to identify associations within inversions. I found that commonly used methods of GWAS appear underpowered in their findings compared to alternatives. These results indicate that inversions have a significant role to play across multiple classes of traits in *D. melanogaster*, and there are available improvements for our current methods of identifying associations within these inversions.

In my third chapter, I performed a series of behavioral studies of a diverse set of crosses to explore the role of In(2L)t on mediating behavior across different environments. Using some of the same DGRP lines from chapter 2, I produced sets of F1 crosses with different genotypes of In(2L)t and observed their behavior using activity quantification hardware. I used a motor to deliver stimuli, and noted activity before and after the startle event. I repeated these experiments at different temperatures to simulate the different thermal environments the inversion is present in across seasons. I observed differences between inversion genotypes in time spent active between genotypes, as well as time spent on the food source. In(2L)t exhibited complex effects on startle response, with the effect on duration and intensity of induced response attenuated or emphasized at different temperatures.

My dissertation illuminates multiple avenues by which populations can adapt to seasonal change, from genetic variation within diet-induced traits, to the ability of inversions to drive temperature-specific changes in behavioral traits. By observing genetically diverse samples across different nutritional and thermal environments, my research gives insight into how genetic variance can lead to variance in phenotypic response. By testing the effect of inversions on traits and comparing different methods of association at understanding inverted loci, I push forward our understanding of how inversions and the variants within them can act on different traits and how to best identify these associations. Adaptation to environmental stressors and challenges lies at the heart of evolutionary studies, and this dissertation takes this research several steps forward into understanding these complex natural phenomena.

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Chapter 1

Improvement of starvation resistance via periodic fasting is genetically variable in *Drosophila melanogaster*

Authors: Adam Lenhart[1], Ayesha Ahsan[1], Margaret McHaty[1], Alan O. Bergland[1]

[1]Department of Biology, University of Virginia, Charlottesville, Virginia, 22554

Contact: Adam Lenhart, benedictlenhart@gmail.com

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Abstract

Organisms subjected to periodic nutrient limitation early in life exhibit improvements in aspects of survival, including resistance to some environmental stressors. Recent findings indicate that forms of periodic fasting such as intermittent fasting and time restricted feeding can improve starvation resistance. However, it remains unclear to what extent this survival improvement persists across different genetic backgrounds. In this study, we examine fasting-induced starvation resistance across a broad survey of wild-derived lineages and document genetic variation within this trait. We adopt a standard dietary intervention and show improvement to starvation resistance within a common laboratory lineage, replicating previous results. Next, we examine fasting-induced starvation resistance across isofemale lines collected across latitudes and in different seasons, and among inbred lines derived from flies collected on different continents. We discover genetic variation of fasting-induced starvation resistance, and show that fasting improved starvation resistance as often as it worsened starvation resistance. Fasted flies generally showed reduced fat concentration, and their starvation survival varied with sex, season of collection, and geographic origin. While specific lineages common to the laboratory can show a specific fasting-induced phenotype, we show that this result is not consistent across genetic backgrounds, reinforcing the idea that phenotypes observed in historic laboratory strains may not be conserved across a species.

Introduction

Limitations to feeding and nutrient intake can benefit lifespan and survival, and this benefit is broadly conserved across species (Kapahi et al. 2017). Dietary restriction can take several forms. Caloric restriction reduces calorie intake although it does not necessarily alter food availability (Heilbronn & Ravussin 2003). Alternatively, dietary restriction assays can function by limiting when food is available, via daily or weekly schedules of periodic fasting (Aly 2014). Short periods of nutrient deprivation are thought to provide physiological resistance to additional stressors (Bubliy et al. 2011) and long-term periodic fasting has recently been shown to benefit starvation resistance (Catterson et al. 2018, Zhang et al. 2018). While these recent studies represent an advancement in our understanding of dietary interventions, it remains unclear to what extent fasting-induced stress resistance is conserved across variable genetic backgrounds.

The well-studied metabolism, powerful genetic tools, and broad global presence of *Drosophila melanogaster* make it an ideal model organism for studies seeking to describe genetic variation of dietary responses and metabolic traits (Faria & Sucena 2017, Lee et al. 2008, p. 200). Fly metabolism is commonly used to gain new insights into broader animal metabolism, as the systems of signaling and regulation present in flies are broadly homologous to many other animals (Kapahi et al. 2017). Decades of research on dietary restriction using *D. melanogaster* have identified traits impacted by fasting, potential genes involved, and shared genetic mechanisms between model and non-model organisms (reviewed in Krittika & Yadav 2019). Periodic fasting studies using flies have identified improvements to lifespan, gut health, and somatic maintenance (Gill et al. 2015, Catterson et al. 2018, Zhang et al. 2018). Several recent studies have independently noted the ability of periodic fasting to impact fat storage and improve starvation resistance. Catterson *et al.* (2018) used a 5-day fasted, 2-day fed model to document improved starvation resistance compared to fully-fed flies. Zhang et al. (2018) confirmed improvement to starvation resistance with fasting for 8 hours of the day, three days a week. Increased storage of triglyceride fats is implicated in starvation resistance (Rion & Kawecki, 2007), however Catterson *et al.* (2018) and Zhang *et al.* (2018) report opposing trends in fat storage among fasted flies, indicating variance in this fat-storage trait. However, these studies use common laboratory lines of Canton-S and w1118, and thus only examine a fraction of the standing genetic diversity present in *D. melanogaster*.

Exploring natural genetic variation present in model systems can greatly inform the understanding of the relevant genetic loci for any trait of interest (Gasch et al. 2016). Many studies, especially those of model laboratory systems, seeking to understand the genetic basis for a disease, phenotype, or pathway would benefit from examining the variation in that trait among naturally occurring populations (Benfey & Mitchell-Olds, 2008). Indeed, several studies have shown that genetic variation of those subjected to dietary interventions significantly alters the success of the interventions (Liao et al, 2010, Jin et al. 2020). For instance, a 40% calorie reduction imposed on a panel of inbred mice reduced lifespan more often than extended it (Liao et al 2010), and considerable variation in the

lifespan and metabolites of fasted *Drosophila* inbred lines has also been observed (Jin et al 2020). Responses to dietary interventions such as intermittent fasting can vary depending on the presence of specific alleles and epistatic interactions among different genetic backgrounds (Heianza & Qi, 2017). Metabolic studies into *Drosophila* are especially able to assay a diverse range of genetic variation, as *D. melanogaster* exist world-wide within a variety of nutrient-specific environments (Grenier et al 2015). However, *Drosophila* periodic fasting studies typically employ a select subset of historic laboratory lineages. In addition, there is evidence that after enough generations raised within the lab, lineages will adapt to laboratory conditions, reducing the extent lab-adapted flies represent natural physiological responses (Matos et al. 2000, Russell & Kurtz, n.d. Linnen et al. 2001, Sgrò & Partridge 2000). While there is a rich history of research on established laboratory models, studies into the effect of periodic fasting on starvation resistance would benefit from employing a more diverse representation of the genetic variation present in nature.

Here, we replicate the intermittent fasting work reported in Zhang *et al.* 2018 and examine the extent to which this dietary regime can alter aspects of survival. Next, we measured the extent of genetic variation of periodic fasting-induced starvation resistance among *D. melanogaster* lineages collected in different seasons, orchards, and continents. The fasting protocol outlined in Zhang *et al.* 2018 can indeed improve the starvation resistance of both male and female *D. melanogaster* when completed in Canton-S / w118 F1 background. However, in more recently sampled lineages with diverse genetic variation this resistance trait varies significantly, with many lines failing to report any fasting-induced starvation resistance.

Materials and Methods

Drosophila stocks and husbandry. Stocks used for this study include a F1 cross of Canton-S and w118, inbred lines obtained from Maine (Behrman et al 2018), the American southeast, the Bahamas (Kao et al 2015), and the Netherlands (Grenier et al 2015), in addition to isofemale lines that were collected at Carter Mountain Orchard in Charlottesville, Virginia (latitude 37.99, longitude -78.47) and the Linvilla Orchard in Media, Pennsylvania (latitude 39.88, longitude -75.41). Flies were kept on a food medium composed of 86% water, 0.574% agar, 6.30% cornmeal, 1.52% yeast, 4.65% molasses, 0.39% propionic acid, 0.15% methylparaben, and 0.52% ethanol.

Fasting assay. To perform the fasting treatment, we constructed replicate vials of 30 flies less than five days old. Only gravid females were used for the isofemale and inbred line experiment, while the F1 experiment also included separate replicates for males. Four replicates of each genotype were either fasted from 9am to 5pm on a nutrient-less agar, or were kept in control conditions on normal media throughout. This fasting protocol was repeated 3 consecutive days out of the week. Starvation resistance was measured at the conclusion of the fasting treatment. For the isofemale line assay, we included additional replicates to measure starvation resistance after each subsequent week of fasting.

Starvation assay. Starvation resistance of flies was measured using the Diamonds method (Seong et al. 2020). Individual flies were transferred to each well of 96 well plates containing 1.5 agar solution. Plates were placed atop flatbed scanners and images were taken every hour until all flies had stopped moving (5-7 days). Images of the wells were processed using Sapphire software to estimate time of death for each fly (Seong et al. 2020) using a neural network trained on fruit fly movement. Sapphire performs semantic segmentation to distinguish between living versus dead flies based on the change-point analysis of location through time. To validate Sapphire's estimates, we performed a test assay in which flies were placed in 96 well plates and starved as described above, with the mortality observed from manual observations of the images confirming the estimates from Sapphire (results not shown).

Metabolite measurement assay. The triglyceride content of fly samples was estimated using coupled calorimetric determination (Tennessen et al, 2014). Each fly sample was homogenized in PBST (PBS + 0.05 % Tween80) using an electric pestle. The samples were centrifuged, and 100µl of supernatant was frozen for future use. The rest of the supernatant was separated, and heat-fixed for 30 minutes at 70°C. 20µl of each sample was added to wells of a 96 well plate. Each sample was paired with a well of 20µl PBST as a negative control. 20µl of triglyceride reagent (Sigma; T2449) was added to each sample on a 96 well plate, as well to a coupled set of PBST controls. The plates were incubated at 37°C for 30 minutes, and then centrifuged. Last, 100µl of glycerol reagent (Sigma; F6428) was added to each sample and control well and incubated for another 5 minutes at 35°C. Absorbance of each well at 540nm was compared to a standard curve to infer fat content. Quantity of protein was measured using the Coomassie blue Bradford assay kit (Fisher 23200). 30ul of each previously frozen sample was mixed with 1.5mL of the Coomassie reagent and incubated for ten minutes. Absorbance of each sample at 595nm was compared to a standard curve to infer protein content.

Statistical inferences. In each fasting-induced starvation resistance assay, we measured time to death for control and fasted experimental groups with at least 3 independent replicate experiments of 20 flies per genotype per treatment group. We assessed the difference in starvation resistance between groups using the Cox proportional hazards test (Coxph), which models the probability of survival as a function of genotype and treatment. For each sex, we built three models and used likelihood ratio tests to assess the statistical significance of each model term using the log-rank test.

Model 1: Survival ~ Time + Treatment

Model 2: Survival ~ Time + Treatment + Genotype.

Model 3: Survival ~ Time + Treatment + Genotype + Treatment * Genotype

In addition, we also examined differences in phenotype (survival time, fat concentration) between two sets of observations using t-tests. We tested the correlation of fat concentration with survival with a Pearson's product moment test.

Results

Starvation resistance improvement can be replicated within a common lab lineage. To confirm the ability of periodic fasting to promote starvation resistance, we replicated a previously published fasting assay described in Zhang *et al*. 2018. This experiment used F1 males and females that are a cross between Canton-S and w118, and performed periodic fasting for three days out of the week. The fasted male flies experienced significantly higher starvation resistance than the control group (**Fig. 1A**, log rank test, $X^2 = 5.21$, df = 1, p= 0.02) confirming the findings of Zhang *et al.* 2018. The female fasted flies demonstrated even stronger improvement to starvation-resistance (**Fig. 1B**; log rank test, $X^2 = 23.51$, df = 1, p= 1.2e-06), as well as higher fasted resistance overall compared to fasted male resistance (**Fig. 1C**; t-test, t = -6.72, df = 84, p = 2.06e-09).

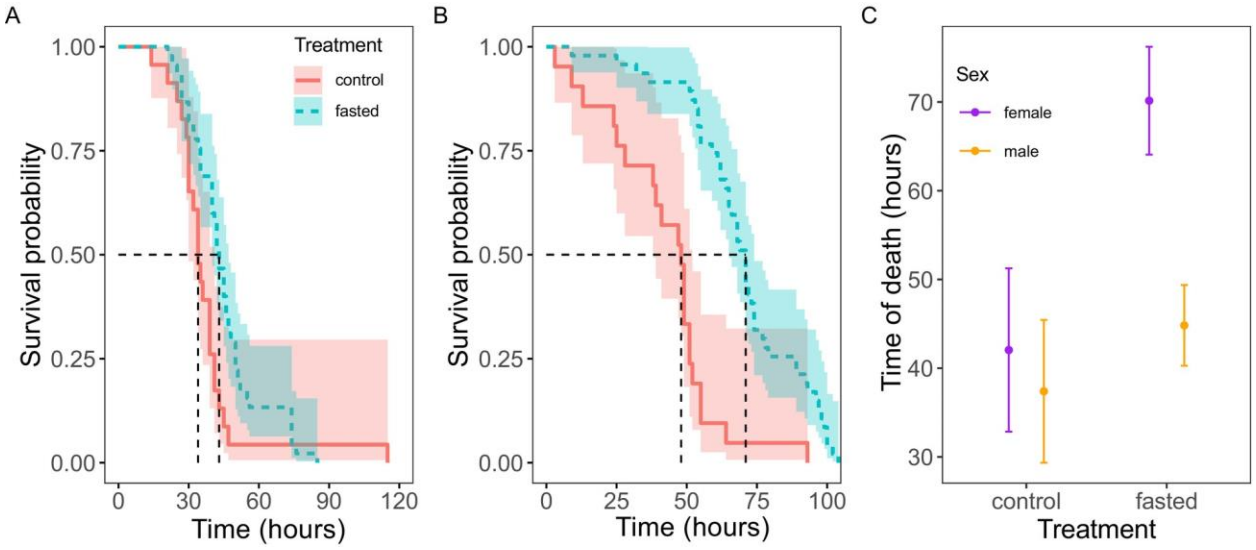

Figure 1. Replication of starvation resistance improvement. A) Comparison of the survival curves of male fasted (blue) and female control (orange) flies. The p value is derived from a log-rank interaction comparing the two groups. B) Survival curves of fasted and control female flies. C) The distribution of starvation times across treatments and sexes. Error bars represent 95% standard errors.

Genetic variation in fasting-induced starvation resistance. We assayed periodic-fasting induced starvation resistance across a global panel of inbred lines to gain a broader understanding of the natural variation within this trait. We used a set of inbred lines from the Netherlands, the Bahamas, Maine, and the southeastern United States, and fasted them in the same method as described above. The inbred panel showed markedly different responses to the fasting regimen compared to the Canton-S/W118 cross. Rather than improving starvation resistance, fasting overall decreased resistance amongst the inbred lines (**Fig. 2A,** log rank test, $X^2 = 6.86$, df = 1 p = 0.008) and we also find significant variation amongst the lines (**Fig. 2B,** log rank test, $X^2 = 109.2$, df = 7, p = 2.2e-16). While there was no

significant gene-by-environment interaction (log rank test, X^2 = 8.2, df = 6, p = 0.22), different lineages responded in opposing ways to fasting. Most lines (ex. USA Southeast 2) exhibited a decrease in starvation resistance with fasting, one with an increase in starvation resistance (ex. Netherlands-2) while others show no difference between treatments (ex. Bahamas-2; **Fig. 2C**). Fat concentration (µg triglyceride/µg body protein) also varied across groups. Control treatment flies contained significantly higher triglyceride concentrations than fasted flies after the treatment (t-test, t = 4.59, df = 61, p = 2.16e-05), with the extent of decrease varying amongst lineages (**Fig. 2D**). The level of prior-fat concentration was a marker of starvation survival success, and positively correlated with hours of survival (r = 0.246, df = 323, p = 6.9e-06).

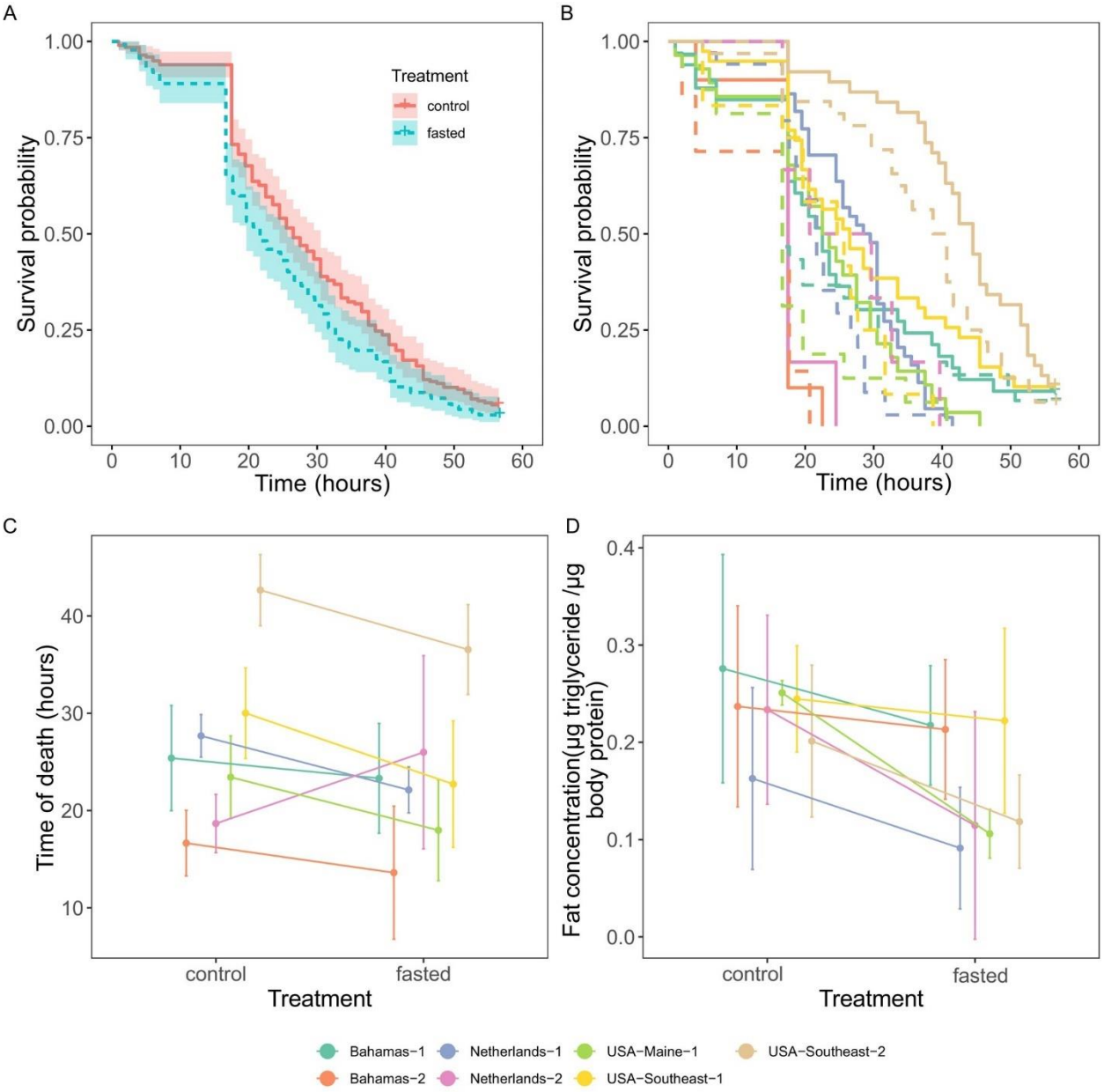

Figure 2. Natural variation in the response of a global panel to fasting. A) Survival curves of control and fasted inbred lines during starvation. B) Survival curves of individual inbred lineages. Color is determined by genotype, type determined by treatment. C) Variation in the impact of fasting on starvation resistance. D) Variation in the impact of fasting on triglyceride fat concentration (µg triglyceride/µg body protein).

Significant variation in fasting-induced starvation also occurs among recently established isofemale lines. To test if fasting-induced starvation resistance could be commonly found in outbred and recently sampled lineages, we examined isofemale lines collected at different times per year in Virginia and Pennsylvania. As with the inbred panel, we do not observe any significant improvement on overall average starvation resistance from the

treatment (**Fig. 3A,** log rank test, X^2 = 1.54, df = 1, p = 0.2). However, we find significant variation in the induced starvation resistance between different isofemale lines (**Fig. 3B**, log rank test, X^2 = 55.69, df = 7, p = 2.33e-11). Flies collected during the summer in Pennsylvania showed significant improvement in fasting-induced starvation resistance (**Fig.3C,** log rank test, X^2 = 4.85, df = 1, p = 0.027) while the other sets of recently collected isofemale lines do not show a change in starvation resistance following periodic fasting. There was no effect of season on starvation resistance among Pennsylvania samples (t-test, t = 1.96, df = 136, p = 0.052), while Virginia samples showed greater resistance in the fall (t-test, t = -4.99, df = 136, p = 1.77e-06) . As an additional assay, we tested starvation resistance after each subsequent week of treatment, to explore any age effect on fasting response. While there was no significant effect of fasting on the starvation survival at any week(week 1: t-test, t = 0.935, df = 302, p = 0.350, week 2: t = 1.21, df = 261, p = 0.223, week 3: t = -0.135, df = 270, p = 0.893) there was a significant effect of age on starvation resistance. 1 week old flies of either treatment resisted starvation longer than 3-week-old flies (**Fig. 3D**, t-test, t = -3.68, df = 549, p = 0.000258).

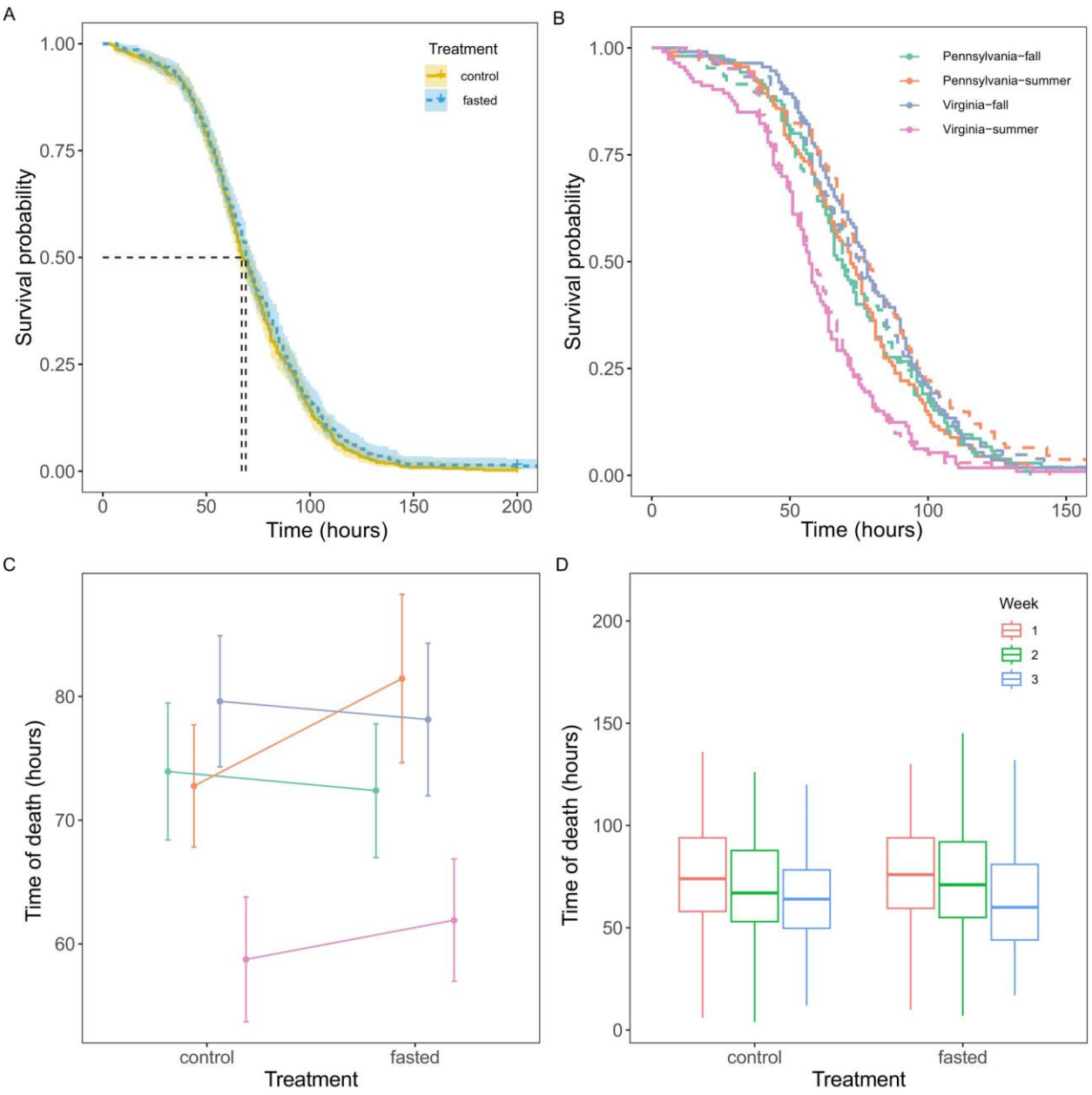

Figure 3. Significant variation in the fasting-induced starvation resistance in natural genetic lineages. A) Survival curves of control and fasted isofemale lines during starvation. B) Survival curves of individual isofemale lineages. Color is determined by genotype, type determined by treatment C) Variation in the impact of fasting on starvation resistance. D) Box and whiskey plot showing the change in hours of starvation survival across weeks of fasting, with color representing the weeks spent fasting prior to fasting.

Discussion

Periodic fasting has elicited changes in fitness traits across multiple species (Aly et al. 2014, Rothschild et al. 2014), and in recent years has been shown to strengthen starvation resistance in fasted *Drosophila* (Catterson et al. 2018, Zhang et al. 2018). However, it remains unclear to what extent these findings are specific to the historic laboratory lineages used, and to what extent natural genetic variation will influence these survival improvements. In this work we leverage the wealth of genetic variation available within *D. melanogaster* to test whether the effects of periodic fasting on survival are conserved within the species.

Periodic fasting promotes starvation resistance in the Cantons / w118 background. We replicated previously reported results (Zhang et al. 2018) of increased starvation resistance following periodic fasting of adult flies, compared to a fully fed control flies (**Fig. 1A-B).** This previous work only examined the males of an F1 cross of two historic lab lineages. Here we show that periodic fasting improves starvation resistance to an even greater extent within females of the same background. Female *Drosophila* have consistently shown greater starvation resistance then males (Rion and Kawecki, 2007, Schwasinger-Schmidt et al, 2012), and the improved starvation resistance of females could be mediated by greater triglyceride stores within the fat body of female flies compared to males (Millington and Rideout, 2018). Taken together, our results confirm that periodic fasting throughout adult life can promote increased starvation resistance.

Periodic fasting's ability to increase starvation resistance significantly varies across genetically diverse lineages. Extending the same fasting model to a global panel of inbred lines revealed significant variation in the effect of fasting on starvation resistance. Lines from as far south as the Bahamas and as far north as the Netherlands exhibited different resistance responses when subjected to periodic fasting (**Fig. 2B**). The general impact of fasting on starvation resistance was detrimental, with control flies out-surviving fasted flies. This finding is surprising given the general view that dietary restriction improves survival (Bubliy et al. 2012; Gill et al. 2015; Kapahi et al. 2017) and seems to contradict our previous results indicating starvation resistance improvement in a historic laboratory lineage (**Fig. 1A-B**). However, other studies have indicated that diverse genetic panels can prove to be exceptions to the commonly held rules of dietary restriction (Gomez et al 2020). For instance, there is no effect of dietary restriction on lifespan when the feeding of mice with natural genetic backgrounds was restricted (Harper et al. 2006). Additionally, dietary restriction both reduced and improved lifespan among a panel of 161 *D. melanogaster* lineages (Wilson et al 2020). In a similar manner, we demonstrate natural genetic variation in the response to dietary intervention (**Fig. 2C**). Some lines improved in starvation resistance, some were unaffected, and some deteriorated in resistance. The overall mean response trended toward lower resistance in fasted flies. Our metabolite findings could offer insight into this result, as fasting treatment appeared to have reduced energy storage amongst fasted flies. Fat concentration significantly increased in the control flies but not the fasted flies, implying the periodic fasting regime impaired the buildup of triglycerides

that generally occurs throughout fly adulthood (Catterson et al. 2018). Post-fasting protein and fat levels amongst our sampled lines varied, as has been previously reported in fly panels (**Fig. 2D,** Jin et al 2020). We show that fat storage positively covaried with starvation survival, as is expected (Hoffmann and Harshman 1999). Additionally, we observe genetic variation in starvation resistance following fasting in a panel of recently caught, outbred isofemale lines **(Fig.3B).** For example, Pennsylvania summer lines contradicted the general trend and improved in starvation resistance with fasting **(Fig.3C)**. Taken together, we document the large breadth of natural variation in fasting-induced starvation resistance amongst lineages originating across seasons and across the globe.

By demonstrating significant genetic variation in fasting-induced starvation resistance, we indicate the extent of unreliability in the success of a starvation hardening treatment. As with cold hardening (Czajka & Lee 1990) or heat hardening (Sejerkilde et al 2003), a short duration of starvation-like conditions is thought to promote later resistance to starvation via a shift in gene expression post-treatment (Bubliy et al 2011). For instance in *Drosophila* on the Indian subcontinent there is not only a latitudinal cline in starvation resistance but sex and latitude specific effects on the ability of hardening to improve starvation resistance (Aggarwal 2014). Starvation resistance clines across latitude indicates different nutritional needs based on local environment (Rion and Kawecki, 2007). Therefore, fasting-induced responses would also differ based on environment of origin. We report variation in starvation resistance improvement in lines collected across latitudes, and further demonstrate that the genetic variation within the fasted animal has a large impact on subsequent survival.

Conclusion

Genetic variation can influence the success of dietary treatments. A long-term goal of some scientists and nutritionists is to identify the schedule and parameters of dietary restriction, intermittent fasting, or nutrition-specific limitation that can consistently improve health across all portions of a population (Stanhope, 2016, García-Montero et al. 2021). But metabolic research including lineages with genetic variation indicates an issue with any one-size-fits-all dietary approach. In *Drosophila* studies, there is significant variance in the impact of dietary restriction on survival. Dietary restriction induced both improvement and reduction in lifespan among a *Drosophila* panel and variation in the metabolite profiles of the same panel (Wilson et al. 2020, Jin et al. 2020). This phenomenon extends to other common model organisms. When a panel of wild isolates of *C.* elegans were treated with dietary restriction, their subsequent lifespan considerably varied with some lineages exhibiting a reduction in lifespan post fasting. (Stastna et al 2015). Among genetically diverse mice, fasting reduced lifespan as often as it extended it (Harper et al. 2006, Liao et al. 2010). Part of the difficulty in identifying a universally beneficial diet is genetic variance amongst the population. Work presented here and elsewhere (Harper et al. 2006, Liao et al. 2010) demonstrate that the phenotypic response to a dietary treatment can assume different directions when examined across lineages. Mounting evidence indicates that an individual's genetic background impacts aspects of metabolic health

such as obesity and diabetes, and how these phenotypes respond to dietary treatment (Heianza & Qi, 2017,Ortega et al. 2017). As medical concerns increase surrounding the rising level of obesity (Dietz 2015), it is important to understand how the genetic background of an individual may influence their susceptibility to dietary intervention.

Historic lab lineages are heavily represented in metabolic research but are not always representative of common dietary responses. Genetic variation of fasting-induced starvation resistance that we observe here highlights the importance of exploring the natural variation present within a trait when seeking to understand responses to potential treatments. Over the last 20 years 16 publications have focused their study on periodic fasting, intermittent fasting or time-restricted feeding in *Drosophila* (Zhang et al. 2018; Catterson et al. 2018; Ulgherait et al. 2021; Villanueva et al. 2029; Livelo et al. 2023; Dissel et al. 2022; Liu et al. 2021; Ratliff et al. 2016; Gill et all, 2015; Oishi et al. 2004; Melkani et al. 2017; Xu et al. 2011; Zhang et al. 2023; Liu et al, 2021; Salgado-Canales et al. 2023; Aggarwall, 2014). Only two of these considered a panel of genetic backgrounds (Ulgherait et al 2021, Aggarwal 2014), and only one used fly lines maintained in labs for less than 50 years (Aggarwal 2014). Canton-S, w118, Oregon R, and their transgenic variants dominate this area of the literature. There is no question of the value of this work nor the tremendous advancements from using historic laboratory lineages, but this study illuminates the limitations resulting from focusing on a small set of lineages. Here we observe the Canton-S\w118 fasting response is just one of many possible responses to intermittent fasting. By using fly lines isolated across latitude, season, and continent we were able to demonstrate the considerable variance in how fasting alters protein accumulation, fat concentration, and starvation resistance. Subsequent work into periodic fasting across model species can use the toolbox of natural variation to fully characterize the response to fasting as specific to and impacted by the genetic variation present.

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Ethics Statement: The authors declare that this paper is completely original and has not been previously submitted to another journal. The paper reflects the authors' own research and analysis in a truthful and complete manner. The paper properly credits the meaningful contributions of co-authors and co-researchers. The results are appropriately placed in the context of prior and existing research. All sources used are properly disclosed.

Conflicts of interest: The authors declare no conflicts of interest.

Data availability

The data for this publication is publicly available for download, and can be found on github at https://github.com/benedictlenhart/StarvationResistance2024/

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Chapter 2

Title: Cosmopolitan inversions have a major impact on trait variation and the power of different GWAS approaches to identify associations

Authors: Benedict Adam Lenhart[1] & Alan Olav Bergland[1]

Affiliations: [1]Department of Biology, University of Virginia, Charlottesville, VA 22904

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To whom correspondence should be addressed:

Adam Lenhart: bal7cg@virginia.edu

Alan Bergland: aob2x@virginia.edu

Abstract

The ability of genomic inversions to reduce recombination and generate linkage can have a major impact on genetically based phenotypic variation in populations. However, the increase in linkage associated with inversions can create hurdles for identifying associations between loci within inversions and the traits they impact. The role of inversions and the genetic architecture within them in mediating genetic variation in complex traits remains to be fully understood. This study uses the powerful genetic tools and rich literature of the fruit fly *Drosophila melanogaster* to investigate the impact of inversions on trait variation. We tested the effects of common inversions among a diverse assemblage of traits including aspects of behavior, morphology, and physiology, and identified that the cosmopolitan inversions In(2L)t and In(3R)Mo are associated with many traits. We compared the ability of different approaches of accounting for relatedness and inversion presence during genome-wide association to identify signals of association with SNPs. We report that commonly used association methods are underpowered within inverted regions, while alternative approaches such as Leave-One-Chromosome-Out improve the ability to identify associations. In all, our research enhances our understanding of inversions as components of trait variation and provides insight into approaches for identifying genomic regions driving these changes.

Introduction

Genomic inversions facilitate adaptation by suppressing recombination and generating linkage between many genes and mutations, therefore affecting the evolution of complex trait change (Charlesworth & Charlesworth, 1973; Dobzhansky, 1937; Kapun, Fabian, et al., 2016a; Kirkpatrick & Barton, 2006; Shanta et al., 2020; Villoutreix et al., 2020). The adaptive importance of inversions for the evolution of novelty, local adaptation, and speciation is clear from a wide variety of organisms across the tree of life (reviewed in (Harringmeyer & Hoekstra, 2022; Lowry & Willis, 2010; Stefansson et al., 2005). Despite the prevalent role of inversions in evolution, efforts to discover the causal mutations that drive adaptive inversions, as well as the general role of inversions on trait variation, remain more elusive (Berdan et al., 2023).

The general importance of inversions in affecting trait variation largely comes from ecological genetics, wherein distinct morphs have been identified in natural populations and subsequently linked to inversions (Wellenreuther & Bernatchez, 2018). For instance, conspicuous behavioral, morphological, phenological and life-history variation has been linked to complex inversion polymorphisms in wild populations of birds (Tuttle et al., 2016), seaweed flies (Mérot et al., 2021), monkey-flowers (Lowry & Willis, 2010), and snails (Koch et al., 2021). In these cases, and many others (Brown & Benson, 1974; Kurlansky, 2011; White et al., 2011), distinct morphs and their patterns of segregation were first characterized (Lowry et al., 2008; Butlin et al., 1982; Johannesson, 1986; Lowther, 1961), prior to identification of inversion genotypes. Therefore, it is less clear if inversions have a major impact on less conspicuous quantitative genetic variation that can be identified through traditional mapping approaches.

There are two main reasons that standard mapping approaches have potentially missed the impact of inversions. The first reflects the design features of mapping approaches that utilize recombinant populations (Churchill et al., 2004; Crombie et al., 2024; Lister & Dean, 1993). These mapping panels have intentionally selected strains with colinear genomes to facilitate recombination and enable efficient QTL mapping. The second reflects statistical techniques of genome-wide association (GWA) studies of outbred wild or laboratory populations. Modern GWA approaches that factor out population structure may have missed important links between inversions and trait variation because inversions can have a major impact on estimates of population structure and relatedness (W. Huang et al., 2014; Li & Ralph, 2019; Price et al., 2008). Thus, the use of population structure as a co-factor in GWA analysis may have led to reduced power to detect association with SNPs linked to inversions. Even when inversion-trait associations can be drawn following GWA (Ayala et al., 2019; González et al., 2020; Harbison et al., 2013; Koch et al., 2021), it is

challenging to identify the specific genetic architecture within inversion driving changes due to the high linkage between loci within inversions (Cáceres & González, 2015; Feuk, 2010). Therefore, the role of inversions in less conspicuous quantitative genetic variation may have been overlooked in many species.

The fruit fly *Drosophila melanogaster* is an excellent model to assess the importance of inversions on quantitative trait variation. *D. melanogaster* possesses large inversions maintained at intermediate frequencies worldwide and are often implicated in local and rapid adaptation (reviewed in Kapun & Flatt, 2019). *D. melanogaster* inversions are known to impact a variety of traits (Aulard et al., 2002; de Jong & Bochdanovits, 2003; Durmaz et al., 2018; García-Vázquez & Sánchez-Refusta, 1988; Hoffmann et al., 2004; Hoffmann & Rieseberg, 2008; Kapun, Schmidt, et al., 2016). For instance, In(3R)P presence is associated with body size, lifespan, and starvation resistance (Durmaz et al., 2018; Kapun, Schmidt, et al., 2016), and In(2L)t is associated with behavioral, stress-tolerance, and morphological traits (Kamping & van Delden, 1999; Nunez, Lenhart, et al., 2024; van Delden & Kamping, 1989).

The Drosophila Genetic Reference Panel (DGRP) provides an excellent resource for identifying the effects of cosmopolitan inversions in *D. melanogaster* on quantitative variation, and for exploring the role of various GWAS methods in discovering association between inversion-linked markers. The DGRP is a collection of 205 inbred and fully genotyped *D. melanogaster* lines, initially collected from a farmer's market in North Carolina (Mackay et al., 2012). The lineages have been inbred, their genomes have been sequenced, and the presence of common inversions has been characterized for each line using a combination of polytene chromosome preparations (W. Huang et al., 2014), principal component analysis (Nowling et al., 2020), and PCR (Corbett-Detig et al., 2012). Due to the availability of these resources, DGRP lines have become a common model for phenotyping studies across many traits (Gardeux et al., 2023). To facilitate association studies with the DGRP, several websites have been developed with a standardized mapping approach that factors out the effects relatedness and also inversions (Gardeux et al., 2023; W. Huang et al., 2014). Indeed, GWA approaches that correct for genome-wide structure or inversions account for approximately 60% of DGRP studies from a representative sample of 36 papers ("curated dataset"; Gardeux et al., 2023), yet few (35%) report testing for associations with inversions or inversion linked markers.

In our study, we test the effects of different GWA mapping approaches to identifying signatures of association with inversions and linked variants. We utilize published studies that measured phenotypic variation in the DGRP. First, we show that several cosmopolitan inversions have large effects on dozens of traits, explaining more trait variation than

expected by SNPs of comparable frequencies. Next, we explored four genome-wide association strategies that differ in their genetic-relatedness matrix (GRMs) and the treatment of inversions as co-factors, and contrast the real GWA signal for each phenotype to 100 permutations. We generated three types of GRMs (i) using the full genome, (ii) using an LD-thinned genome, (iii) and using a leave-one-chromosome-out (LOCO) approach. In addition, we performed association analysis and permutations using the full-genome based GRM and factored out the effect of inversions following methods outlined in (Gardeux et al., 2023; W. Huang et al., 2014). We show that the result of the GWA greatly depends on the mapping strategy, and that only the LOCO approach resolves association signals that exceed permutations. Finally, we use the output of the LOCO-GWA to test whether SNPs identified as top candidates under the different mapping strategies show different levels of enrichment for signatures of local adaptation, and whether signals of pleiotropy are resolvable at specific loci inside the inversion.

Materials and Methods

Selection of trait data. For the analysis in this paper, we reanalyzed trait data collected on the DGRP (Mackay et al., 2012). We made use of the DGRPool resource, which has consolidated the inbred line means from many publications (Gardeux et al., 2023). We used the "curated" data, and removed traits from this dataset that describe genomic features or used less than 75 unique samples, ending up with 409 unique traits derived from 36 publications (Supplemental Table 1). Of these 36 studies, 19 of them were also represented in the phenotype aggregation study reported in Nunez et al., 2024 (Nunez, Lenhart, et al., 2024). We annotated these traits by classifying each trait into 5 general groups: "Behavior", "Life-History", "Morphology", "Physiology", and "Stress-resistance."

Linear model association of traits with genomic inversions. We characterized the effect of cosmopolitan inversions In(2L)t, In(2R)NS, In(3L)P, In(3R)K, In(3R)P, and In(3R)Mo on these traits. For this analysis, we used the inversion classifications provided by (Huang et al 2014). For each trait, we used a simple linear model to test the effect of inversion status on strains that were homozygous for either the inverted or standard allele, and counted the number of traits with significant association with any of the inversions with p-value < 0.05. Next, we evaluated whether the extent of association between traits and inversion status is greater than expected relative to other SNPs in the genome. For each inversion, we performed linear regression on 100 SNPs identified at the same frequency as each inversion (±1%), and on the same chromosome arm, but at least 2mb from the inversion breakpoints to avoid areas of highest linkage disequilibrium. Using the matched-allele models we recorded the number of traits that are associated with inversions at $p < 0.05$. Next, calculated we R^2 for the observed and matched-allele models to ask whether the

inversions explain more variation than expected by other comparable SNPs in the genome. If a trait was significantly associated with an inversion both within the linear model results and the R^2 of that model surpassed the 95% quantile of permutations, we designated that trait as part of a group of "inversion-associated traits" used in downstream analysis. We identified three sets of inversion-associated traits, each containing the traits associated with either In(2L)t or In(3R)Mo.

Principal component analysis of trait data. We ran PCA analysis on each of the inversion-associated trait sets. Missing data for any trait was imputed using the *imputePCA* function from missMDA v1.19 (Josse & Husson, 2016), before calculating the principal component loadings and amount of variance explained using the *PCA* function from FactoMineR v2.8 (Lê et al., 2008). We separated the principal component loadings by inversion presence using the inversion genotype of the DGRP lines and then compared the PCs of "inverted" and "standard" groups using Student's t-test.

Principal component analysis of genomic data. To understand how inversions impact general patterns of genomic structure, we performed a series of PCA on the DGRP genetic polymorphism data. We used three SNP selection strategies for this principal component analysis that mirror SNP selection strategies used for the construction of the GRM (see below). The first version of SNP selection ("Full") used all SNPs across the autosomal and X chromosome with minor allele frequency (MAF) greater than 5% and sites with missing genotype data in less than 20% of DGRP lines. The second version ("LD") used SNPs with MAF > 5% and missing rate < 15%, and a low pairwise linkage disequilibrium ($R^2 < 0.2$). To ensure that SNPs were at least 5000 base pairs apart we used the *snpgdsLDpruning* function of the R-package SNPrelate v3.17 (Zheng et al. 2012) with the *slide.max.bp* parameter set to 5000. The third version used a leave-one-chromosome out ("LOCO") approach that used the same filtering and thinning strategy as the LD-pruning approach but in four parts, each missing one of the main chromosomal arms. We used the *snpgdsPCA* function from *SNPrelate* v3.17 (Zheng et al. 2012) for PCA. To quantify the effect of inversions on PC1 and PC2, we constructed linear models in the same manner described above, recording the R^2 of both the linear models and a set of permutations with the lines' inversion genotype shuffled. We designated a model outcome significant if its R^2 surpassed 95% of permutations.

Construction of GRMs. We developed three genomic relatedness matrixes (GRM) to address population structure in different ways. For the "Full" method, we use the GRM matrix that is supplied by the DGRP website and is commonly used in DGRP GWAS studies (http://dgrp2.gnets.ncsu.edu/). This GRM uses all SNPs with a MAF > 0.05 and a missing rate < 20% (W. Huang et al., 2014). For the "LD" method, we used LD pruning using the

same parameters that we used for the LD-pruned PCA, described above, and constructed a GRM from the whole genome using the *snpgdsGRM* function in SNPRelate based on the Genome-wide Complex Trait Analysis (GCTA) method (Yang et al. 2011). For the "LOCO" method, we generated sub-GRMs, each one drawing from the DGRP genome but ignoring one chromosome arm ("2L", "2R", "3L", "3R", and "X") and using the same steps as described for the LD-thinned approach.

GWA Analysis. We performed association mapping using mixed-effect models implemented in the R package *GMMAT* v1.3.2 (Chen et al. 2019). This approach used the "Full", "LD", and "LOCO" GRMs as a random effect to control for population structure. In addition, we performed a fourth association mapping approach based on the GWA approach developed by Huang *et al*. 2014, which we refer to as the "Factored-out" approach. The Factored-out approach first standardizes each trait by the effects of the inversions by regressing line mean data against inversion status using the model:

$$\text{trait} \sim \text{In(2L)t} + \text{In(2R)NS} + \text{In(3R)P} + \text{In(3R)K} + \text{In(3R)Mo}$$

Next, the residuals of this model are used as the trait to be used for association analysis. We used the "Full" GRM with the Factored-out approach to replicate the association model implemented in by Huang et al. 2014 and available on the DGRP online GWA tool (http://dgrp2.gnets.ncsu.edu/).

For each of the four GWA approaches, we compared a "reduced model" to a "full model." The reduced model is described by the formula:

$$y \sim \text{Wolbachia} + \text{GRM},$$

where *y* represents the line means for a particular trait (or residuals in the case of the factored-out model). Wolbachia is a fixed effect corresponding to the Wolbachia infection status encoded as a binary presence or absence, and GRM is a random effect genetic relatedness matrix. Wolbachia status is based on the tables published in W. Huang et al., 2014. The reduced model is compared to a full model defined as:

$$y \sim \text{variant}_i + \text{Wolbachia} + \text{GRM},$$

where variant_i is the fixed effect of the i^{th} SNP or small-indel reported for the DGRP. We contrasted the full and reduced models using the *glmm.score* function in the GMMAT package (v1.4.2).

For each trait and GWA method, we conducted 100 permutations by randomly shuffling the trait data prior to fitting and comparing the reduced and full models.

GWA summary statistics. We compared overall genomic signal from the GWA of each trait using statistics for the observed and permutated GWA models. We calculated the proportion of SNPs with a p-value less that 10^{-5} ("hits"), a common p-value threshold in DGRP studies (Durham et al., 2014; Krefl & Bergmann, 2022; Marriage et al., 2014; Mitchell et al., 2017; Vaisnav et al., 2014; Vonesch et al., 2016; Watanabe & Riddle, 2021). We compared the number of hits in the observed data to the distribution of hit counts from the permutations for each trait and GRM method, reporting the proportion of traits where the observed hit count exceeds the 95th percentile of the trait's permutation-based distribution." We also partitioned the genome into bins based on whether SNPs are inside or outside inversions as defined using coordinates in Corbett-Detig et al. 2012 (Corbett-Detig et al., 2012).

Enrichment tests. We tested if GWAS hits identified via different GRM approaches prioritize SNPs that are potentially subject to temporally or spatially variable selection. We used data from the DEST dataset to obtain allele frequencies from *D. melanogaster* populations sampled across the North American east coast (Kapun et al., 2021), and Charlottesville, Virginia across multiple years (Nunez, Lenhart, et al., 2024). BayPass software identifies polymorphisms that are more differentiated than expected given population structure (XtX* outliers) and identifies association between variants and environmental variables after correcting for population structure (Olazcuaga et al., 2020). Using this framework, we identified differences in allele frequencies between populations (XtX*) and Bayes Factor (BF) of association with environmental variables. We used latitude for the association of East Coast variants and maximum temperature two weeks prior to collection for Charlottesville variants (Nunez, Lenhart, et al., 2024). In order to improve stability, we ran the software five times and report the mean statistic per SNP (Blair et al., 2014). Overall, we generated a null distribution of XtX* and BF using the POD framework for 10-times the number of SNPs as the observed data, ran BayPass with five replicate iterations, and calculated empirical p-values for XtX* and BF using these POD simulations.

We identified the level of enrichment between top GWA variants and top BayPass variants. We identified the top hits within each GWA study by identifying the 500 hits with the lowest P-value, and top XtX* and BF variants as those that surpass 95% of the corresponding distribution from the simulated POD data. We computed Fisher's Exact test by contrasting the odds that top association hits for any trait are enriched for top XtX* and BF hits. We compared these Fisher's Exact test odds-ratios to odds-ratios constructed in the same way using the permuted GWA.

COLOC analysis: We tested if inverted regions of the genome are likely to have pleiotropic effects on phenotypic variation using a COLOC analysis. By treating the top principal

component loadings as a dimensionally-reduced trait, we sought to identify regions in the genome with a shared association with multiple inversion-linked traits. We used the Factored-out and LOCO GWAS frameworks to score the impact of SNPs genome-wide on the PC1 and PC2 loadings for the In(2L)t and In(3R)Mo associated traits. We identified areas of colocalized signal on PC1 and PC2 using a sliding window analysis across the genome with window size 10Kb and step size 5Kb. Within each window we used the *coloc.abf* function from coloc v5.2.3 (Giambartolomei et al., 2014), with the SNP-wise p-values for PC1 and PC2. This analysis identified regions of SNPs likely associated with the traits differentiated along only PC1, only PC2, or regions of SNPs with a colocalized association across PC1 and PC2.

Results

Cosmopolitan inversions impact phenotypic variation. To study the role of inversions on genetically-based trait variation in *D. melanogaster*, we reanalyzed data from publications that measured trait variation in the DGRP and were curated in the DGRPool database (Gardeux et al., 2023). We analyzed 409 traits, categorizing them into five groups: morphological, life-history, stress resistance, physiological, and behavioral. (Supplemental Table 1). We found that In(2L)t and In(3R)Mo are associated with more traits than expected given SNPs of the same frequency (**Fig. 1A**). In(2L)t is especially associated with many behavioral traits including startle response, sleep, and movement, while In(3R)K and In(3R)Mo are associated with morphological traits such as femur and abdomen size (Supplemental Table 2). We found that the inversions explain ~10% of the variation in traits, and that dozens of traits are explained better by inversion status than expected from random SNPs in the genome (**Fig. 1B**).

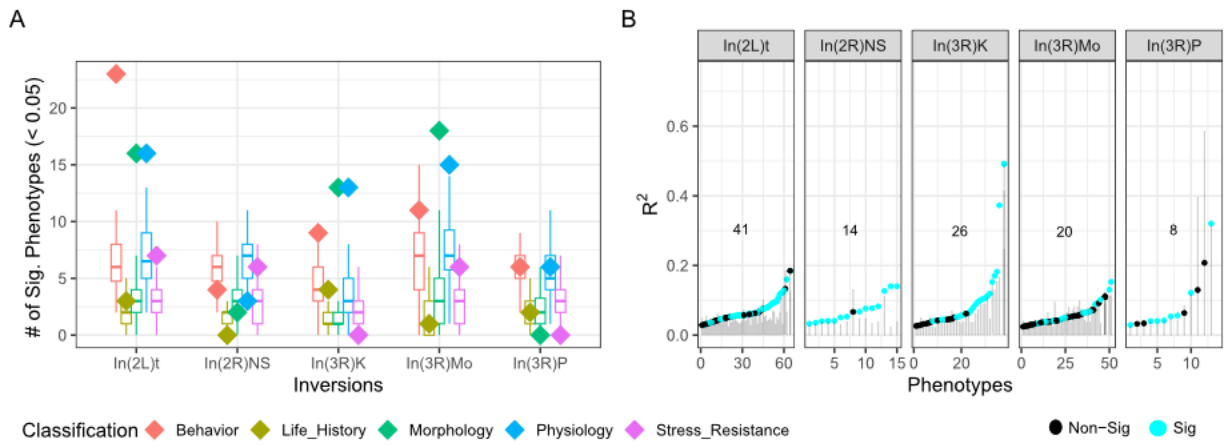

Figure 1. Cosmopolitan inversions have a major impact on trait variation. **A)** Diamonds, colored by trait category, indicate the number of traits significantly affected by inversion presence, overlayed over the same-frequency models shown with box plots. **B)** The proportion of variation explained by each inversion (R^2) for traits significantly associated at p<0.05 with each inversion, compared against the distribution of corresponding same-frequency models in grey. Statistically significant inversion model values that surpass the null distribution are colored cyan.

Principal component analysis of trait's associated with In(2L)t and In(3R)Mo. We performed PCA on the traits that are associated with In(2L)t and In(3R)Mo and also explain more variation than expected by chance (In(2L)t: n=41, In(3R)Mo: n=20)(Fig. 1). The top two principal components (PC1 and PC2) explain over one third of the trait variation for both In(2L)t and In(3R)Mo (**Fig. 2A**). Therefore, we restrict further analysis to these two principal components. In(2L)t significantly loads into PC1 (t-test, t = -4.38, df = 16.68, p = 4.29e-4) of its associated trait set, while In(3R)Mo significantly loads onto both PC1 (t-test, t = -5.18, df = 18.03, p = 6.21e-5) and PC2 (t-test, t = 2.27, df = 15.54, p = 0.038; **Fig. 2B**) of its associated trait set. In the In(2L)t PCA traits like body size and ethanal sensitivity have positive loadings on PC1 and traits like startle response and negative geotaxis have negative loading. Lines homozygous for In(2L)t have lower values of PC1, thus have higher startle response and higher activity levels (**Fig. 2C**), amongst other differences (Supplemental table 3). In the In(3R)Mo PCA traits like body size have positive loadings on PC1 and traits like feeding and chill coma recovery have negative loading. Lines homozygous for In(3R)Mo have lower values of PC1, thus have lower body size (**Fig. 2D**), amongst other differences (Supplemental table 3).

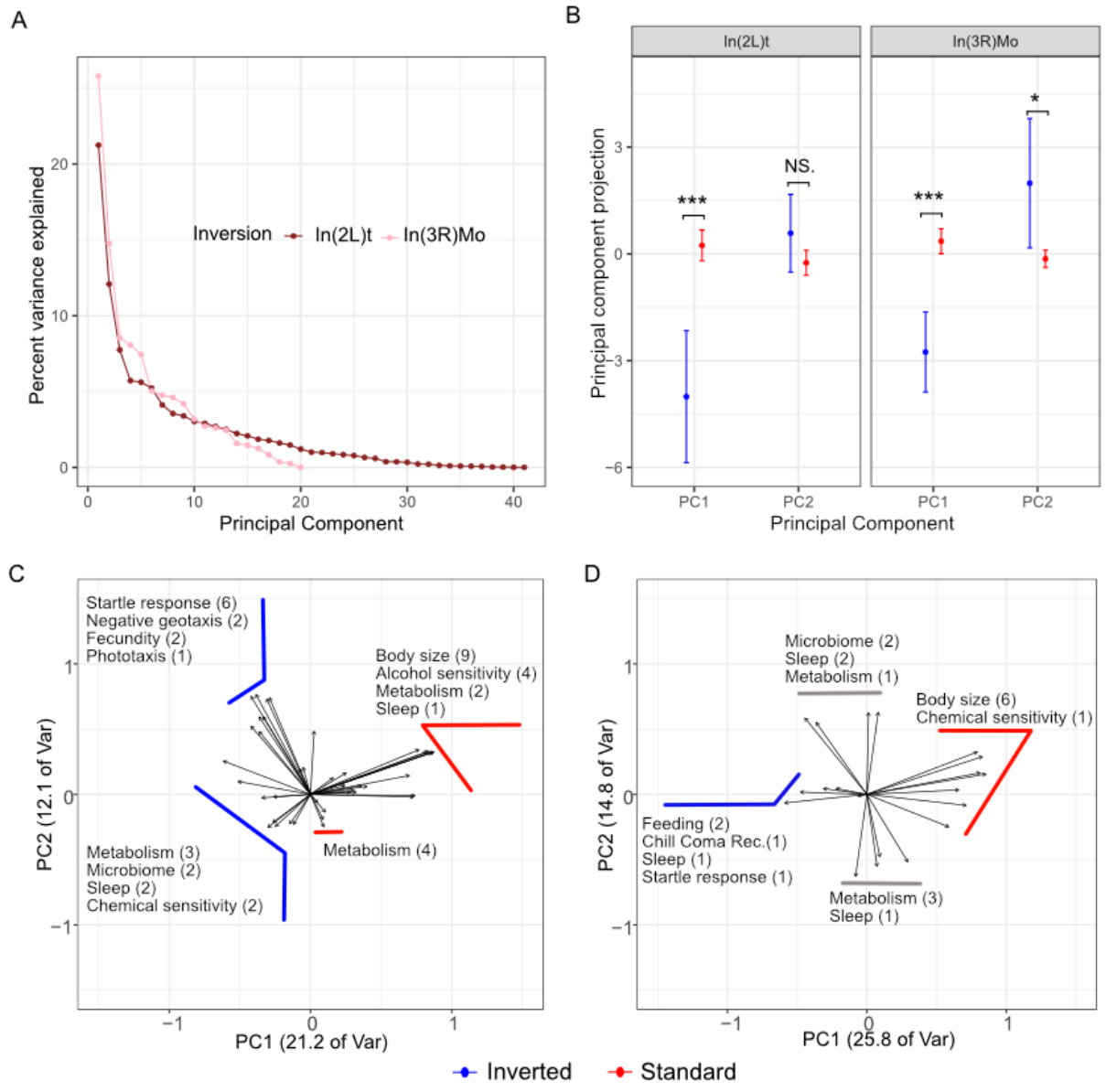

Figure 2. Principal component analysis of In(2L)t and In(3R)Mo. **A)** Scree plot showing the variance explained by principal components, colored by their associated inversion **B)** The loading of In(2L)t and In(3R)Mo genotype onto PC1 and PC2 of their respective PC analyses. **C)** PCA for the traits significantly impacted by In(2L)t. Labels are aggregated to show similar traits together, e.g. "Sleep (2)" corresponds to two sleep traits. Variance explained by each PC is given on the axis title. **D)** PCA for the traits significantly impacted by In(3R)Mo.

Controlling the effect of inversions on PC and GRM space. Cosmopolitan inversions in *D. melanogaster* have been shown previously to have an impact on genome-wide patterns of genetic variation as summarized by principal components and genetic relatedness matrices (Li & Ralph, 2019; Price et al., 2008; Seich al Basatena et al., 2013). Therefore, we

tested if different SNP selection strategies can mitigate this impact. As previously reported (W. Huang et al., 2014), PCA of the "Full" genome shows that inversions strongly impact PC space. In(2L)t primarily impacts PC1 ($F_{1\,179} = 870$, $p = 2.89e\text{-}70$) and In(3R)Mo primarily impacts PC2 ($F_{1\,195} = 331$, $p = 5.54e\text{-}44$, **Fig. 3A**). The "LD" SNP-set reduces the impact of inversion status somewhat for In(2L)t ($F_{1\,179} = 175$, $p = 2.89e\text{-}28$) and In(3R)Mo ($F_{1\,195} = 90.71$, $p = 6.93e\text{-}18$, **Fig. 3A**). In contrast, PCA of the LOCO genome shows a sharply reduced impact of In(2L)t on PC1 ($F_{1\,179} = 5.84$, $p = 0.017$) and In(3R)Mo on PC2 ($F_{1\,195} = 0.02$, $p = 0.88$, **Fig. 3A**). To quantify these relationships, we calculated the proportion of variation in the genetic PC1 and PC2 that is explained by inversion status, and contrasted that to a null distribution made via 100 permutations. We found in the Full and LD methods, In(2L)t and In(3R)Mo explained more variation for PC1 and PC2 (**Fig. 3B**) than expected by chance, with In(2L)t explaining the most variance within PC1 within the Full method and less within the LD method, while In(3R)Mo explained the most variance for PC2 within the Full method and less within the LD method. Meanwhile, within the LOCO method each inversion explains a near zero amount of variance for PC1 or PC2, and PC2 is no longer significantly impacted by either inversion (**Fig 3B**).

To identify how the presence of inversions impacts patterns of relatedness, we compared the relatedness estimation of the DGRP lines using different SNP selection strategies. Across all approaches, relatedness is low within standard genotype lines with no cosmopolitan inversions (**Fig. 3C**). This replicates observations in Huang et al. 2014. However, within the Full and LD-thinned approaches we see that relatedness is driven largely by the presence of the cosmopolitan inversions. In contrast, the LOCO approach can drive relatedness for inverted lines on a given chromosome near zero while still

accounting for inversions on the other chromosomal arms (**Fig. 3C**).

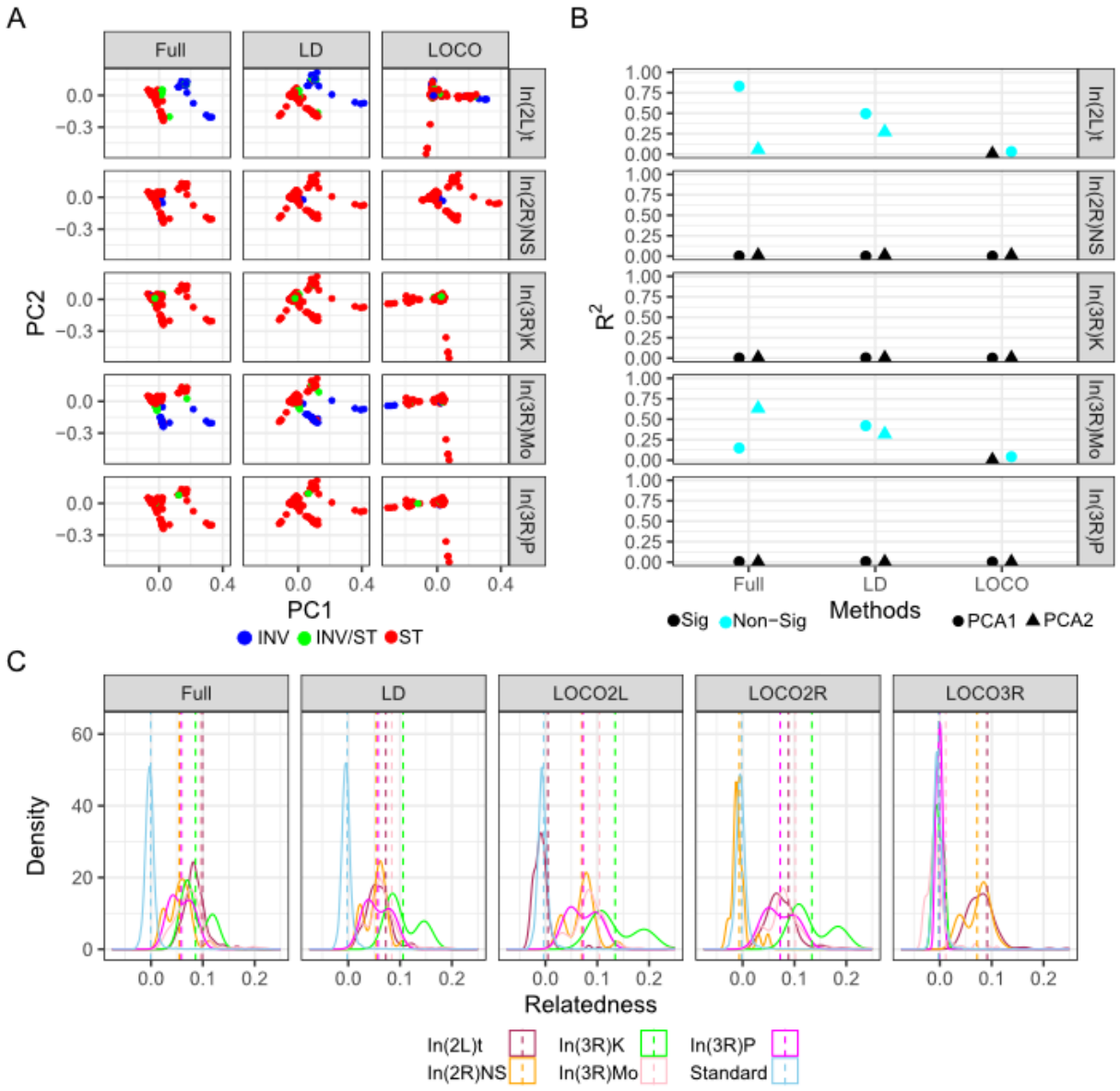

Figure 3. Presence of inversion drives variance in the sample genomes. **A)** The first and second PCs for each sample colored by the genotype of that sample. **B)** The R squared values for models comparing PC1 and PC2 to inersion, colored by which values exceed a distribution of permutations. **C)** The distribution of pairwise relatedness values between each of the samples, colored by genotype of the samples and split across different methods.

The LOCO approach can better capture signal for inversion-associated traits. After characterizing the impact of different GRM methods and presence of inversion in the DGRP data, we tested the strengths and weaknesses of the four GWA strategies (Factored-out, Full, LD, LOCO) on the magnitude of association signal. We compared the summary

statistics from the observed trait GWA against permutations to see how many traits identified more signal than expected by chance. Using the Factored-out approach, we found that about 6% of traits have “hit-counts” that exceeds the largest 95% of the null distribution generated by permutation (**Fig. 4**). In other words, for over 375 traits, the Factored-out approach performed no better than chance. We found that the Full and LD-thinned approaches reported similarly. The LOCO method surpasses permutation significantly more often than the Factored-out method within inverted regions (Fishers Exact Test - FET , 2L: p = 9.47e-9, 2R: p = 5.17e-4, 3L: p = 6.66e-10, 3R: p = 1.61e-7) , as well as outside the inverted region (FET, 2L: p = 1.2e-9, 2R: p = 7.71e-4, 3L: p = 1.61e-5, 3R: p = 9.19e-13) (**Fig. 4**).

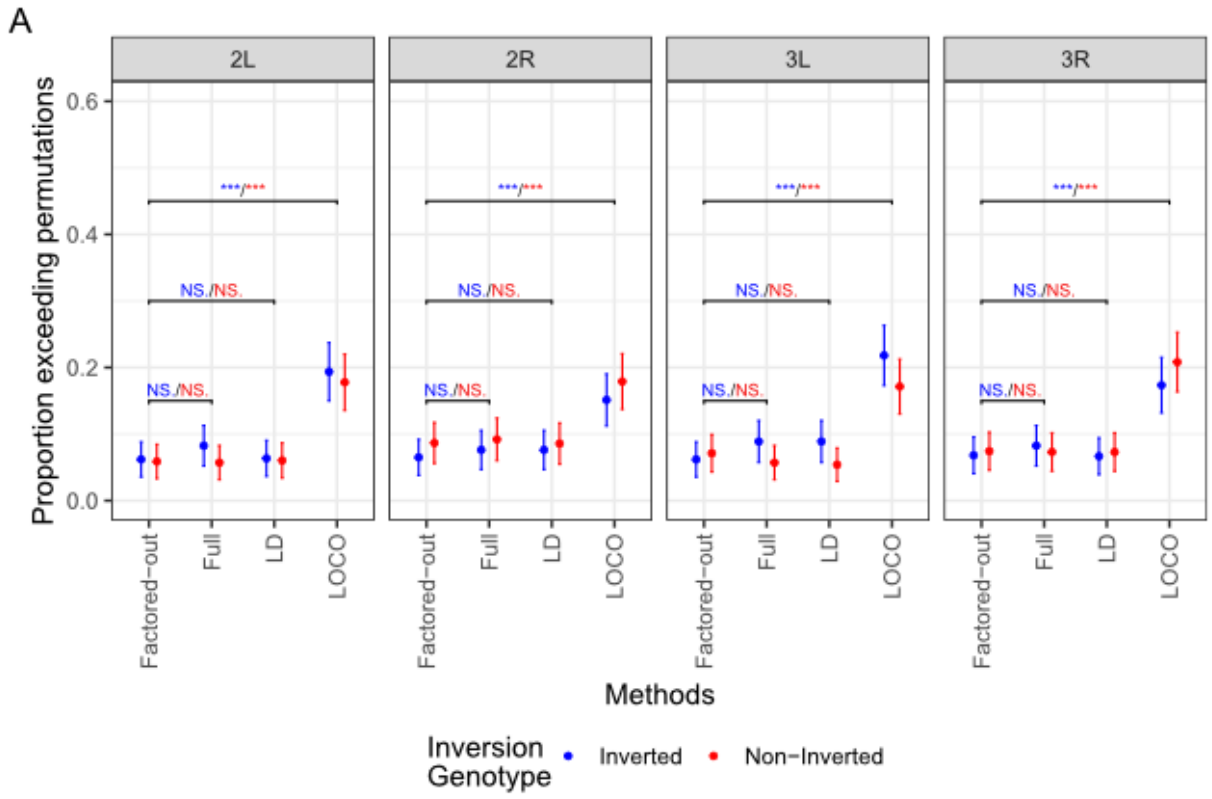

Fig 4. The LOCO approach can better capture signal genome-wide. **A)** The proportion of significant hits for each GWAS output are compared across each method and colored by location relative to inversions. The proportion of traits that exceed their corresponding traits is given on the x axis, along with binomial confidence intervals. The color of the significance annotation refers to the inversion genotype under comparison. (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, NS. = p >= 0.05)

Enrichment tests. To compare the utility of the four approaches to identify biologically meaningful loci, we characterized the ability of each SNP selection method to identify loci thought to be important for local adaptation. Using estimates of allele frequencies of *D. melanogaster* collected across seasons and across latitudes (Kapun et al., 2021, Nunez et

al., *Genetics* 2024, Nunez et al., *biorxiv* 2024), we used the Baypass (Olazcuaga et al., 2020) software to identified SNPs that are more strongly differentiated across the North American east coast, or within Charlottesville, VA through time (XtX* outliers). In addition, we identified the strength of association between SNPs and latitude for the East Coast samples and between SNPs and temperature in the two weeks prior to sampling for the Charlottesville samples. To understand which methods could successfully identify enrichment within inverted regions, we (i) examined the enrichment on SNPs within inversions in 2L and 3R and (ii) compared enrichment between traits associated and not associated with the corresponding inversion. There was a significant jump in enrichment between GWAS hits and max temperature bayes factor for the LOCO method on 2L (FET: p = 0.019), but not for hits derived from the Factored-out method (**Fig. 5**). Correspondingly, there was an increase in enrichment between GWAS hits and differentiation across max temperatures for the LOCO methods on 3R (FET, p = 0.011) but not for the Factored-out method (**Fig. 5**). There was no difference reported within the East Coast enrichments with GWAS hits between inverted and non-inverted associated traits.

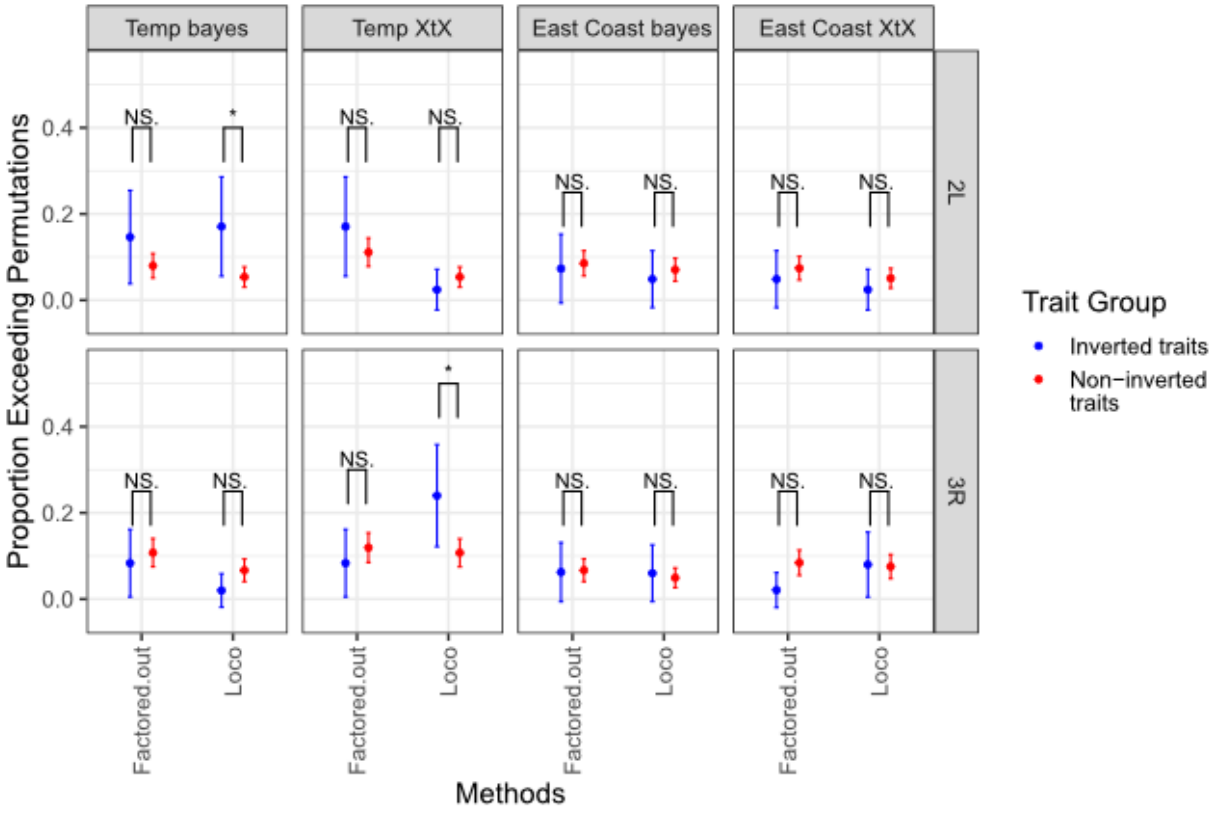

Fig 5. Enrichment of top bayes loci from two populations with top LOCO and Factored-out GWAS hits. The proportion of traits for whom their enrichment exceeds 95% of permutations is shown with error bars from binomial confidence intervals. Color indicates whether the traits are associated with inversion. (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, NS. = p >= 0.05)

COLOC enrichment within the genome. As an additional test to compare the ability of approaches to identify association signal within inversion, we calculated the probability that regions of the genome share SNPs that affect multi-dimensional traits (co-localization). Using the top principal component loadings from Fig. 2 as dimensionally-reduced traits, we scored effect of SNPs genome-wide on PC1 and PC2 using the LOCO and Factored-out GWAS approaches. We followed with a sliding window analysis performing Bayesian association to identify loci within the genome are likely associated with traits differentiated along only PC1, the traits differentiated along only PC2, or for both PC1 and PC2. With the LOCO method, we identified variants within the In(2L)t inverted regions and near the breakpoints that have high association likelihood with PC1 of the In(2L)t-linked traits (**Fig. 6A**). In contrast, for In(3R)Mo areas of likely association were identified across 3R for both PC1 and PC2, with the peaks aligning with other inversion breakpoints on 3R (**Fig. 6B**). Notably, the peaks of highest likelihood of association differed between PC1 and PC2, suggesting that distinct loci within the inverted regions influenced different sets of traits. In contrast, SNPs scored using the Factored-out method failed to capture any signal of likely association with either PC1, PC2, or both. (Supplemental Figure 1).

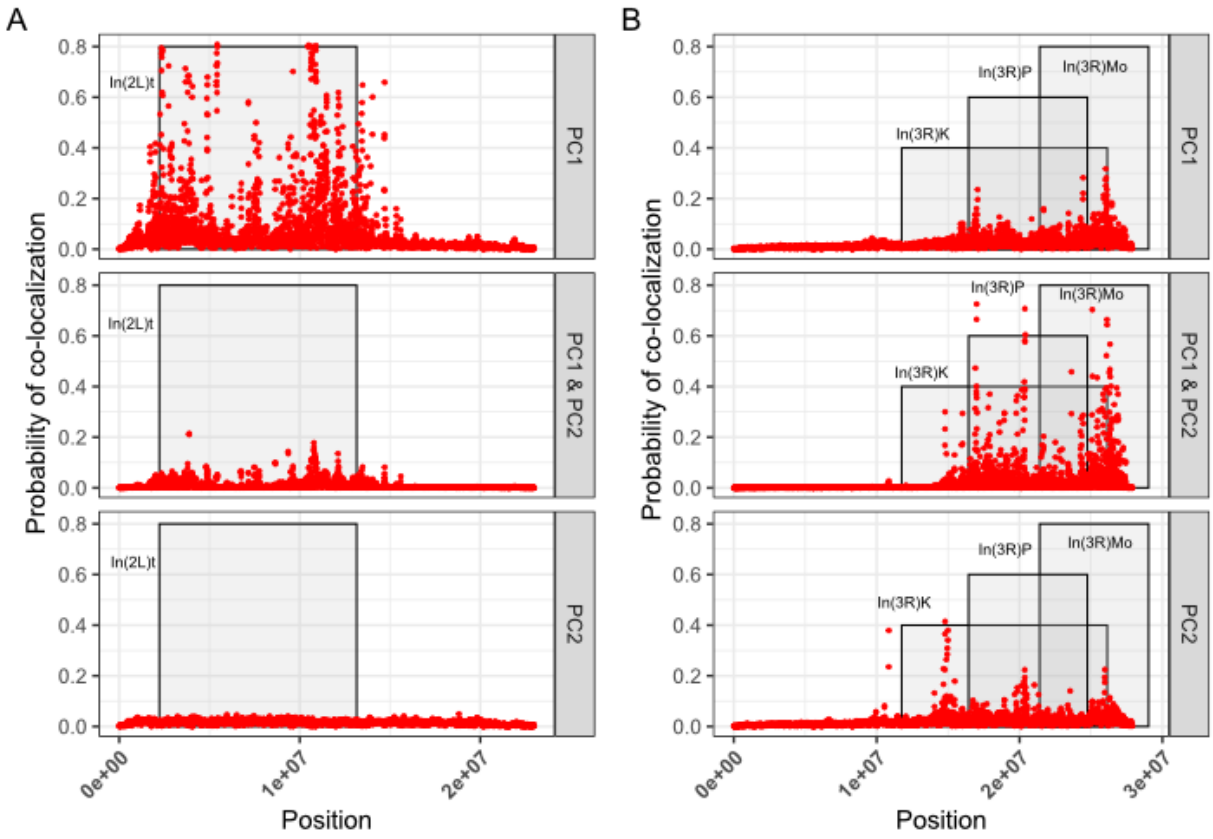

Fig. 6. LOCO method reveals areas of likely colocalization of association for inversion-related traits **A)** Results of a sliding window analysis examining enrichment between SNPs scored using LOCO for PC1 and PC2 of In(2L)t. The y-axis shows the likelihood of association, and the x-axis shows position on the genome. The grey shaded regions show the zone of cosmopolitan inversions on the chromosome arm. **B)** Same analysis as in A, but for traits associated with In(3R)Mo.

Discussion

Genomic inversions can simultaneously alter multiple traits and provide a mechanism for adaptation. Associations between inversions and traits have been identified across life, along with evidence for natural selection acting upon these genomic features (Harringmeyer & Hoekstra, 2022; Lowry & Willis, 2010; Stefansson et al., 2005b). We find here that inversions within *D. melanogaster* impact a suite of diverse traits (**Fig. 1A, Fig. 2C, Fig. 2D**), and specific statistical methods are better equipped to map associations between inversion-linked loci and these traits. Inversions should be considered as major players in association studies, as their presence here is shown to explain not only large parts of trait variance (**Fig. 1B, Fig. 2B**), but variance within the genome as well (**Fig. 3**). We illustrate that different GWAS approaches have broadly different power in the number and

strength of associations they can identify (**Fig. 4**). Compared to several commonly used methods, the LOCO approach is better able to identify variants within inversions associated with key traits, associated with alleles changing across environments (**Fig. 5**), and associated with variants underlying variance in multiple inversion-linked traits (**Fig. 6A, Fig. 6B**).

The trait-inversion associations we document illustrate a clearer picture of how inversion mediates adaption in *Drosophila*. Years of previous work have found numerous insights into the effects of inversion on *D. melanogaster* traits, implicating these mutations in changes to body size, pigmentation, and more (Aulard et al., 2002; de Jong & Bochdanovits, 2003; Durmaz et al., 2018; García-Vázquez & Sánchez-Refusta, 1988; Hoffmann et al., 2004; Hoffmann & Rieseberg, 2008; Kapun, Schmidt, et al., 2016). Despite these known impacts, only 13 out of the 26 publications aggregated here report any test for association between inversion and their trait(s) of study (Supplemental Table 1). Here we reexamined the impact of inversions on a large body of diverse trait data, and show that inversions like In(3R)Mo and In(2L)t significantly affect more traits than would be expected by chance, including inversion-trait associations not previously identified (Supplemental Table 1; **Fig. 1**). In(3R)Mo varies across latitudinal clines in multiple continents (Kapun, Fabian, et al., 2016a), and the overlapping inversion In(3R)P is thought to allow for these north-south delineated populations to adapt to different environment (de Jong & Bochdanovits, 2003; Kapun, Fabian, et al., 2016b; Kapun & Flatt, 2019; Rane et al., 2015). In our analysis, In(3R)Mo is likely more enriched than the commonly researched In(3R)P due to its 3x higher presence than In(3R)P within the DGRP. Here we confirm that inversions on 3R impact body size (**Fig. 2D**). Similarly, highlighting the association between In(2L)t and activity (**Fig. 1A, Fig. 2C**) provides new avenues for investigation for the ongoing link to this inversion and seasonal adaptation (Machado et al., 2021; Nunez, Lenhart, et al., 2024). We show that inversion presence explains much trait variance within specific traits (**Fig. 1B, Fig. 2B**), indicating these inversions should be a major factor in consideration for association studies.

Inversions provide a challenge for association studies, as the increased linkage disequilibrium and relatedness within inverted samples can elevate the false discovery rate (Price et al., 2008; Seich al Basatena et al., 2013). Many modern GWAS techniques thus seek to compensate for the impact of relatedness by using top principal components as cofactors (Price et al., 2006) or factoring our relatedness using GRMs as a random-effect (Yu et al., 2006). The Factored-out approach described here employs these last two methods uses genome-wide GRMs and additionally factors out the effect of inversions prior to genome-wide association mapping. Of the studies we analyzed, 21/36 used this method or an equivalent for GWA with the DGRP (Supplemental Table 1). However, we

report that only about 5% of GWA using this method find more hits than from random permutations (**Fig. 4**), indicating a lack of power and a potentially high false-positive rate amongst many published DGRP studies. Other popular GWAS methods such as thinning the relatedness matrix for linkage disequilibrium fare little better (**Fig. 4**). In contrast, LOCO is designed to identify association within instances of high LD within the genome, by avoiding proximal contamination between highly linked SNPs while still partially accounting for population structure (Yang et al., 2014). Recent association studies have using LOCO methods of establishing relatedness while investigating association studies within inversions and other areas of high LD (Baran et al., 2013; Koch et al., 2021). For example, Calboli 2022 established an association between an agriculturally relevant trout disease and an inversion using a LOCO method, but not with their accompanying "Full" genome method (Calboli et al., 2022). Here we provide evidence that LOCO can outperform other methods at identifying association signal within inverted regions (**Fig. 4**).

Further evidence indicates that the LOCO method provides advantages in identifying loci involved in inversion-mediated adaptation. Recent research indicates that In(2L)t could mediate seasonal adaption (Machado et al., 2021), and implicated that behavioral traits could be contribute to rapid adaptation to temperature (Machado et al., 2021; Nunez, Lenhart, et al., 2024). We illustrate high enrichment within In(2L)t between the most temperature associated alleles and those GWAS hits for traits such as sleep and startle response (**Fig. 5**). Crucially, the overlap of environmentally-varying alleles and top GWAS loci can be identified from LOCO-based GWAS, but not from the Factored-out approach (**Fig. 5**).

We identify differences in the ability of GWAS approaches to identify regions within the genome that are likely associated with multiple inversion-linked traits. Dimensional reduction of multiple traits can potentially indicate some shared mechanism or biological component that explains variance in aspects of morphology (Furusawa & Kaneko, 2018; Mei et al., 2007). For example, if calf length, thigh length, and foot length all load onto the top principal component, this component likely reflects some unifying aspect of body size development (Berner, 2011). Therefore, the loading of a mutation onto multiple principal components can reflect a broader pleiotropy beyond that of a single mechanism. We thus characterized areas of high Bayesian association between PC1 and PC2 of the inversion linked sets in order to illustrate such pleiotropy. LOCO identifies the areas of highest likely association with multiple inversion-linked traits are near their corresponding inversions, as one might expect (**Fig. 6A, Fig. 6B**). However within the inverted regions there are peaks of higher likelihood of association, similar to the finding in Nunez et al., 2024 of peaks of SNP-phenotype enrichment within In(2L)t (Nunez, Lenhart, et al., 2024). Peaks of association with PC1 of In(3R)Mo may indicate loci relevant to traits such as body size, while a peak for

PC2 may indicate different loci relative to traits such as metabolic storage and sleep, and the peaks for PCs indicate areas of likely pleiotropic effect (**Fig. 2D**). While LOCO identifies these areas of likely association, this signal cannot be recapitulated via Factored-out (Supplemental Figure 1). Characterization of pleiotropic effects of In(2L)t and In(3R)Mo enables us to further understand the adaptive identify of inversions (Kirkpatrick & Barton, 2006).

Inversions have the potential to be a fruitful area of investigation within association studies rather than a statistical nuisance. Despite the evidence across taxa that inversions can alter many classes of traits (Harringmeyer & Hoekstra, 2022; Lowry & Willis, 2010; Stefansson et al., 2005), inversions are sometimes presented as a statistical hindrance (Price et al., 2008; Seich al Basatena et al., 2013). Efforts such as the creation of popular mapping populations from largely co-linear genotypes (Churchill et al., 2004; Crombie et al., 2024; Lister & Dean, 1993), and the use of multiple methods to factor out inversion presence within the DGRP (W. Huang et al., 2014) represent steps to account for these mutations. To be clear, the phenotyping and association studies from the DGRP and other mapping populations have produced many important and foundational insights. However, models that remove or ignore inversions miss a valuable opportunity. Methods like LOCO offer tools toward building association studies to identify relevant loci within inverted regions (Baran et al., 2013; Calboli et al., 2022). Inversions can play a significant role in the traits of humans and across many forms of life (García-Ríos et al., 2019; Giner-Delgado et al., 2019; K. Huang & Rieseberg, 2020; Merrikh & Merrikh, 2018). Improving our ability to connect inversion to traits will motivate future work to better understand how these complex mutations contribute to trait regulation and formation.

Data Availability

The DGRP's genomic data, Wolbachia infection status, "Full" GRM, and inversion genotype table are all available from the DGRP website (http://dgrp2.gnets.ncsu.edu/data.html). The aggregated DGRP phenotype data is available from the DGRPPool website (https://dgrpool.epfl.ch/) or from their original publications. All other data is available for download at (https://github.com/benedictlenhart/InversionGWAS)

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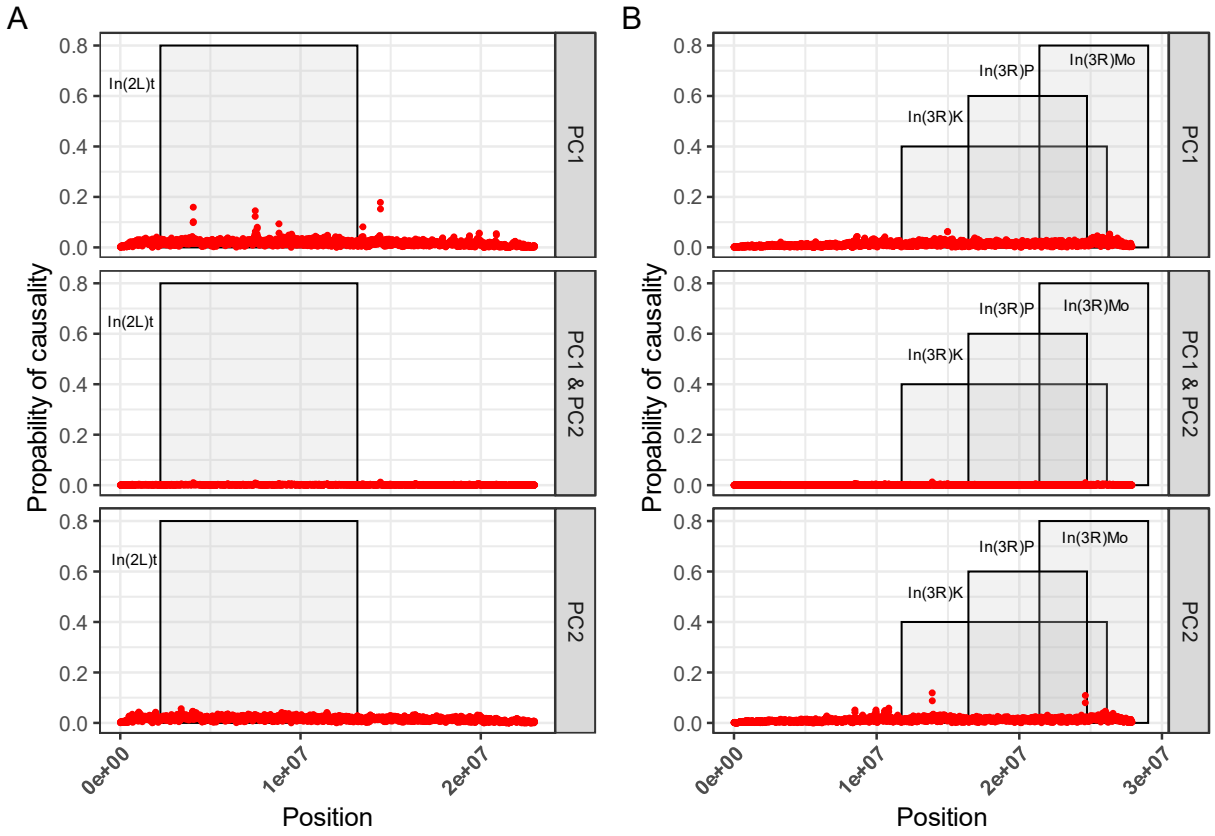
Supplemental Figures



Supp. 1: Factored-out method reveals little enrichment. **A)** Results of a sliding window analysis examining enrichment between SNPs scored using Factored-out for PC1 and PC2 of In(2L)t, the y axis showing the strength of enrichment and the x showing position on the genome. Grey shaded region showthe zone of cosmopolitan inversions on the chromosome arm **B)** Same analysis as in A, but considering In(3R)Mo

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Chapter 3

The inversion In(2L)t impacts complex and environment-specific behaviors in *Drosophila melanogaster*

Authors: Benedict Adam Lenhart[1] & Alan Olav Bergland[1]

Affiliations: [1]Department of Biology, University of Virginia, Charlottesville, VA 22904

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To whom correspondence should be addressed:

Adam Lenhart: bal7cg@virginia.edu

Alan Bergland: aob2x@virginia.edu

Abstract

Changes in behavior allow animals to respond to environmental change. In part, genetic variance in behavioral traits enables populations to evolve and adapt to environmental challenges. One type of mutation found to impact behavior are genomic inversions; these structural variants are also sometimes found to change in frequency across latitudinal and seasonal environments. However, how inversions regulate behavioral changes across different environments remains incompletely understood. This study uses modern behavioral assays and a genetically diverse panel of the fruit fly *Drosophila melanogaster* to investigate how the inversion In(2L)t impacts different aspects of behavior in an environment-sensitive manner. We test the activity, foraging, and startle-induced behavior of flies with different In(2L)t genotypes across each sex and different temperatures. We observe that *Drosophila* homozygous for In(2L)t are more active, spend more time away from a food source, and have a longer duration of startle response. Additionally, the impacts of In(2L)t on aspects of behavior are often sex-specific and sensitive to temperature. Taken together, our research demonstrates that inversions can regulate aspects of behavior, and suggests hypotheses explaining the distribution of In(2L)t across space and time.

Introduction

Animals adjust their behavior in response to environmental change that occurs on multiple time-scales. Long term changes like climate change influence habitat use and the timing of reproduction (Beever et al., 2017; Miller-Rushing et al., 2008), short-term environmental changes like predator and competitor presence affect cooperation and male-male contests (Groenewoud et al., 2016; Taylor et al., 2001). Some behavioral changes are cyclical, like the seasonal changes in migratory behavior (Chapman et al., 2015) or mating behavior (Riters & Stevenson, 2022). In general, behavioral variation is considered plastic in that a single individual modifies its behavior in response to environmental change (Snell-Rood, 2013). However, natural populations exhibit genetic variation in behavioral traits influencing both baseline behaviors (Fleury et al., 1995; Wong et al., 2019) and how sensitively behavior responds to environment factors (Flint, 2003; Niepoth & Bendesky, 2020).

Advancements in technology have improved our ability to quantify genetic and environmental effects on behavior (T. D. Pereira et al., 2020), and to do so at large scale (Schaefer & Claridge-Chang, 2012). For example, radio tags attached to mice track behavioral responses to variation in the nutritional environment and social cues (Peleh et al., 2019). Microscope recordings of *C. elegans* monitor responses to chemical and motor stimuli (Likitlersuang et al., 2012; Swierczek et al., 2011). In Drosophila melanogaster, automated methods have evolved from early beam-break systems that inferred circadian activity and sleep (Pfeiffenberger et al., 2010; Chiu et al., 2010; Cichewicz & Hirsh, 2018) to video tracking software that quantifies speed (Faville et al., 2015), spatial positioning (Donelson et al., 2012), and responses to sensory stimuli (Werkhoven et al., 2019).

The unique genomic character and rich literature of *Drosophila melanogaster* makes *Drosophila* a tractable model for studies into the natural genetic variation in behavior. One particularly interesting genetic variant is the large (10Mb) structural inversion, In(2L). This inversion is linked to elevated startle response duration (Mackay et al., 2012; Nunez et al., 2024) foraging behavior (Lee et al., 2017).This inversion is found at intermediate frequencies in populations worldwide (Stalker, 1980; van Delden & Kamping, 1989) and appears to mediate seasonal adaption in *D. melanogaster* as its frequency shifts seasonally . Previous studies that report the role of In(2L)t on behavior use the beam-break model, creating the opportunity for newer models better characterize its behavioral impact across a range of environments.

To explore how In(2L)t alters behavior in an environment-specific manner we employed automated behavioral tracking. We generated F1 offspring with different In(2L)t genotypes through controlled crosses . We found that In(2L)t increases the duration of startle

response, the frequency of activity, and the length of time spent away from food. Furthermore, these inversion-driven differences are sex-specific, and modulated by certain thermal environments.

Methods

Fly stocks and husbandry. We used F1 crosses between inbred strains of the Drosophila Genomic Research Panel (Supp. Table 1) (Mackay et al., 2012). Flies were kept on standard cornmeal media and maintained within a 25°C incubator set to a 12:12 light/dark schedule and 50% relative humidity. For each behavioral measurement, we used 2-5 day old non-virgin flies.

Behavioral assays. We recorded the behavior of the F1 offspring using the Drosophila Arousal Threshold (DART) device (BFK Labs, Hertford, UK) (Faville et al., 2015). Flies were placed within plastic tubes with a 1.5% agar, 5% sucrose solution to prevent desiccation and starvation and a cotton plug to prevent asphyxiation. Before assays, we acclimated the flies in the DART overnight. The flies were kept in constant darkness, 50% relative humidity, and at a three temperatures (20°, 25°, and 30°C). We recorded the basal activity and induced activity following mechanical stimulus. We performed this experiment in three replicate blocks, each time redoing the DGRP crosses and using the same behavioral quantification methods. The replicate experiments tested 414, 693, and 709 F1 individuals respectively. We measured male and female fly behavior at 25C°, then compared behavior of female flies across a range of temperatures.

Behavioral quantification. We used the DART MATLAB software to track the motion of individual flies within videos and to quantify behavior (version 811f772; Faville et al., 2015). We recorded the base speed of flies (mm/s) and the proportion of time moving (minutes per hour) in the hour prior to mechanical stimulus. We characterized startle response by assessing the change in speed following mechanical stimulus. For each fly, we estimated startle-induced activity as the speed in the minute following stimulus, minus that individual fly's base speed. We estimate startle response duration as the time between the stimulus to when the individual fly's speed returns to its base speed. To estimate foraging behavior, we split the tubes into 8 even-length regions, then found the ratio of time spent in the nearest-food region vs in the other seven regions.

Statistical analysis. We analyzed data using mixed effect models implementing the *lmer()* function from lme4 (version 1.134)(Bates et al., 2015). The full model is described by the formula:

$$\text{Trait} \sim \text{In(2L)t} + \text{temperature} + \text{sex} + \text{experiment}$$

Where In(2L)t is the fixed effect of inversion genotype, temperature is the fixed effect of temperature, sex is the fixed effect of sex, and experiment is a random effect of the replicate experimental block. We assessed statistical significance of the fixed effect terms using the *anova()* function from lmerTest (version 3.1-3) (Kuznetsova et al., 2017). Anova results for each phenotype are found in (Supp. Table 2_Anovas).

If a significant difference was found from one of the fixed effects, post-hoc pairwise Student's t-tests were used to find differences between pairs of groups.

To check for the presence of gene by environment interaction within In(2L)t's impact on a trait across temperatures, we created two models.

Additive model:

Trait ~ In(2L)t + temperature

Interactive mode:

Trait ~ In(2L)t * temperature

We compared the two models using the aov() function from Base R to identify significance of interaction.

Results

Flies with the inversion In(2L)t are consistently more active across temperatures

Inversion genotype had a significant effect on the proportion of time that flies are active ($F_{2\ 4616}$ = 47.96, p = 3.49e-21). Female flies homozygous for the inversion move around more than heterozygous flies (t-test, t = 3.93, df = 743 p = 9.28e-5), and heterozygote females move around more than homozygous standard females (t-test, t = -4.65, df = 811, p = 3.85e-6; **Fig. 1A**). Standard male flies were also active less than heterozygotes (t-test, t = -2.9, df = 106, p = 0.0045). Sex had a significant effect overall on proportion of time moving ($F_{1\ 3610}$= 30.64, p = 3.33e-8), as did temperature ($F_{1\ 4616}$ = 399, p < 2.2e-18). In general, differences in activity between genotypes were preserved across temperatures (**Fig. 1B**), with inverted homozygotes being more active than standard homozygotes at 20°C (t-test, t = -3.89, df = 690 p = 1.11e-4) and 30°C (t-test, t = -6.74, df = 709 p = 3.35-11). At 20°C, the heterozygotes had the same activity as inverted homozygotes (t-test, t = -0.27, df = 606, p = 0.78), suggesting that the inverted allele is dominant, whereas at 30°C the heterozygotes had an intermediate level of activity, with standard flies exhibiting less activity than inverted flies (t-test, t = -6.74, df = 709, p = 3.35e-11). We do not observe significant difference in base speed between genotypes ($F_{2\ 4128}$ = 0.25, p = 0.78; **Fig. 1C**) or between the sexes ($F_{1\ 101}$

= 1.7, p = 0.19). However, there was a significant effect of temperature on speed ($F_{1\,4128}$ = 0.25, p < 2.2e-16; **Fig. 1D**).

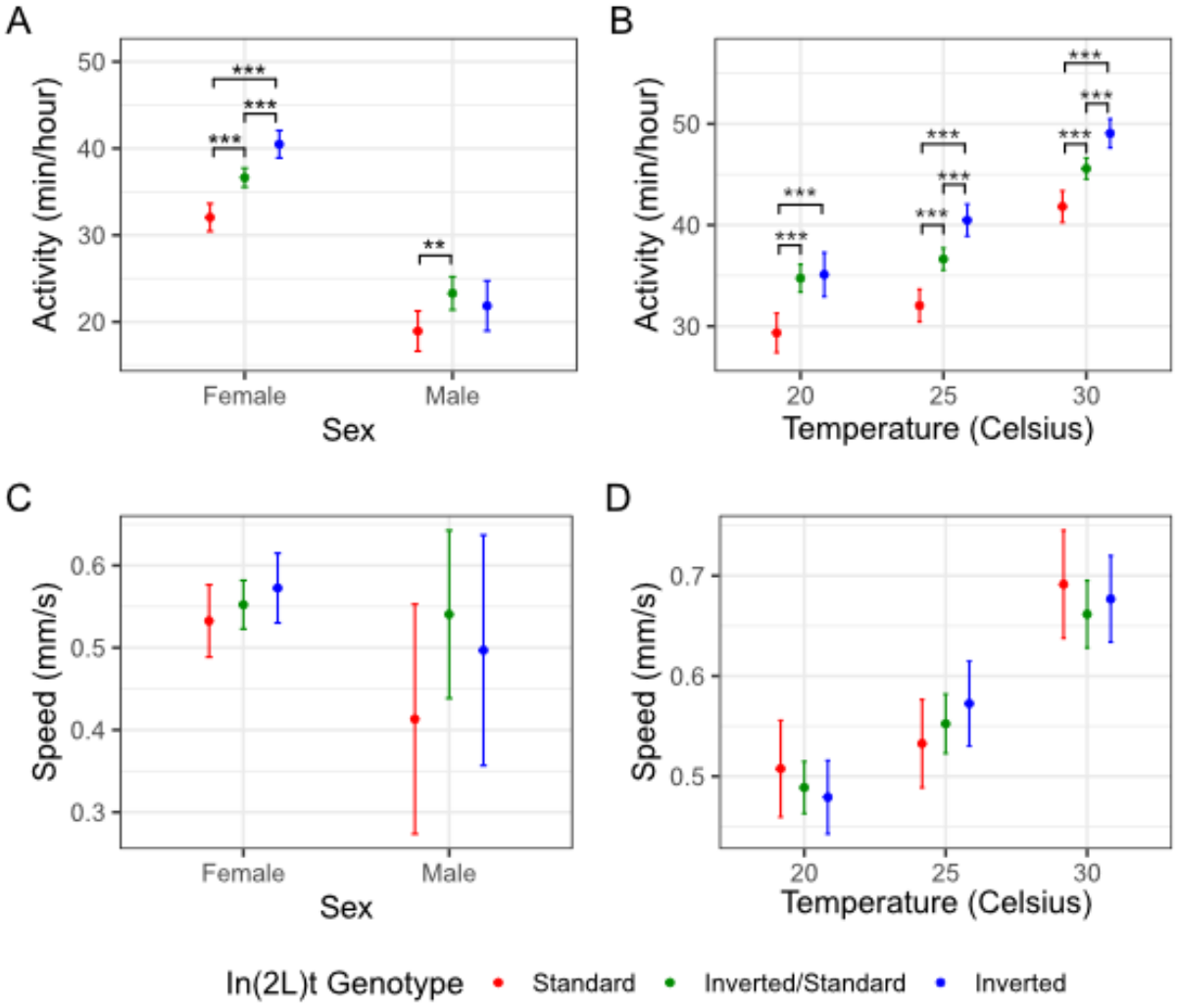

Fig. 1: In(2L)t presence associated with more time spent active A) Measurements of mean time spent active per hour across sexes at 25C°, colored by the In(2L)t genotype. B) Comparison of mean time spent active over a range of temperatures. C) Baseline speed at 25C° measured in mm/s across sexes, colored by presence of In(2L)t. D) Same as in C, but comparing across temperatures. (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, no * = p >= 0.05)

Inversion genotype affects startle response duration and intensity

We observe on average no effect of genotype on the duration of startle response ($F_{2\,4126}$ = 0.09, p = 0.91), though we do observe an effect of temperature on this trait ($F_{1\,4126}$ = 91.33, p < 22e-16). Specifically, we can identify that inverted female flies are startled for longer than

standard females post-stimulus at 25C° (t-test, t = -2.23, df = 662, p = 0.0258; **Fig. 2A**). There is no observed effect of sex on startle duration ($F_{1\ 3710}$ = 2.02, p = 0.16). We reanalyzed existing datasets in Mackay et al., 2012 and found a significant increase in startle response duration for inverted females ((Mackay et al., 2012), t-test, t = -3.38, df = 10, p = 0.007) and for inverted males ((Mackay et al., 2012), t-test, t = -2.86, df = 9, p = 0.02).

In(2L)t-significantly impacts startle induced speed ($F_{2\ 6188}$ = 9.72, p = 0.0001), and there is a genotype by environment interaction ($F_{2\ 3921}$ = 3.8, p = 0.022). Heterozygote females have higher induced speed than inverted homozygotes at 25°C (t-test, t = 2.5, df = 700, p = 0.013; **Fig. 2C**), while at 20°C heterozygote females have higher induced speed than standard homozygotes (t-test, t = -2.23, df = 662, p = 0.026; **Fig. 2D**). Inverted homozygotes have higher induced activity at 30C° than heterozygotes (t-test, t = -4.04, df = 790, p = 5.91e-5) or standard flies (t-test, t = 3.40, df = 612, p = 7.01e-4; **Fig. 2D**). There is an effect of sex on startle induced speed ($F_{1\ 6034}$ = 5.82, p = 0.016).

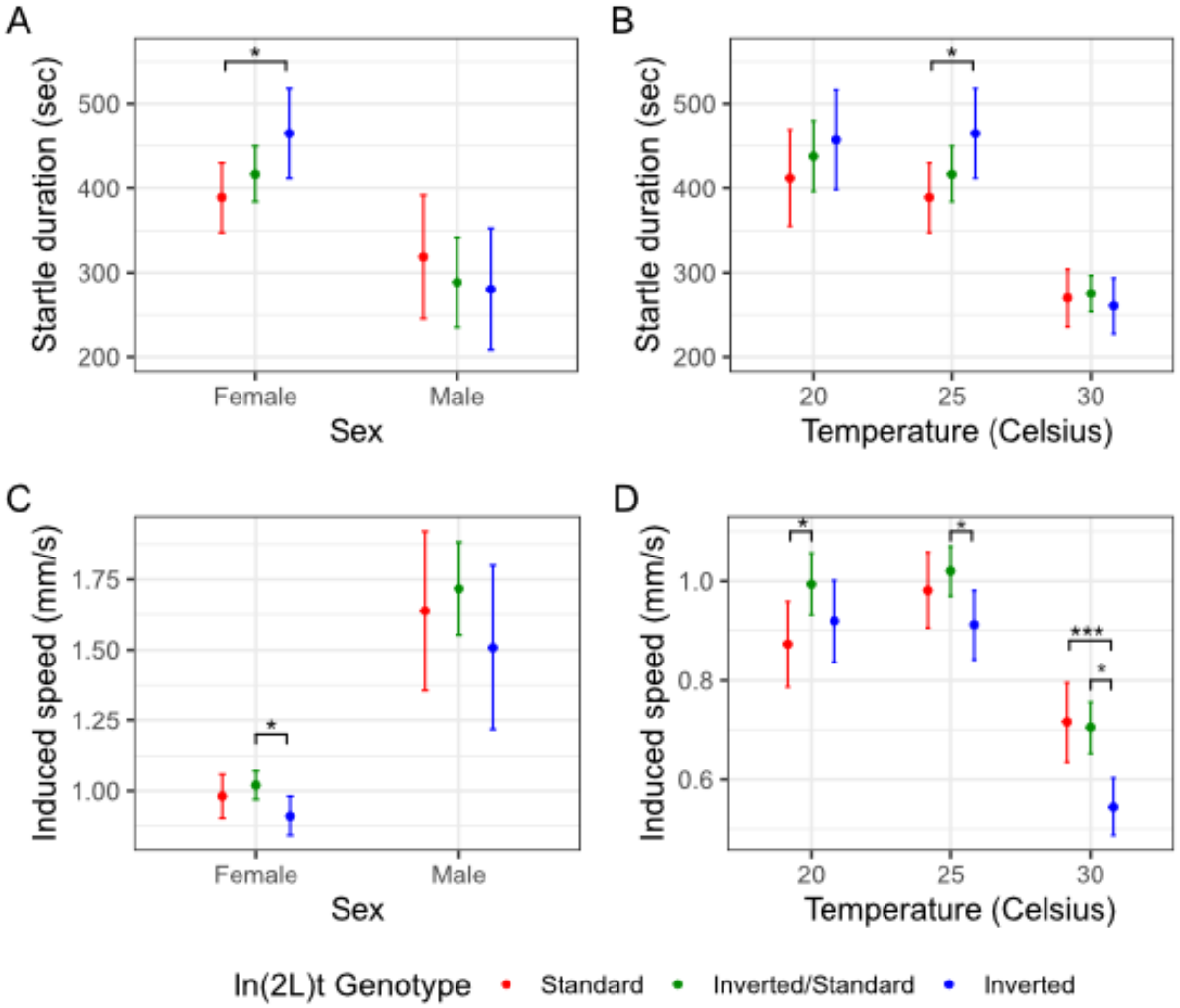

Fig. 2: Inverted flies exhibit changes in induced behavior A) Startle response at 25C° measured in number of seconds moving after stimuli, colored by In(2L)t presence. B) Same as in A, but across temperatures. C) Induced activity at 25, defined as the difference in speed between startled state and baseline, again colored by In(2L)t genotype. D) Same as in C, but across temperatures. (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, no * = p >= 0.05)

In(2L)t associated with more time spent away from food

We observed a significant effect of In(2L)t genotype on the location of flies over time relative to their food ($F_{2\,2745}$ = 16.83, p = 5.42e-8). Inverted flies were near food less than standard flies both in the case of females at 25°C (t-test, t = 2.04, df = 438, p = 0.042) and males at 25°C (t-test, t = 2.48, df = 9.12, p = 0.043; **Fig. 3A**). Across temperatures, female inverted flies spent less time near food than heterozygote flies, including at 20C° (t-test, t = 4.2, df = 326, p = 3.44e-5) and at 30C° (t-test, t = 3.49, df = 398, p = 5.46e-5; **Fig. 3B**). While

there was no effect of sex on ratio of time spent near food ($F_{1\ 2627}$ = 2.47, p = 0.12), there was an effect of temperature on this foraging-related trait ($F_{1\ 2745}$ = 37.35, p = 1.12e-9).

Interestingly, Lee et al., 2017 also observed that In(2L)t is associated with variance in foraging within the DGRP (Lee et al., 2017a). We reanalyzed their data and found that flies homozygous for In(2L)t were significantly worse at finding the food source, and subsequently starved sooner, than standard genotyped flies (t-test, t = 3.14, df =19, p = 0.005).

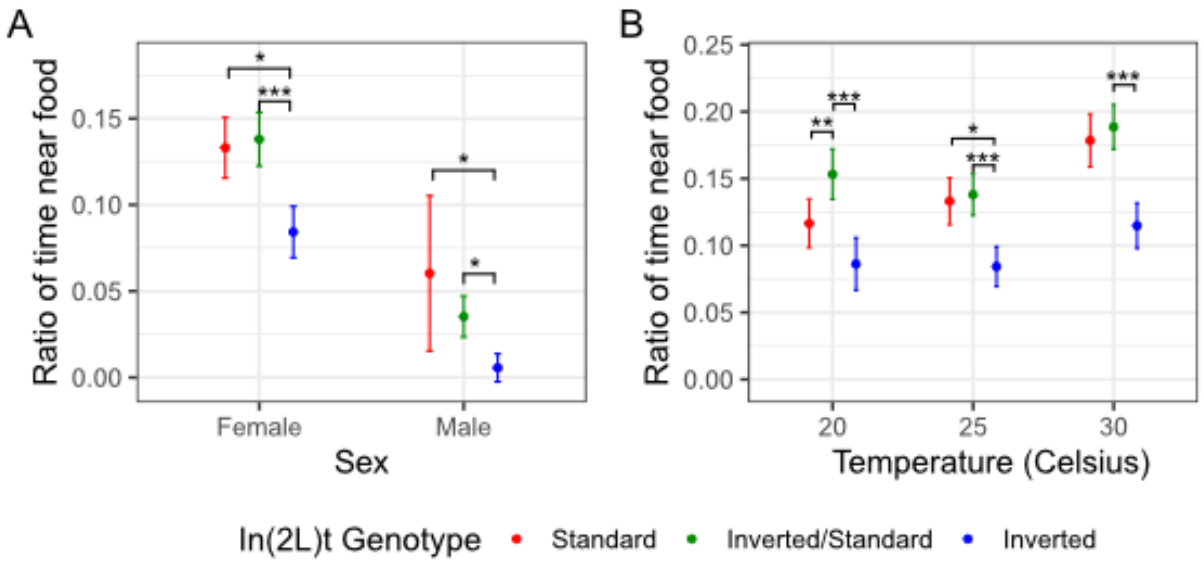

Fig.3: Inverted flies spend more time away from food source A) Ratio of time spent near food vs away from food at 25C within the DART experiment°, colored by presence of In(2L)t. B) Same as in A, but across temperature. (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, no * = p >= 0.05)

Conclusion

In this study, we use automated behavioral observation to further characterize how the inversion In(2L)t impacts multiple aspects of behavior in an environment and sex specific manner. By quantifying motion and activity of *D. melanogaster* from a set of genetically diverse crosses, we show that presence of In(2L)t has an impact on time spent active (**Fig. 1A, Fig.1B**), the duration and intensity of startle response (**Fig. 2**), and the amount of time spent near the food (**Fig 3A**). In(2L)t's impact on behavior is mostly consistent across temperatures, though we do note evidence of gene by environment interaction between for startle response intensity. With these findings, we emphasize the importance of inversions

as a model for investigating the genetic variation in complex traits such as behavior, and the sensitivity of these variants to changing environments.

In(2L)t has a complex role in the characterization of induced and un-induced activity, and contains genetic architecture thought to regulate aspects of behavior. In general, homozygous In(2L)t flies are more active than homozygous standard flies (**Fig. 1A**), and more often venturing out from the food source (**Fig. 3A**). Additionally, while In(2L)t flies may exhibit a reduced intensity of response to stimuli (**Fig. 2C**), they respond with elevated speed for longer than the standard genotype counterparts (**Fig. 2A**). This ability of In(2L)t to affect how flies respond to motor stimuli resembles In(2L)t's impact on startle response observed in Mackay et al., 2012 (Mackay et al., 2012). *D. melanogaster* has imperfect linkage within inversions (Kennington et al., 2007; Kennington & Hoffmann, 2013), resulting in polymorphisms between those with the inverted genotype. It is thus possible that specific regions or genes within the inversion may have an outsized role on behavioral changes (Nunez et al., 2024, Chapter 2). The *For* gene has a large impact on the range of travel while foraging (Nagle & Bell, 1987; H. S. Pereira & Sokolowski, 1993), and resides within In(2L)t. Previous work has identified that In(2L)t can explain part of the variance in foraging behavior (Lee et al., 2017). *Vglut* is in high linkage with In(2L)t (Kapun & Flatt, 2019; Nunez et al., 2024). This gene resides near the 5' In(2L)t breakpoint, regulates transport of glutamate, and is linked to sleep and startle response (Birgner et al., 2010; Hamasaka et al., 2007). Alleles for these genes and others within or near the inversion are subjected to suppressed recombination (Dobzhansky & Epling, 1948; Kirkpatrick & Barton, 2006), which may explain the impact of In(2L)t genotype on different aspects of behavior.

The effect of In(2L)t on behavior is largely consistent across temperatures. This insensitivity to changes in environment implicates In(2L)t's role in seasonal modification of traits. Inverted flies are more active than standard across temperatures (**Fig. 2B**), as well as spend more time away from food (**Fig. 3B**). This consistent impact on behavior is relevant considering there is mounting evidence that In(2L)t is involved in mediation of seasonal adaptation. The frequency of In(2L)t changes cyclically across the year, becoming highest in the winter months (Machado et al., 2021; Nunez et al., 2024). This implies that the phenotypic variance explained by In(2L)t could be under directional selection within a given season. This argument is supported by the identification signals of selection for loci within the inversion (Nunez et al., 2024). A resilient effect of In(2L)t on traits, such as we observe, can lead to consistent action of selection, potentially driving the yearly fluctuation in In(2L)t frequency. If higher activity is adaptive within colder months, then this could drive In(2L)t frequency higher, while high activity becoming maladaptive in warmer months could drive the In(2L)t frequency lower.

Season-specific regulation of behavior is essential for successful adaptation to new environments (Chapman et al., 2015; Riters & Stevenson, 2022). Strategies of inactivity versus activity are dependent upon the nutritional abundance (Wang et al., 2006), access to light (Welsh, 1938), and temperature stressors (Buchholz et al., 2019) that come with changing seasons. For example, circadian control of activity and sleep is closely regulated by light and temperature, two elements of environment that change from summer to winter (Boothroyd et al., 2007). There is strong evidence that natural variation in sleep exists across different environments, with nighttime duration of inactivity increasing at lower latitudes (Svetec et al., 2015). Given that the length of sleep can be selected for (Harbison et al., 2017), this indicates selection could be acting on genetic variation regulating periods of inactivity in different latitudinal environments. Even startle response varies depending on the benefit of strong/weak responses. For examples, sticklebacks change their response intensity depending on the temperature of their local environment (Guderley et al., 2001). Selection acts on many aspects of behavior in different directions across different seasons, but genetic variation in these traits must be preserved for this yearly adaptation to progress.

The impact of In(2L)t on *D. melanogaster* behavior demonstrated how fitness-conferring alleles can be maintained across seasons. In(2L)t pleiotropically impacts time spent active, duration and magnitude of induced activity, and aspects of foraging within *D. melanogaster* (**Fig. 1A, Fig. 2A, Fig. 2C, Fig. 3A**), and there is evidence of regions within the inversions being especially impactful on behavior (Nunez et al., 2024, Chapter 2). Additionally, we report evidence that In(2L)t's effect on behavior is sometimes environment-specific (**Fig. 2B, Fig. 2D**) or environmentally resilient (**Fig. 1B, Fig. 3B**). Selection acts on different aspects of behavior, sometimes in a seasonal manner (Chapman et al., 2015; Riters & Stevenson, 2022). The activity levels impacted by In(2L)t could thus be under seasonally varying selection. In(2L)t's impact on activity levels is largely robust across temperature changes, indicating that selection could drive changes in the inversion's frequency across seasons. The potential complex and shifting selection on the inversion can help explain both why In(2L)t frequency fluctuates yearly (Machado et al., 2021), and how this inversion continues to be identified at intermediate frequency within populations (Nunez et al., 2024; Stalker, 1980; van Delden & Kamping, 1989). Together, the balancing selection driven by In(2L)t's pleiotropic effects and its interactions with thermal environment could serve to maintain the genetic variation in these traits across seasons, thus enabling repeated adaptation to seasonal environments.

Data Availability

The DGRP lines used here are available from the Bloomington stock center in Indiana (https://bdsc.indiana.edu/). Inversion genotype tables for the lineages are all available from the DGRP website (http://dgrp2.gnets.ncsu.edu/data.html). The reanalyzed phenotype data from previous studies can be found from their original publications. All other data is available for download at (https://github.com/benedictlenhart/In-2l-t_Behavior)

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