Evolution of Intrapopulation Genetic Variation and Genomic Basis of Diversity-driven Ecological Emergence

集団内遺伝的変異の進化と多様性が駆動する生態的創発特性の遺伝基盤

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Graduate School of Science and Engineering,
Chiba University

Takahisa Ueno

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General Introduction

Biodiversity is the variety of life and the interactions between organisms on Earth. Evolutionary biologists and ecologists have long studied the mechanisms underlying the evolutionary maintenance and ecological pattern of such tremendously high biodiversity. In general, biodiversity can be considered at three main levels: genetic diversity, species diversity, and ecosystem diversity. A fundamental source of biodiversity is genetic diversity, which is defined as the extent of genetic variation within a species. Genetic variation is often referred to as genetic differences in many traits among conspecific individuals, which has traditionally been important in evolutionary processes. That is, genetic variation provides the raw material for evolution via natural selection and gene flow—a change in the genetic composition of a population. Evolutionary biologists have accumulated evidence for evolution made by genetic variation that influences fitness and is supplied by geographically other populations (e.g., Fisher, 1930; Kottler et al., 2021). Such intraspecific genetic variation was ignored by ecologists for a long time, though it had long contributed to understanding evolutionary mechanisms in classical studies. This is because the classical ecological theory focused on mainly predicting the dynamics of species' abundances over time, assuming that individuals are homogeneous within a population, i.e., intraspecific genetic variation is noise around the average characteristic of a species. However, genetic diversity can no longer be neglected in ecological dynamics (Fig. G-1), because interindividual genetic variation is ubiquitous (Nakazawa, 2020). Heterogeneity within populations has been gradually revealed to unpredictably affect population demography (Hughes et al., 2008; Bolnick et al., 2011). Similarly, ecologists have progressively unveiled that species diversity drives ecological dynamics before

paying attention to the ecological importance of genetic diversity (e.g., Naeem et al., 1994; Cardinale et al., 2012). Overall, understanding the ecological consequences of biodiversity passing throughout evolutionary processes is actively advancing in ecology and evolution, as described below.

Until the early nineties, numerous studies had focused on the causes of the establishment of high biodiversity and pursued the mechanism of coexistence and interactions among species (e.g., Hutchinson, 1959; Macarthur & Levins, 1967). In this paradigm, biodiversity was thought to just respond to environmental change and ecosystem functioning. Approximately three decades ago, a major paradigm shift happened in ecological thinking for biodiversity. Numerous experimental and theoretical studies presented evidence supporting that biodiversity could actively drive the nature of populations, communities, and ecosystems (see reviews by Tilman et al., 2014; van der Plas, 2019). These results built a new ecological subfield, conceptualizing the relationships of "biodiversity and ecosystem functioning" (BEF hereafter). BEF research generally investigates "ecological properties", defined as any measurable component of ecosystems, communities, and populations, in order to assess their responses. In previous studies, ecological properties in ecosystems often rephrase ecosystem properties (de Bello et al., 2021). According to various BEF studies, species diversity (e.g., species number) contributes to non-additively increasing ecosystem properties: (i) ecosystem functioning such as productivity (Naeem et al., 1995; Tilman et al., 1996) and nutrient cycling (Hättenschwiler et al, 2005), (ii) stability (Isbell et al., 2015), and (iii) resistance (Tilman and Downing, 1994). The emergent effects on ecological properties and their stability driven by biological diversity have been well known as the "diversity effect" in ecology. Today, we face biodiversity loss caused by

global anthropogenic perturbations, which prompt many ecologists to elucidate its effects on planetary health, such as the quality of ecosystem services and the risk of infectious diseases (Cardinale et al., 2012). Understanding the role of biodiversity in various levels of ecological organization will be increasingly necessary to provide the answers to our global issues of the twenty-first century.

Recent studies have established an important facet of the non-additive effects caused by genetic diversity (Forsman, 2008; Hughes et al., 2008; Wennersten and Forsman, 2012; Wolf and Weissing, 2012; Raffard et al., 2019). Research on these effects is noteworthy because genetic diversity can have a more pronounced impact on regulating ecological dynamics than species diversity (Des Roches et al., 2018). An increase in the number of genotypes/phenotypes in a population improves ecosystem functioning in eelgrass, tall goldenrod, and crayfish (Reusch et al., 2005; Crutsinger et al., 2006; Raffard et al., 2017) and population properties: (i) population performance, such as survival rate in pygmy grasshoppers (Caesar et al, 2010), productivity in weedy herbs and fruit flies (Crawford and Whitney, 2010; Takahashi et al., 2018), and nutrient acquisition in diatom (Sjöqvist and Kremp, 2016), (ii) population persistence (Forsman et al., 2015), and (iii) range shift (Forsman et al., 2016). Genetic diversity effects are suggested to require a variety of population processes via improved use of resources in fish (Dyer et al., 2009) and reduced predation and sexual harassment in insects (Ahnesjö and Forsman, 2006; Takahashi et al., 2014). On the other hand, genetic diversity sometimes negatively affects ecological properties. For example, genotype richness in the riparian tree did not improve forest productivity in an ecosystem-scale common garden experiment (Fischer et al., 2017). Individual genotypes in this study were not collected from sympatric populations and were likely to be insufficient in detecting

niche complementarity as previous studies expected. This finding suggests a lack of resource-use complementarity among such genotypes (Zuppinger-Dingley et al., 2014). Depending on environmental conditions and indices relating to sociality, diversity effects on population properties could occur negatively in some cases (Jousset et al., 2011; Ellers et al., 2011; Takahashi et al., 2018; Wuest and Niklaus, 2018; Turner et al., 2020; Fisher et al., 2021). Nevertheless, most studies focused on the mechanisms that cause positive diversity effects and, therefore, the strength and ubiquity of negative ones are currently controversial. Further research is required to investigate both directions of diversity effects, considering environmental conditions and types of population properties.

In many empirical and theoretical studies, as described above, diversity is generally quantified as the richness (i.e., the number) of genotypes and species. It is assumed that richness in an ecological organization facilitates niche differentiation in traits/genes among species and conspecific individuals, and that niche differentiation reflects the extent of niche complementarity, which determines the direction of diversity effects. These advanced disciplines of diversity effects raised another question: which "diversity of traits/genes" is responsible for diversity effects? However, our understanding of causal drivers of diversity effects remains poor for several reasons. Firstly, previous studies rarely focused on the dissimilarity of traits influencing the population properties. The dissimilarity of traits is generally regarded as niche differentiation among species and individuals. Studies of microcosm communities revealed that, in some cases, the diversity effects of species on ecosystem properties were determined by dissimilarity in species traits rather than species number (Heemsbergen et al., 2004; Wojdak and Mittelbach, 2007). On the other hand, we lack

sufficient data across a broad range of traits relating to an increase or decrease in various population properties. According to a previous discussion, it also remains unpredictable whether the diversity of traits is associated with diversity effects positively or negatively (see Bolton et al., 2015, 2016; Forsman, 2016). For example, color variation was expected to enhance the survival and the expansion of geographical distribution in insects (Caesar et al., 2010; Takahashi and Noriyuki, 2019), whereas reducing resource use in two reptile species (Broennimann et al., 2014). Variations in behavioral traits that occurred by sexual diversity facilitated faster detection of novel food patches in guppies (Snijders et al., 2019, 2021) while decreasing female survival probability due to sexual harassment by male aggression in lizards (Le Galliard et al., 2005). A theoretical model predicted that phenotypic variation under positive frequency-dependent selection, where the rare phenotype is unlikely to be advantageous, affected a population harmfully (Takahashi et al., 2018). This prediction suggests that variation in traits passing through evolutionary processes does not always affect population performance positively. To reveal which dissimilarity of traits is relatively important for positive or negative diversity effects, we need to consider variation in various traits simultaneously.

Secondly, only a limited number of studies have investigated the causative genes of diversity effects. Several recent studies have expanded the idea of the traditional approach that associates genotypes with phenotypes among genetically different individuals and verified the association of genome information with ecological properties among diverse populations in *Arabidopsis thaliana* (Wuest and Niklaus, 2018; Turner et al., 2020; Wuest et al., 2022, 2023). In another example, the dimorphism of foraging behavior in *Drosophila melanogaster*, which is maintained by

allelic variation in the single-gene *foraging* under balancing selection, has a positive diversity effect on population biomass (Takahashi et al., 2018). However, many previous studies just used the diversity of only a few genotypes. Moreover, genetic regions controlling positive diversity effects on plant productivity were different between the two studies because the original two genotypes differed in establishing genetically diverse populations that shaped positive diversity effects (Wuest and Niklaus, 2018; Wuest et al., 2023). These findings suggest that the mechanism underlying diversity effects is complicated and that it is technically challenging for us to target the specific traits/genes responsible for diversity effects.

Finally, candidate diversity-effect traits/genes would change depending on the number of genotypes. Turner et al. (2020) used 60 genotypes of *A. thaliana* across the Eurasian range and experimentally assembled non-native mixtures of their combinations to measure population properties. This study also identified candidate loci whose allelic diversity was associated with population productivity, although genetic richness did not increase it. However, identified specific genetic loci in Turner et al. (2020) were different from those in Wuest and Niklaus (2018) and Wuest et al. (2023). We might have overlooked both positive and negative diversity effects that occurred among genotypes that were not considered previously. Moreover, the problem of false positives arising from genetic architecture population could remain in Turner et al. (2020) because assembled individuals across the habitats and measured productivity in such non-native mixtures. Hence, sufficient assessments of causative traits/genes were not demonstrated yet. To promote an understanding of the genomic basis of the ecological consequence of genetic diversity, it is worth experimentally combining various genotypes that result in the diversity effects by various mechanisms and investigating

traits/genes whose diversity was associated with them.

My thesis constructed five chapters, which followed the concept of evolutionary ecology: from evolutionary process to ecological consequence (Fig. G-1). In all chapters, I used *Drosophila* species, which is one of the most powerful, experimentally tractable model organisms for studying ecology and evolution. In animals, including fruit flies, intrapopulation behavioral variation, including personality variation, is thought to exert a considerable effect on ecological and evolutionary dynamics and eco-evolutionary feedback (Dall et al., 2012; Wolf and Weissing, 2012; Takahashi et al., 2018). Chapter 1 starts with a verification of to what extent interindividual variation in daily activity is found within a natural population. The behavioral daily rhythms in *Drosophila* have been extensively investigated under laboratory conditions (Tauber et al., 2003). Adults of *D. melanogaster* have a bimodal activity distribution that is described as “crepuscular”: the flies move actively in the early morning and evening and are less active during mid-day (Helfrich-Förster, 2001). Similar patterns in daily activity are observed in other *Drosophila* species (Beauchamp et al., 2018). Therefore, the crepuscular activity is thus believed to be ubiquitous in *Drosophila*. However, it is unclear whether there is intrapopulation variation in activity patterns and their daily rhythms in *Drosophila* species. In the chapter, I investigated the genetic variation in larval and adult activities in a single natural population of *D. immigrans*, a close relative of *D. melanogaster*.

In Chapter 2, I focused on the relationship between behavioral variation and mito-nuclear gene interaction. Genetic variation in activity often has been captured in the nuclear genome with candidate-gene approaches and genome-wide association analysis (Shorter et al., 2015; Li et al., 2016; Watanabe et al., 2020). However, genetic

variation in mitochondria could play an important role in variation in metabolic phenotypes and behavioral, life-history, and reproductive traits (Gemmell, Metcalf and Allendorf, 2004; Arnqvist et al., 2010; Løvlie et al., 2014; Novičić et al., 2015), because the mitochondrial genome is known to encode key regulators of cellular energy-producing pathways in metabolic processes (i.e., oxidative phosphorylation). In the chapter, I examined the mitochondrial haplogroups on differences in larval and adult activity in natural populations of *D. immigrans*. I then explored the effect of mito-nuclear genetic interaction on individual behavior.

Chapter 3 focused on the evolutionary change of genetic diversity within a population over time. The extent and causes of genetic variation have been debated for more than half a century. An important implication in a recent study was that mutation-selection balance, balancing selection, and the interaction of spatially/temporally fluctuating selection are all essential to maintain genetic variation in nature (Charlesworth, 2015). Out of them, contemporary evolution, or adaptive evolution in populations on timescales of less than a few hundred years, is mainly driven by such fluctuating environments. Seasonality is one of the ubiquitous drivers of fluctuation selections in organisms within a year. Studies on the seasonal changes in genetic variation will contribute to a better understanding of the mechanisms underlying the maintenance of genetic diversity and, in turn, can have an impact on the comprehension of diversity effects. In *Drosophila* having multiple generations in a year, environmental changes across seasons could create different selection regimes, remarkably leading to different phenotypes with respect to several life-history traits and desiccation tolerance (Behrman et al., 2015; Rudman et al., 2022). However, many studies that demonstrated seasonal changes in phenotypes cannot fully reject the possibility that environmental

factors, such as conditions in which the experiments were conducted, accidentally generated an apparent seasonal variation (Stone et al., 2020). In the chapter, I investigated the evolutionary response to seasonal environmental changes by simultaneously measuring thermal tolerance and morphological traits in *D. lutescens* of spring and autumn periods collected in a single location.

I have pursued the role of genetic variation mainly in behavior and thermal tolerance within the context of the evolutionary process, as described so far in the preceding paragraphs. The intrapopulation genetic variation could be maintained through evolutionary processes, and the resulting impacts can potentially influence subsequent consequences for population dynamics. In Chapters 4 and 5, I investigated "which diversity of traits/genes" affect diversity effects in two *Drosophila* species stemming from a wild population. In my experiments, I used two types of omics data: phenome and genome. Until now, complex emergent properties induced by biodiversity did not necessarily seem genetically tractable because genome information was limited. *Drosophila* is useful for my question: it exhibits natural phenotypic/genetic variation in diverse types of traits, diverse genotypes with re-sequenced genomes are handleable in some species (*Drosophila* 12 Genomes Consortium, 2007), and it is a model for ecological and quantitative genetics. The two chapters would give us more precise evidence for the genomic basis of diversity effects. Chapter 4 focused on which phenotypic diversity out of various traits non-additively affects population properties. I exhaustively examined the associations of diversity effects with phenotypic variation in various traits. On the other hand, Chapter 5 focused on which nucleotide diversity out of various genes non-additively affects population properties. I adopted a data-driven approach to survey genes contributing to diversity effects using a natural genetic

variation in *D. melanogaster*. To pursue the genomic basis of diversity effects at levels of traits and genes, I assessed the dissimilarities quantified by Euclidian distances of phenotypes per trait and the number of nucleotide differences per genetic site between two inbred lines of *Drosophila* species. I also measured the diversity effects that occurred by the two lines. Different dissimilarity of various combinations is expected to increase or decrease diversity effects (Fig. G-2). Then, I identify drivers of diversity effects and propose “ecological genomics” combining ecological properties and genetic backgrounds in populations. This thesis could be an important milestone in the pursuit of non-additive ecological dynamics, opening up new horizons in ecology.

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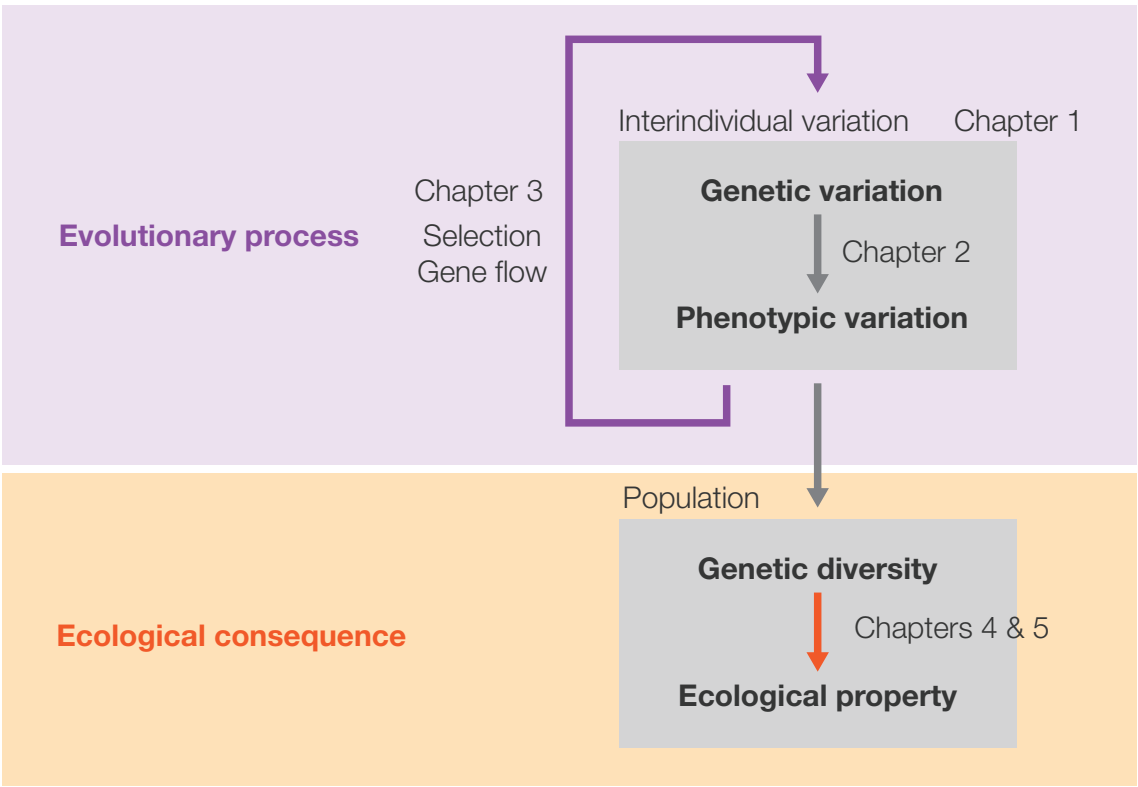

Figure G-1. Evolutionary process and ecological consequence underlying genetic diversity. The concept of evolutionary ecology mainly consists of two frameworks. Each chapter focuses on each research interest.

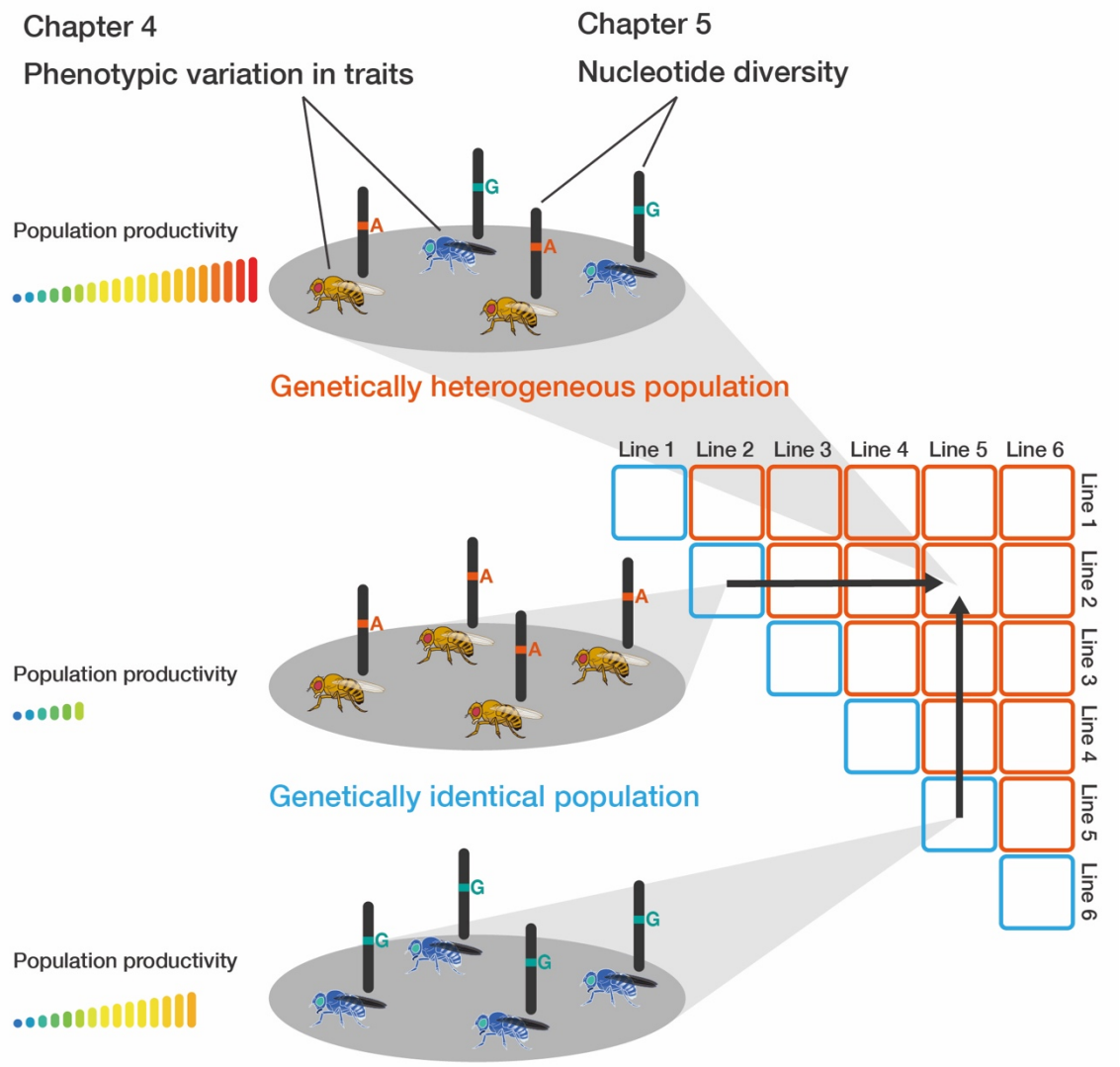

Figure G-2. The strategy of my experiment in Chapters 4 and 5. The various combinations (i.e., genetically diverse populations) and monocultures (i.e., genetically identical populations) were established to measure the dissimilarity (assessed by qualitative phenotypic variation and nucleotide diversity) and population properties (e.g., population productivity). Traits and genes were identified by the associations of the dissimilarity with diversity effects calculated from a population property.

Chapter 1

Intrapopulation genetic variation in the level and rhythm of daily activity in *Drosophila immigrans*

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Abstract

Genetic diversity within a population, such as polymorphisms and personality, is considered to improve population performance because such intraspecific variations have the potential to alleviate the competition for a limited resource or the risk of predation and sexual harassment at a population level. Variation in the level and rhythm of daily activity in a population could also affect population performance by directly altering ecological, social, and sexual interactions among individuals. However, it remains to be elucidated whether such intra-population variation in the level and rhythms of daily activity exists in a natural population. Here, I investigated the genetic variation in daily activity within a single natural population of *Drosophila immigrans*. I established 21 isofemale lines from a single natural population and measured larval activity level and the level and daily pattern of adult activity over a 24 h period. Larval activity level significantly varied among isofemale lines. Likewise, the activity level in the adult stage significantly varied among lines. The significant variation was also found in the daily pattern of adult activity; some lines showed greater activity level in the daytime, and others showed greater activity level in the night. My results consistently suggest that there is a genetic variation in behavioral activity in a natural population, probably contributing to shaping the population performance.

Keywords: daily rhythm; fruit fly; natural population; quantitative variation

Introduction

Individuals within a population vary in many traits, including both continuous and discontinuous characteristics such as sex, color, size, morphology, behavior, and personality. Studies on intraspecific genetic variation had focused on two areas fundamental to evolutionary ecology: the evolutionary processes generating genetic variation and the ecological consequences of the evolution of genetic variation in a species/population (Forsman, 2008; Bolnick et al., 2011). For example, population genetics revealed that intraspecific genetic variation is maintained in populations through balancing selection or a migration-selection balance (Slatkin, 1973; Mallet and Barton, 1989). Genetic variations are then suggested to enhance speciation and adaptive radiation over an evolutionary time scale (Slatkin, 1973; Mallet and Barton, 1989). On the other hand, ecological consequences of the genetic variation are getting a lot more attention (Wolf et al., 2007; Wolf and Weissing, 2012). Intraspecific (genetic and phenotypic) variation is suggested to affect ecological dynamics, such as population processes, community structure, and ecosystem function (Forsman and Wennersten, 2016; Des Roches et al., 2018). Such intraspecific variations have the potential to enhance population performance through improved use of resources (Dyer et al., 2009), parasite resistance (Sih et al., 2012), and reduced predation (Ahnesjö and Forsman, 2006) and sexual harassment (Takahashi et al., 2014) at a population level. In general, variation in traits relating to predator–prey interactions or resource utilization (e.g., body colors, feeding organs, and foraging behaviors) are expected to have larger ecological effects than less functional or nonfunctional traits.

Variation in activity level and rhythms of daily activity within a population could also affect population performance by directly altering ecological, social, and

sexual interactions among individuals. Variation in activity has been implicated in shaping interindividual interactions and social networks within populations (Mizumoto et al., 2017), but the ecological function of within-population variations in daily rhythm is still controversial. In *Drosophila*, behavioral and physiological daily rhythms have been well studied under laboratory conditions (Tauber et al., 2003). Adult individuals of *D. melanogaster* have a bimodal activity distribution that is described as "crepuscular": the flies move actively in the early morning and evening and are less active during mid-day (Helfrich-Förster, 2001; Tauber et al., 2003). Similar daily patterns in activity are known in other *Drosophila* species (Beauchamp et al., 2018), and such obvious crepuscular activity is thus believed to be ubiquitous in *Drosophila*. However, it remains unclear whether there are intrapopulation variations in activity patterns and their daily rhythms in *Drosophila* species. In the present study, I investigated the genetic variation in larval activity level and the level and daily pattern of adult activity within a single natural population in *Drosophila immigrans*.

Material and Methods

Study species

Drosophila immigrans is a globally distributed generalist which can oviposit on a wide array of substrates such as fungi, fruits, sap fluxes, and flowers (Markow and O'Grady, 2008). Their developmental time is within the range of 11 to 17 days, depending on temperature (Markow and O'Grady, 2006). In Japan, this species is extremely common during May and December (Beppu, 2014).

Fly strain

The adults of *D. immigrans* were collected in the Ecology Park of the Natural History Museum and Institute of Chiba, Japan (35° 59′ 8″ N, 140° 13′ 7″ E) in 2018. Each collected female was isolated to establish isofemale lines. Their siblings were maintained with media used by Fitzpatrick et al. (2007) (500 ml H_2O, 50 g sucrose, 25 g active yeast, 8 g agar, 5.36 g $KNaC_4H_4O_6 \cdot 4H_2O$, 0.5 g KH_2PO_4, 0.25 g NaCl, 0.25 g $MgCl_2$, 0.25 g $CaCl_2$, 0.35 g $Fe_2(SO_4) \cdot 6.9H_2O$) in 170 ml bottles (AS-115, Thermo Fisher Scientific). The flies were reared under a 12L:12D cycle at 23°C, at which temperature the population of *D. immigrans* is empirically known to develop well. In total, 21 isofemale lines were established in 2018. Before examining larval and adult activity, all isofemale lines were reared for three generations to reduce genetic variation within a line and to remove environmental and maternal effects. Of the 21 isofemale lines, 13 and 19 lines were used to measure the larval and adult activity, respectively (Table 1-S1). Eleven lines were used in common for both larval and adult experiments.

Larval activity

Larval activity was measured between 16:00 and 18:00 during April and May in 2019. To determine the larval activity level of each isofemale line, a larva was placed on a wet filter paper (φ31 mm) without food and filmed using a digital video camera (960 × 540 pixels at 30 fps, Panasonic HC-V480MS) for 15 min under LED light at 25°C, at which temperature I expected that larval activity is maximized (see Anreiter et al., 2016). A border around the filter paper made using a water repellent pen prevented a larva from escaping the filter paper stage. Before tracking individuals, 15-min-long videos were trimmed to 10-min-long videos. Each video was time-compressed to a 1-min-long video as larval movements are very slow. Locomotor behavior was tracked using a real-time

tracking system, UMATracker. Two-dimensional coordinate values from UMATracker were used to estimate larval activity level as the average path length of larval locomotion.

Adult daily rhythms

The daily activity of adult males and females of each isofemale line was examined during July 2018 and May 2019. The activity was observed for 24 hours and scored as the number of infra-red beam breaks in 10-min intervals using the DAM2 *Drosophila* Activity Monitor System (Trikinetics, Inc., Waltham, MA). The activity monitor was placed in an incubator with the same settings as the rearing conditions (i.e., 12L:12D cycle at 23°C). Individual flies were anesthetized with CO_2 and transferred into a recording tube made from a transparent straw (φ5 mm × 65 mm), one end of which was closed with rearing medium, and the other by an air-penetrable plug. Flies were allowed to recover from the anesthetic for at least 30-min before the recording started. I had confirmed that the LED lighting do not critically affect air temperature in an incubator.

I calculated the level of adult activity per hour by summing the number of activity counts during each hour. To resolve the problem of temporal autocorrelation in time series data, principal components analysis (PCA) was conducted for the number of activity counts in each hour. PCA condensed the 24 hours of data on adult activity into a small number of uncorrelated variables, which were the possible indices of the level and patterns of daily activity. To interpret each principal component score (PC score), isofemale lines were divided into three classes using the scores of each PC: top 4 lines, bottom 4 lines, and others.

Statistics

All analyses were conducted in R version 3.5.3 (R Core Team, 2019). The difference in larval activity level among isofemale lines was analyzed by one-way ANOVA. The differences in adult PC scores among isofemale lines were analyzed by two-way ANOVA with isofemale line ID and sex as independent variables. The *p*-values of the two-way ANOVA were calculated using the *F* test from the "*car*" package. The correlation between males and females for each PC (PC1–PC5) score was analyzed with Pearson's correlation test. Note that, Spearman's rank correlation test was used for PC1 and PC2 scores because these males were not normally distributed (Shapiro-Wilk *W* test, PC1: $p < 0.05$; PC2: $p < 0.01$). The consistency of activity level between larval and adult stages (PC1 score) was analyzed with linear regression analysis.

Results

Larvae moved approximately 0.5 mm per second on average on the wet filter paper (Fig. 1-1). Within-line variation in locomotion was relatively small. The locomotion was significantly different among the 13 isofemale lines ($F_{10,387} = 16.8$, $p < 0.001$), indicating genetic variation in locomotive activity of larvae.

For adults, the level of daily activity peaked in the early morning and evening (Fig. 1-2). Daily activity across all measured isofemale lines across 24 hours was illustrated in Fig. 1-S1. Using 19 isofemale lines, the PCA identified two important PC axes: the first axis of the PCA explained 26.9% of the total variance (PC1) and the second axis explained 15.3% (PC2). Other PCs were less important and had a low contribution (<10%). Isofemale lines showing a high PC1 score were more active compared to those with low PC1 scores throughout a day (Fig. 1-3a). On the other hand,

lines with a higher PC2 score were more active in the light than in the dark and lines with a lower PC2 score were more active in the dark than in the light (Fig. 1-3b). Thus, the PC1 and PC2 illustrate the overall level and daily rhythm of behavioral activity, respectively. PC1 was significantly different among the isofemale lines, while no difference was found between sexes (line: $F_{18,228} = 2.35$, $p < 0.01$; sex: $F_{1,228} = 0.44$, $p = 0.51$; interaction; $F_{18,228} = 0.92$, $p = 0.56$). PC2 also varied among the isofemale lines, but did not between sexes (line: $F_{18,228} = 1.67$, $p < 0.05$; sex: $F_{1,228} = 0.01$, $p = 0.91$; interaction; $F_{18,228} = 1.31$, $p = 0.19$). Other PCs except PC4 were also not significantly different among lines and between sexes (Table 1-S2). Correlation between males and females was found for PC1, but not for PC2 to PC5 (PC1: $\rho = 0.47$, $p = 0.04$; PC2: $\rho = 0.17$, $p = 0.48$; Fig. 1-4a, b; Table. 1-S3). For 11 isofemale lines of which both adult and larval activity levels were measured, the mean PC1 including both males and females was not significantly related to mean larval locomotive activity ($F_{1,9} = 1.29$, $p = 0.28$). Other mean PCs except PC4 were also not significantly related to mean larval locomotive activity (Table 1-S4).

Discussion

In *Drosophila*, daily rhythms in behavior and physiology have been well documented all over the world. Numerous studies suggest that the adults of drosophilid species show a bimodal distribution in activity (Ferguson et al., 2015; Helfrich-Förster, Bertolini and Menegazzi, 2020). For example, a bimodal activity pattern was reported in *D. melanogaster* (Helfrich-Förster, 2000; Dubowy and Sehgal, 2017), *D. suzukii* (Plantamp et al., 2019), *Zaprionus indianus* (Prabhakaran and Sheeba, 2013). Even in the present study, I showed that adult *D. immigrans* showed a bimodal distribution in activity,

indicating that the daily rhythm of this species is consistent with known adult activity patterns in *Drosophila*. On the other hand, few studies had examined intrapopulation variation in adult daily activity. I here revealed the presence of intrapopulation genetic variation in the level of activity in both larvae and adults. I also found the intrapopulation genetic variation in the daily pattern of activity in the adult stage.

The activity level of each line was consistent between sexes. A previous study demonstrated that the adult activity level was positively correlated between males and females in *D. melanogaster* (Watanabe et al., 2020). The consistency of activity levels between sexes could be general in *Drosophila*. On the other hand, I showed no positive correlation between larval and adult activity of *D. immigrans.* The similar pattern has been reported in various holometabolous insects such as a red flour beetle, a mealworm, and a fruit fly; that is, no positive correlation of larval and adult activity was found (Anderson, Scott and Dukas, 2016; Matsumura, Fuchikawa and Miyatake, 2017; Monceau et al., 2017). A different pattern has also been reported in some hemimetabolous insects such as a damselfly and a firebug (Brodin, 2009; Gyuris, Feró and Barta, 2012), in which larval and adult activity tended to positively correlate each other. However, exceptionally in a behavioral polymorphism governed by *foraging* gene in *D. melanogaster*, the activity level is suggested to be consistent throughout their life including both larval and adult stages (Edelsparre et al., 2014). The consistency of activity level throughout the life is still controversial in insects.

I also found that the PC2 score (the rhythm of daily activity) was not correlated between males and females, while there was significant genetic variation in the rhythm of daily activity among lines. This suggests that the daily rhythm of activity tends to differ between opposite-sex siblings in nature. However, since few studies have

addressed the variation in the rhythm of daily activity, I cannot ascertain whether such a pattern was ubiquitous at this time.

Recent studies suggest that adult *D. melanogaster* may show a different daily rhythm in semi-natural environments. In such environments, adults dramatically increased their afternoon activity, suggesting that *Drosophila*'s bimodal activity distribution may only appear under strictly controlled laboratory conditions (Vanin et al., 2012; Green et al., 2015). Thus, the variation in daily rhythms that I found under laboratory conditions may not reflect the pattern of those in a natural condition, though my findings still suggest that variation in activity rhythms themselves may exist in natural populations. The intrapopulation variation in activity rhythms may have evolved to reduce interindividual competition.

Intraspecific behavioral variation has been suggested to affect population dynamics. For example, in *D. melanogaster*, intrapopulation variation in activity improves population performance by reducing resource competition (Takahashi et al., 2018). Thus, the variation in activity that I found in the present study may be linked to the reduction of resource competition. In addition, in the present study, I found that some isofemale lines were active in the daytime and while others were active at night. The variation of activity patterns among individuals could potentially function to reduce the rate of encounters among individuals at population level. A reduction in inter- and intrasexual interactions, which negatively affect survival and reproduction, is likely to enhance mating success and reduce interindividual competition for resource (Reebs, 2002; Kronfeld-Schor and Dayan, 2003; Závorka et al., 2016; Hau et al., 2017). Thus, the variation of both the level and daily pattern of activity is hypothesized to affect individual performance and thus population dynamics.

At the current moment, it is technically challenging for us to quantify the rhythm of larval daily activity. However, I have to examine the rhythm of larval daily activity to test whether its consistency with that of adult daily activity in the future. In addition, in the present study, I have not tested the effect of the presence of variation on the interindividual interactions, reproduction, and population growth. Further studies are needed to test the effect of the presence of variation in daily activity on individual performance and population process, as described above.

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Figures

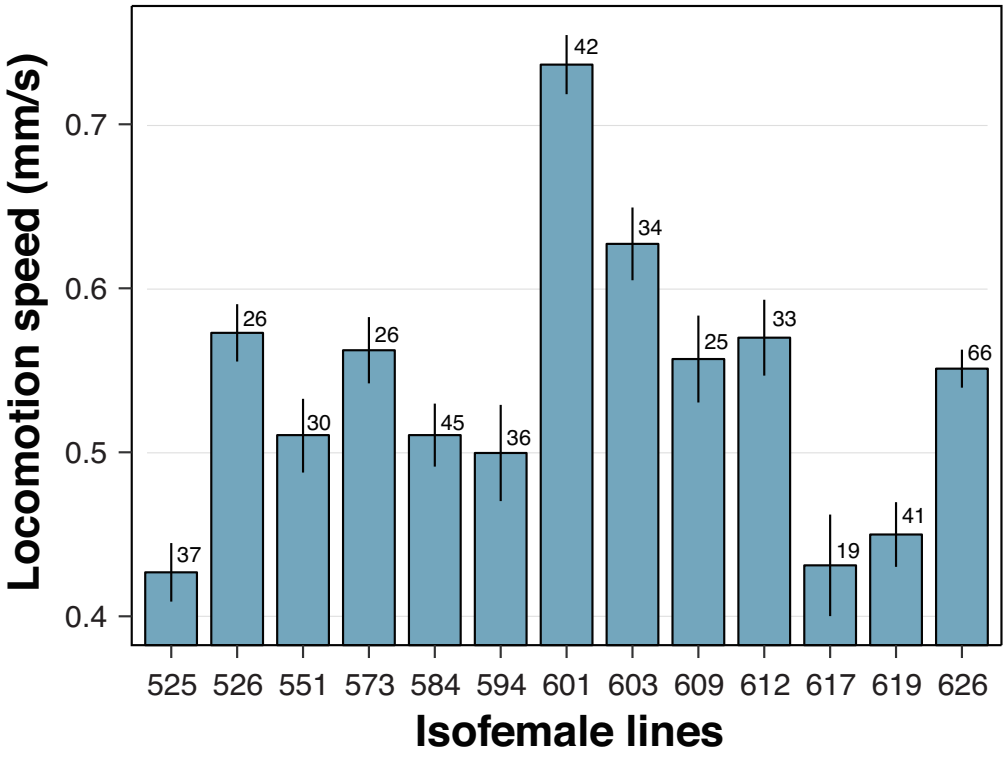

Figure 1-1. The variation in larvae locomotion speed among isofemale lines of *Drosophila immigrans*. The x-axis labels are isofemale line ID, and the numeral on each bar is a sample size. Error bars are SEM.

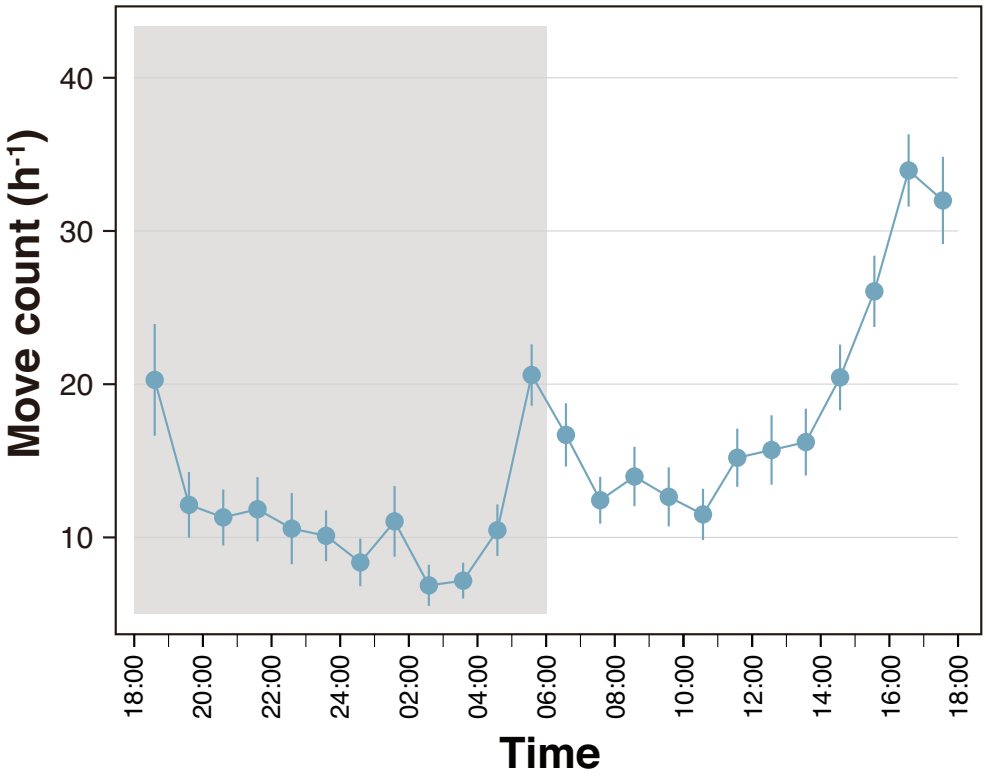

Figure 1-2. The average daily activity of all adults examined ($N = 266$). The activity of flies peaked during morning and evening. Gray area in a panel represents dark conditions. Error bars are SEM.

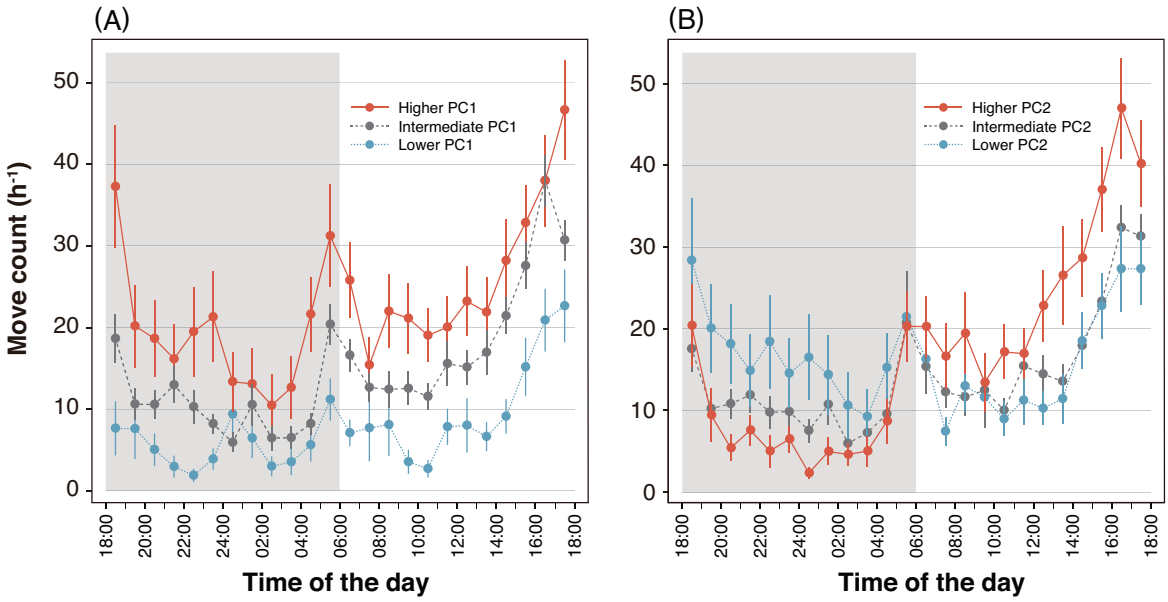

Figure 1-3. The average daily activity of adults in each isofemale line showing higher, intermediate and lower PC1 (A) and PC2 (B) values. Black and white bars represent light / dark conditions. Solid lines (red), broken lines (green), and dotted lines (blue) represent a pattern of isofemale lines with higher PC scores, isofemale lines with intermediate PC scores and isofemale lines with lower PC scores, respectively. Error bars are SEM.

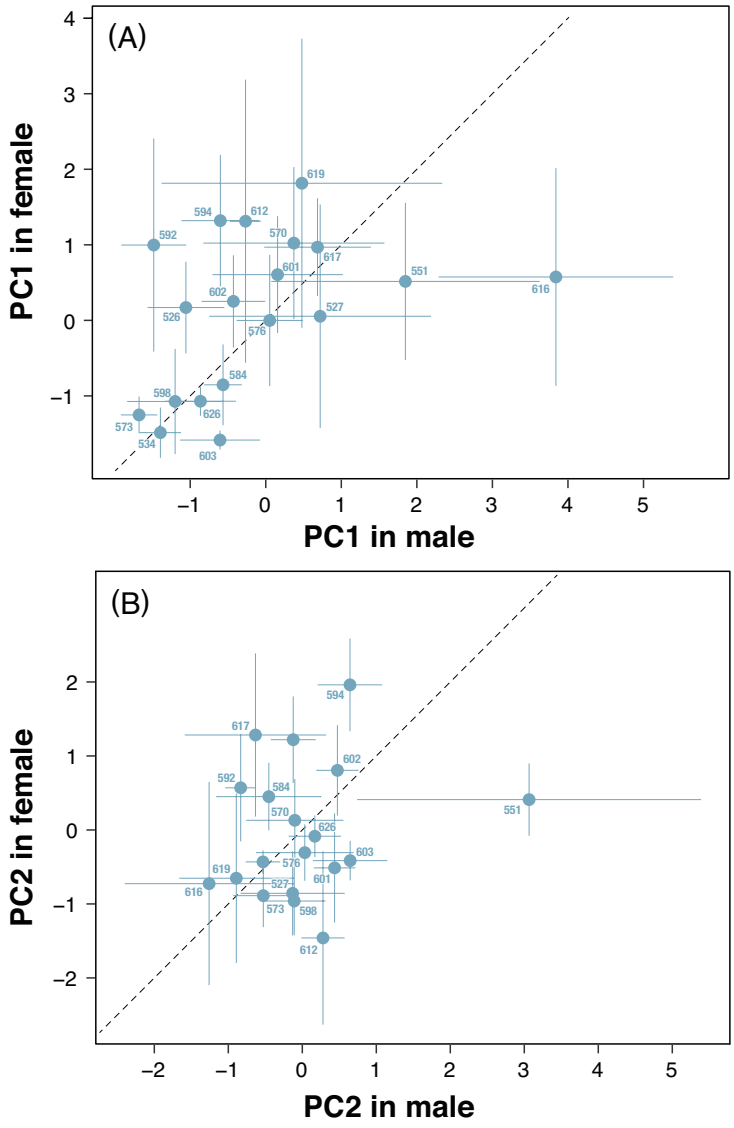

Figure 1-4. The variation in PC1 (A) and PC2 (B) and the correlations of between sexes. Dashed line represents a diagonal line. Sample sizes of male and female in each isofemale line are 6.6 ± 1.3 and 7.4 ± 1.8 (mean ± SD), respectively. Labels on the shoulder of each plot mean isofemale line ID. Error bars are SEM.

Supplemental material

Table 1-S1. The list of isofemale lines of which larval and adult activity were measured.

Line ID	Larval activity	Adult activity
525	Measured	Not measured
526	Measured	Measured
527	Not measured	Measured
534	Not measured	Measured
551	Measured	Measured
570	Not measured	Measured
573	Measured	Measured
576	Not measured	Measured
584	Measured	Measured
592	Not measured	Measured
594	Measured	Measured
598	Not measured	Measured
601	Measured	Measured
602	Not measured	Measured
603	Measured	Measured
609	Measured	Not measured
612	Measured	Measured
616	Not measured	Measured
617	Measured	Measured
619	Measured	Measured
626	Measured	Measured

Table 1-S2. Results of two-way ANOVA for PC3 to PC5. The values in parentheses are the contribution (proportion of variance) of each PC score.

PC3 (6.2%)

	F	***p***
line	1.10	0.35
sex	3.2	0.07
line × sex	0.57	0.92

PC4 (5.3%)

	F	***p***
line	1.68	0.04
sex	0.72	0.40
line × sex	1.14	0.31

PC5 (4.7%)

	F	***p***
line	1.48	0.10
sex	12.1	<0.001
line × sex	1.43	0.12

Table 1-S3. Correlation tests between males and females for PC3, PC4, or PC5 scores analyzed by Pearson's correlation.

	r^2	p
PC3	0.12	0.15
PC4	0.03	0.48
PC5	0.0007	0.91

Table 1-S4. Simple linear regression analyses between larval activity and each PC scores (PC2–PC5).

independent variable	dependent variable	*F*	*p*
larvae activity	**PC2**	0.19	0.67
	PC3	0.17	0.69
	PC4	7.03	0.026
	PC5	0.04	0.84

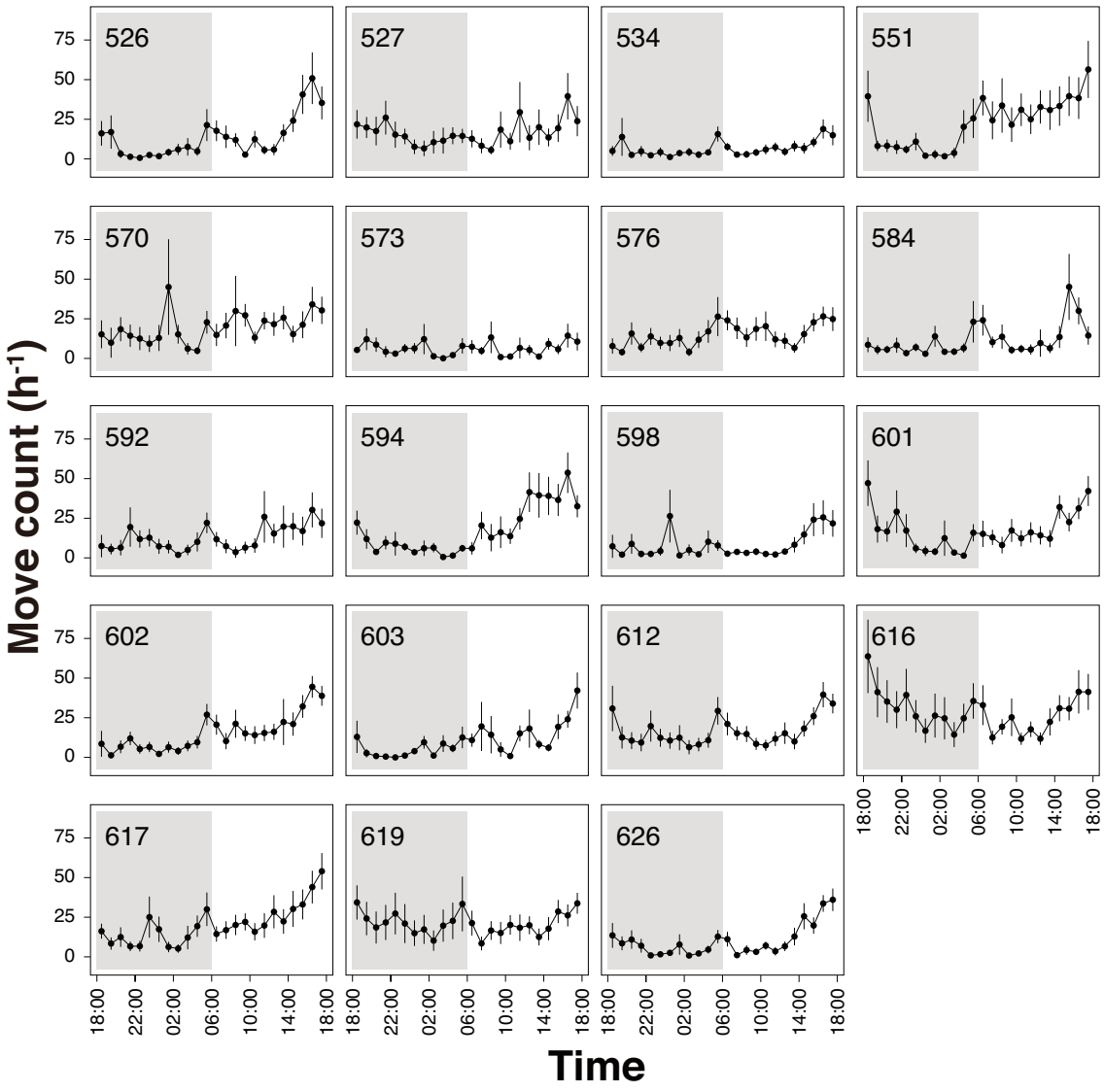

Figure 1-S1. The daily activity of adults of each isofemale line. The activities of flies were varied among lines. Gray area in a panel represents dark conditions. Error bars are SEM.

Chapter 2

Mitochondrial polymorphism shapes intrapopulation behavioural variation in wild *Drosophila*

Published in *Biology Letters* (Ueno and Takahashi, 2021).

Abstract

Intrapopulation variation in behaviour, including activity, boldness and aggressiveness, is becoming more widely recognized and is hypothesized to substantially affect ecological and evolutionary dynamics. Although previous studies used candidate-gene approaches and genome-wide association analyses to identify genes correlated with variations in activity and aggressiveness, behavioural variation may not be fully captured in the nuclear genome, as it does not account for mitochondrial genomes. Mitochondrial genes encode products that are key regulators of the cellular energy-producing pathways in metabolic processes and are thought to play a significant role in life history and reproductive traits. In this study, I considered many isofemale lines of *Drosophila immigrans* established from two wild populations to investigate whether intrapopulation variation in the mitochondrial genome affected activity level within this species. I identified two major haplogroups in these populations, and activity levels in both larvae and adults differed significantly between the two haplogroups. This result indicated that intrapopulation variation in activity level may be partially controlled by mitochondrial genes, along with the interaction between nuclear and mitochondrial genes and the age of individual organisms.

Keywords: age dependence, *Drosophila immigrans*, intrapopulation difference, mito-nuclear interaction, mtDNA haplogroups, activity level

Introduction

Intrapopulation behavioural variation including personality variation has received increased attention within ecology and evolutionary biology (Dall et al., 2012), and variation in behaviour, such as activity level, boldness, and aggressiveness, is thought to exert a considerable effect on ecological and evolutionary dynamics and eco-evolutionary feedback (Dyer et al., 2009; Takahashi et al., 2018). Recently, candidate-gene and genome-wide association studies have revealed that certain nuclear genes contribute to variation in activity and aggressive behaviour (Li et al., 2016; Watanabe et al., 2020), but understanding of the genetic basis of behavioural variation within animal populations remains limited.

However, awareness of the influence of mitochondrial haplotypes on behavioural characteristics is growing. Because the mitochondrial genome is known to encode products that are key regulators of cellular energy-producing pathways (i.e., oxidative phosphorylation) in metabolic processes, mitochondrial genetic variation could play a significant role in interindividual variation within whole-organism metabolic phenotypes and in behavioural, life-history, and reproductive traits (Gemmell, Metcalf and Allendorf, 2004; Arnqvist et al., 2010; Løvlie et al., 2014; Novičić et al., 2015). In seed beetles, epistatic effects between the mitochondrial haplotype (or mitogenome) and nuclear gene(s) were suggested to govern geographic variation in activity level (Løvlie et al., 2014). However, it remains to be elucidated whether mitochondrial genomes affect behavioural variations within a population. Quantifying the effect of mitochondrial polymorphisms on intrapopulation behavioural variation represents a first step toward understanding the evolutionary origin, maintenance, and ecological functions of behavioural variation within populations. In the present study, I

examined the effect of mitochondrial haplogroups on intrapopulation differences in larval and adult activity in two natural populations of *Drosophila immigrans*. I then explored the effect of mito-nuclear genetic interaction on individual behaviour.

Materials and Methods

Study species

Drosophila immigrans is a globally distributed generalist that can oviposit on a wide array of substrates, such as fungi, fruits, sap fluxes, and flowers. Their developmental time ranges from 11 to 17 days, depending on the temperature (Markow and O'Grady, 2006, 2008). In Japan, this species is widespread from May to December (Beppu, 2014) and presents genetic variations in larval and adult activity levels and the daily rhythm of adults within a population (Ueno and Takahashi, 2020). The species clusters into two extremely divergent haplogroups in Indian populations (Sarswat, Dewan and Fartyal, 2016).

Fly strain

I used *D. immigrans* collected from two natural populations in Japan, which were obtained from the Ecology Park of the Natural History Museum and Institute of Chiba (35° 36 01″ N, 140° 08 11″ E, Chiba City) in 2018 and Hokkaido University (43° 04 44″ N, 141° 20 13″ E, Sapporo City) in 2019. For the Chiba population, I used the isofemale lines established by Ueno and Takahashi (2020). Isofemale lines for the Sapporo population were established in this study. Each line was maintained with media used by Fitzpatrick et al. (2007) in plastic vials (KFB-1M, Chiyoda Science). The flies were reared under a 12L:12D cycle at 23°C. Before examining individuals in these

lines, all isofemale lines were reared for at least three generations to reduce genetic variation within the lines and to remove environmental and maternal effects. Table 2-S1 describes the lines used to measure larval and adult activity, respectively.

Mitochondrial DNA haplogroup analysis

To identify the mitochondrial DNA haplotypes of isofemale lines, the mitochondrial cytochrome *c* oxidase I (COI) DNA barcode region was considered (Hebert et al., 2003). DNA from an adult in each line was extracted using MightyPrep reagent (Takara, Japan). Each DNA extract was amplified with a COI primer (Fwd-5′TCCTGGATTCGGAATAATTTCTCA-3′; Rev-5′AACGTCGAGGTATTCCAGCT-3′) using MightyAmp DNA polymerase (Takara, Japan). The PCR products were purified and sequenced, with lengths of 486bp (accession no. LC623772-LC623813).

To classify the haplotypes of each line, the COI sequences of *D. immigrans* and *D. melanogaster* were obtained from GenBank (GenBank accession numbers in Table 2-S2), and a phylogenetic tree of aligned sequences was constructed using the maximum likelihood method and the Tamura-Nei model (Tamura and Nei, 1993). I then constructed the minimum spanning haplotype network in PopART v. 1.7. For its presentation, I took into account the haplotype's population and frequencies.

Larval activity assessment

The larval activity levels of each isofemale line for Chiba and Sapporo were measured between 16:00 and 18:00 during April and May 2019 (see Ueno and Takahashi, 2020) and during September 2019 and November 2020, respectively. Each larva was placed on wet filter paper (φ31 mm) without food and filmed for 15 min (960 × 540 pixels at 30

fps) under LED light at 25°C, at which point I expected that larval activity would be maximized (Anreiter et al., 2016; Ryan et al., 2016). A border was created around the filter paper using a water-repellent pen to prevent the larvae from escaping the filter paper. Each video was trimmed to 10 min and time-compressed to 1 min to account for the slow movements of the larvae, which were tracked using a real-time tracking system (UMATracker; Yamanaka and Takeuchi, 2018). The trajectory data of two-dimensional coordinate values generated by UMATracker were used to estimate larval activity levels based on the average path length of larval locomotion.

Adult activity assessment

The daily activity levels of adult males and females in the Chiba and Sapporo isofemale lines were examined during July 2018 and May 2019 (see Ueno and Takahashi, 2020) and during October 2019 and February 2020, respectively. The activity was observed for 24 h and scored as the number of infrared beam breaks in 10-min intervals using the DAM2 *Drosophila* Activity Monitor System (Trikinetics, Inc., Waltham, MA). The activity monitor was placed in an incubator maintained with the same parameter values as the rearing conditions (i.e., 12 L:12D cycles at 23°C). Individual flies were anaesthetized with CO_2 and transferred into a recording tube made from a transparent straw (φ5 mm, 65 mm length), one end of which was closed with rearing medium and the other by an air-penetrable plug. Flies in the recording tubes were allowed to recover from the anaesthetic for at least 30 min before recording commenced. I confirmed that LED lighting did not critically affect the air temperature in the incubator.

Statistical analyses

All analyses were performed in R version 4.0.3 (R Core Team, 2020). To analyse the differences in larval activity levels among haplogroups, I implemented linear mixed models (LMMs) using the *lme4* package with isofemale lines and the assay month and year as random effects and haplogroups and populations as fixed effects. The differences in the total 24 h scores of adults in different haplogroups were analysed using generalized linear mixed models (GLMMs), where I fitted isofemale lines and the assay month and year as random effects and haplogroups, populations, and sex as fixed effects. The *p*-values of each fixed effect were calculated from the χ^2-test using the *Anova* function in the *car* package. Further, I implemented the LMM and GLMM and analysed the differences in both larval and adult activity among haplogroups within each population separately.

Results

Six haplotypes were identified and fell into two major haplogroups (haplogroup 1 and 2). Both mitochondrial haplogroups were found in the two natural populations investigated (Fig. 2-1 and Fig. 2-S1) and corresponded to those that had been previously reported in populations in India and Japan.

In larvae, the activity level of haplogroup 2 was higher than that of haplogroup 1 in both populations (Fig. 2-2a and Fig. 2-S2a). The difference between haplogroup 1 and 2 was significant (haplogroup [H]: $\chi^2 = 4.41$, $p = 0.04$; population [P]: $\chi^2 = 0.06$, $p = 0.81$; H × P: $\chi^2 = 0.71$, $p = 0.40$). This tendency was obvious in the Chiba population ($\chi^2 = 3.37$, $p = 0.07$) but less so in the Sapporo population ($\chi^2 = 0.30$, $p = 0.58$). For adults, the effect of haplogroup and the interaction effect between haplogroup and population on 24 h activity-level scores was significant (haplogroup [H]: $\chi^2 = 4.04$, $p = 0.04$;

population [P]: $\chi^2 = 0.00005$, $p = 0.99$; sex: $\chi^2 = 126.7$, $p < 0.001$; H × P: $\chi^2 = 4.84$, $p = 0.03$, Fig. 2-2b and Fig. 2-S2b, see also Fig. 2-S3 and 2-S4 for the details). In the Sapporo population, the activity level of haplogroup 2 was significantly higher than that of haplogroup 1 ($\chi^2 = 5.89$, $p = 0.02$, Fig. 2-2b), while the difference in activity level between haplogroups in the Chiba population was not obvious ($\chi^2 = 0.06$, $p = 0.80$).

Discussion

Sequence variation in the mitochondrial genome has traditionally been considered selectively neutral (Ballard and Kreitman, 1995) and has been extensively used as a genetic marker in population biology and ecology. However, recent evidence suggests that the pattern of genetic variation in mitochondria is often shaped by natural selection (e.g., Rand, Clark and Kann, 2001). This has been verified by comparative genomic data and theory, showing that positive or negative frequency-dependent selection (NFDS) can drive mitochondrial genetic variation (Bazin, Glémin and Galtier, 2006; Liu and Asmussen, 2007; Kazancioğlu and Arnqvist, 2014). A few remarkable observations in insects also suggested balancing selection by NFDS (Dowling, Friberg and Lindell, 2008). In practice, the natural populations of *Drosophila* spp. show widespread geographical homogeneity with mitochondrial polymorphism (James and Ballard, 2003; Oliver et al., 2005; Andrianov et al., 2008), suggesting that NFDS maintains mitochondrial haplotype variation in natural and laboratory populations. I also showed that two haplogroups coexisted in four natural populations of *D. immigrans*, suggesting the generality of balancing selection for mitochondrial polymorphism in *Drosophila*. Although I only analysed a small part of the mitogenome, my data and that of previous studies suggest that many genes of the mitogenome display

intrapopulation polymorphism owing to maternal inheritance without gene exchange among haplotypes, probably leading to variation in metabolic traits within populations.

In practice, I found that mitochondrial genetic variation can explain the variation in activity levels between haplogroups within a population, while my experimental design did not fully isolate the effects of mitochondrial genes from that of nuclear genes. I also noted that activity level was differently affected by mitochondrial haplotypes between populations. The mitochondrial haplogroups in the Chiba population mainly affected larval activity, whereas the mitochondrial haplogroups in the Sapporo population significantly affected adult activity, suggesting an interaction between the mitochondrial and nuclear genomes during different developmental stages. In a previous study, mitochondrial genomes in seed beetles (*Callosobruchus maculatus*) were introgressed into controlled nuclear genetic backgrounds across lines established from different populations, which demonstrated that the mito-nuclear interaction affected behavioural variation (Løvlie et al., 2014). My results also showed that within-haplogroup activity levels in larvae and adults differed between populations. This difference suggests that membership in haplogroup 2 consistently increased the activity levels of larvae and adults in both populations, but that mitochondrial haplogroups affected activity levels at different ages between populations owing to the interaction between nuclear and mitochondrial genomes. To confirm epistasis effects on the activity level, I should control for nuclear genetic background between populations. Still, my results suggest that variation in mitochondrial genomes may also play an important role in ecological functions via variations in activity levels. The influences of mitochondrial genome on variation in functional traits, especially in the metabolic and behavioural, personality traits, should be investigated in various animals in the future.

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Figures

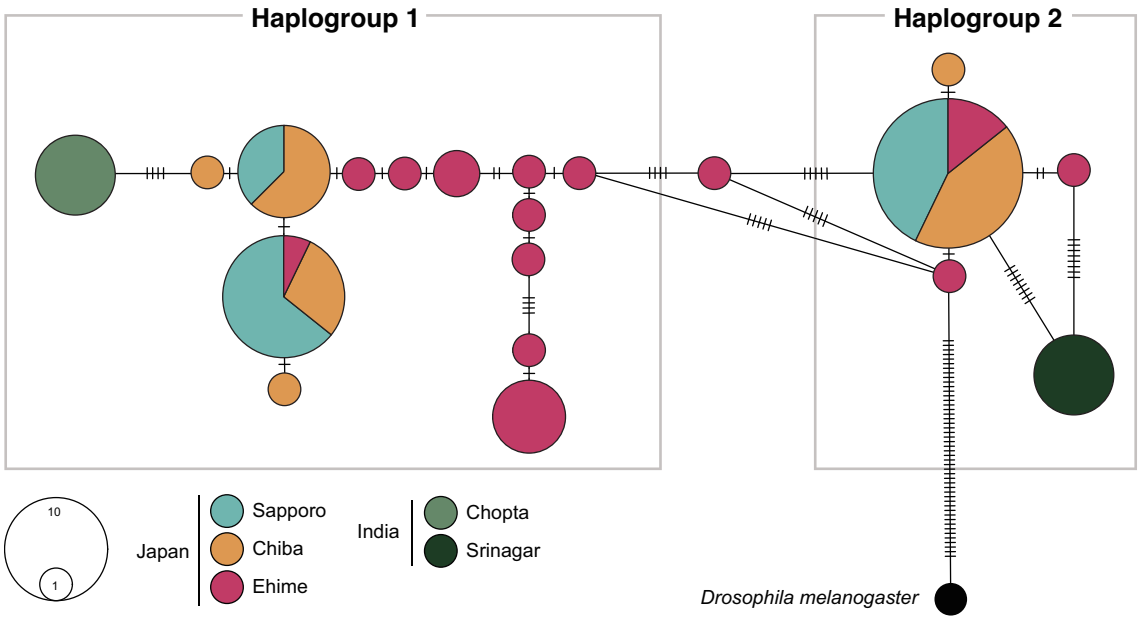

Figure 2-1. Minimum spanning haplotype network constructed in PopART. Major haplogroups in two wild populations. Haplotypes are represented by circles whose sizes are proportional to the number of individuals. Different colors represent populations. Mutational steps between haplotypes are indicated by hatch marks.

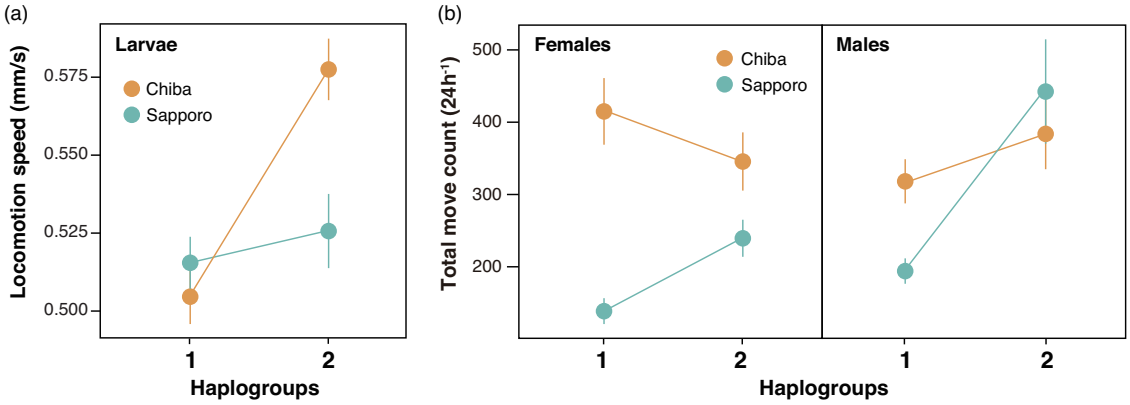

Figure 2-2. Activity levels of *Drosophila immigrans*. (a) Larval activity levels in two haplogroups and two populations. (b) Total 24 h adult activity level scores of females and males in two haplogroups and two populations. Plots represent average activity levels, and error bars indicate standard error of the mean.

Supplemental material

Table 2-S1. The list of isofemale lines of which larval and adult activity were measured.

Population	Line ID	Larval activity	Adult activity	Remarks
Chiba	525	Measured	Not measured	Ueno et al. (2020)
Chiba	526	Measured	Measured	Ueno et al. (2020)
Chiba	527	Not measured	Measured	Ueno et al. (2020)
Chiba	534	Not measured	Measured	Ueno et al. (2020)
Chiba	551	Measured	Measured	Ueno et al. (2020)
Chiba	570	Not measured	Measured	Ueno et al. (2020)
Chiba	573	Measured	Measured	Ueno et al. (2020)
Chiba	576	Not measured	Measured	Ueno et al. (2020)
Chiba	584	Measured	Measured	Ueno et al. (2020)
Chiba	592	Not measured	Measured	Ueno et al. (2020)
Chiba	594	Measured	Measured	Ueno et al. (2020)
Chiba	598	Not measured	Measured	Ueno et al. (2020)
Chiba	601	Measured	Measured	Ueno et al. (2020)
Chiba	602	Not measured	Measured	Ueno et al. (2020)
Chiba	603	Measured	Measured	Ueno et al. (2020)
Chiba	609	Measured	Not measured	Ueno et al. (2020)
Chiba	612	Measured	Measured	Ueno et al. (2020)
Chiba	616	Not measured	Measured	Ueno et al. (2020)
Chiba	617	Measured	Measured	Ueno et al. (2020)
Chiba	619	Measured	Measured	Ueno et al. (2020)
Chiba	626	Measured	Measured	Ueno et al. (2020)
Sapporo	N03	Measured	Measured	
Sapporo	N13	Measured	Measured	

Sapporo	N14	Measured	Measured
Sapporo	N16	Measured	Measured
Sapporo	N17	Measured	Measured
Sapporo	N19	Measured	Measured
Sapporo	N21	Measured	Measured
Sapporo	N25	Measured	Measured
Sapporo	N27	Measured	Measured
Sapporo	N30	Measured	Measured
Sapporo	N33	Measured	Measured
Sapporo	N34	Measured	Measured
Sapporo	N36	Measured	Measured
Sapporo	N41	Not measured	Measured
Sapporo	N49	Measured	Measured
Sapporo	N53	Measured	Measured
Sapporo	N56	Measured	Measured
Sapporo	N60	Measured	Measured
Sapporo	N66	Measured	Measured
Sapporo	N68	Measured	Measured
Sapporo	N77	Measured	Measured

Table 2-S2. List of species, country, population, and GenBank accession number of COI sequences used in this study.

Species	**country**	**population**	**GenBank Accession Number**
D. immigrans	Japan	Ehime	AB824775
D. immigrans	Japan	Ehime	AB824777
D. immigrans	Japan	Ehime	AB824778
D. immigrans	Japan	Ehime	AB824779
D. immigrans	Japan	Ehime	AB824780
D. immigrans	Japan	Ehime	AB824782
D. immigrans	Japan	Ehime	AB824784
D. immigrans	Japan	Ehime	AB824785
D. immigrans	Japan	Ehime	AB824786
D. immigrans	Japan	Ehime	AB824787
D. immigrans	Japan	Ehime	AB824788
D. immigrans	Japan	Ehime	AB824789
D. immigrans	Japan	Ehime	AB824791
D. immigrans	Japan	Ehime	AB824792
D. immigrans	Japan	Ehime	AB824793
D. immigrans	Japan	Ehime	AB824795
D. immigrans	Japan	Ehime	AB824797
D. immigrans	Japan	Ehime	AB824798

D. immigrans	Japan	Ehime	AB824800
D. immigrans	Japan	Ehime	AB824801
D. immigrans	Japan	Ehime	AB824802
D. immigrans	India	Srinagar, Uttarakhand	KP730843
D. immigrans	India	Srinagar, Uttarakhand	KP730844
D. immigrans	India	Srinagar, Uttarakhand	KP730845
D. immigrans	India	Srinagar, Uttarakhand	KP730846
D. immigrans	India	Srinagar, Uttarakhand	KP730847
D. immigrans	India	Srinagar, Uttarakhand	KP730848
D. immigrans	India	Chopta, Uttarakhand	KP730849
D. immigrans	India	Chopta, Uttarakhand	KP730850
D. immigrans	India	Chopta, Uttarakhand	KP730851
D. immigrans	India	Chopta, Uttarakhand	KP730852
D. immigrans	India	Chopta, Uttarakhand	KP730853
D. immigrans	India	Chopta, Uttarakhand	KP730854
D. melanogaster			M57910

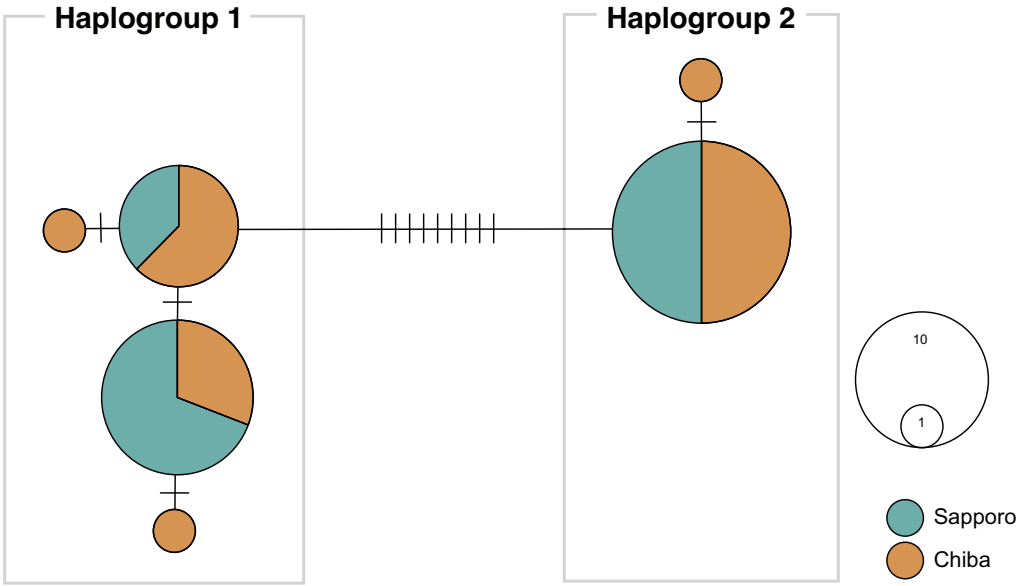

Figure 2-S1. Minimum spanning haplotype network constructed in PopART, using data that I obtained in the present study. Haplotypes are represented by circles whose sizes are proportional to the number of individuals. Different colors represent populations. Mutational steps between haplotypes are indicated by hatch marks.

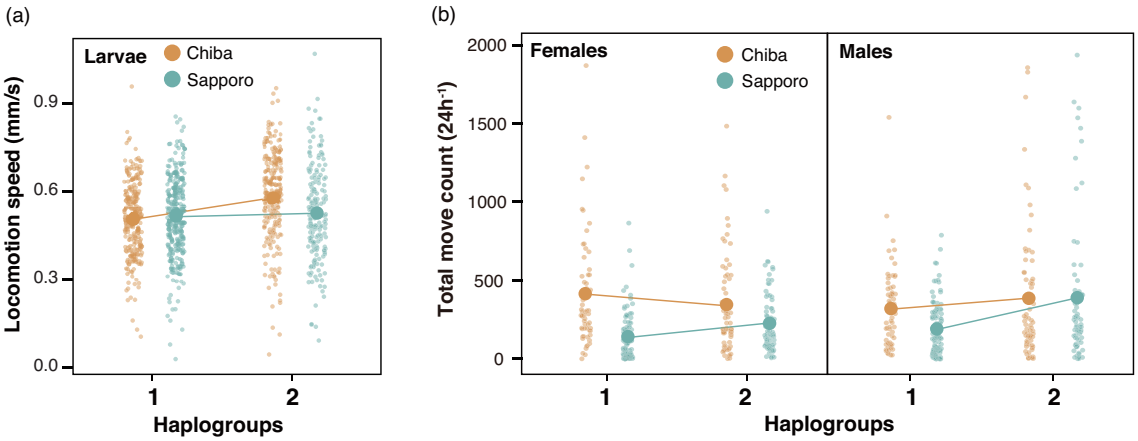

Figure 2-S2. Average activity levels of *Drosophila immigrans* with all the raw data. (a) Larval activity levels in two haplogroups and two populations. (b) Total 24-h adult activity level scores of females and males in two haplogroups and two populations. Large plots represent average activity levels.

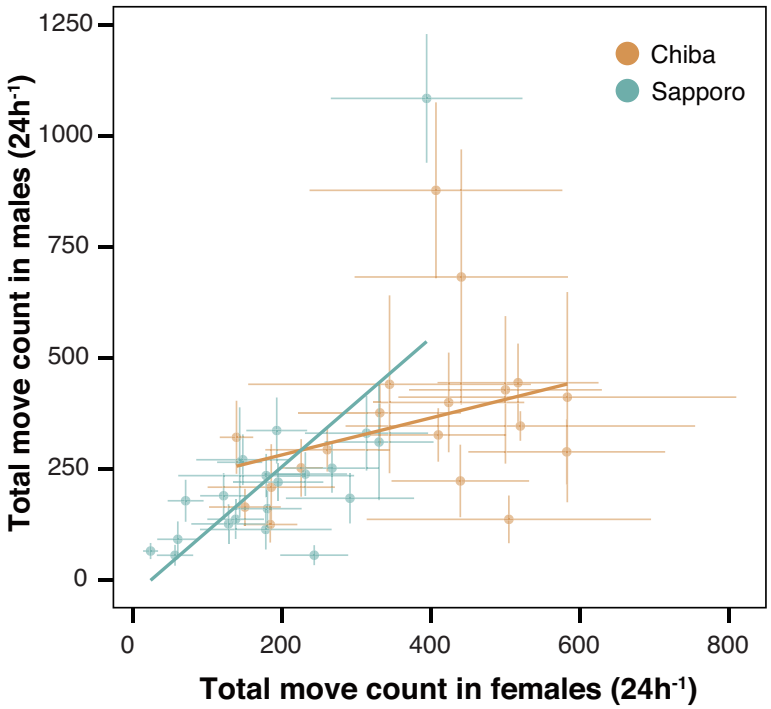

Figure 2-S3. The variation in adult activity levels and the correlation between sexes. A Plots represent the mean value of each isofemale line. Error bars represent SEM. Pearson's product-moment correlation showed that the correlation in Chiba was not significant ($r = 0.33$, $p = 0.16$), but significant in Sapporo ($r = 0.66$, $p < 0.01$).

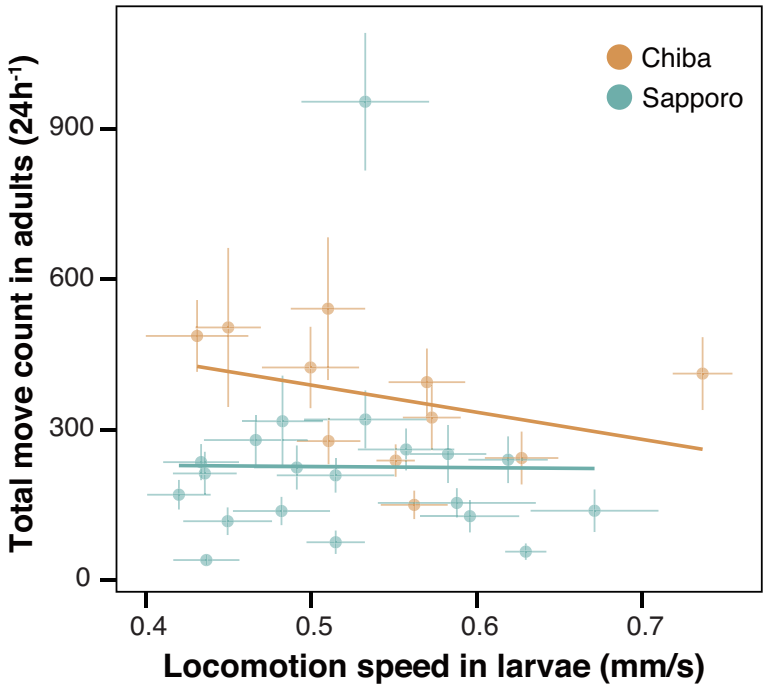

Figure 2-S4. The correlation of larval and adult activity levels. A total move count in adults in an isofemale line was calculated by the mean of total move counts of males and females in a line. Plots represent the mean value of each isofemale line. Error bars represent SEM. Pearson's product-moment correlation showed that the locomotion speed in larvae was not significantly correlated with the total move count in adults Chiba and Sapporo (Chiba: $r = -0.36$, $p = 0.27$; Sapporo: $r = -0.009$, $p = 0.97$).

Appropriate evaluation of rapid evolutionary response to seasonal environmental variability in a wild *Drosophila* population

Under revision.

Abstract

Seasonal environmental change is one of the most rapid and remarkable environmental variables. Although relatively rapid adaptation to environmental changes over several years or several decades has been described in many taxa, rapid responses to seasonal environments are delicate, and therefore the detection of the evolutionary responses requires delicate methods. Here, I examined the evolutionary response to seasonal environmental changes in terms of thermal tolerance and morphological traits, using *Drosophila lutescens* of spring and autumn periods collected in a single location. I first demonstrated that flies in the two seasonal periods were almost genetically identical using double-digest restriction site-associated DNA sequencing and analysis. After my experimental design eliminated the effect of possible confounding environmental factors which affect thermal tolerance, I showed that the heat tolerance of *D. lutescens* was significantly higher in the autumn period than in the spring period. Furthermore, cold tolerance was slightly higher in the spring period than in the autumn period. Although wing length and thorax length did not change significantly between seasons, the ratio of wing length to thorax length changed significantly between the spring period and the autumn period. These results indicate that seasonal environmental heterogeneity can induce evolution within a year. The present study demonstrates rapid evolutionary responses to environmental fluctuations.

Keywords: adaptation, seasonal variation, heat tolerance, cold tolerance, *Drosophila lutescens*

Introduction

Almost all organisms live in environments that vary spatiotemporally. Such environmental heterogeneity can impose highly variable selection on populations and regulate the evolution and adaptation of organisms. Understanding how populations adapt to environmental variability has contributed to revealing the history of range expansion, the evolutionary process, and the maintenance of biodiversity (Sexton et al., 2009; Williams et al., 2015). Most previous studies focused on continuous variation in space, such as latitude, longitude, and altitude, and disclosed adaptive evolution in terms of morphological and life-historical traits (Blanckenhorn and Fairbairn, 1995; Heibo, Magnhagen and Vøllestad, 2005; Sørensen et al., 2005). On the other hand, recent studies revealed that organisms evolve various traits in response to environmental changes occurring on ecological time scales, i.e., several years to several decades (Carroll et al., 2007). These evolutionary changes are referred to as rapid or contemporary evolution. Rapid evolutionary responses across seasons within a year also were explored for decades in various studies (Dobzhansky, 1956; Stalker, 1980; Rodríguez-Trelles, Tarrío and Santos, 2013). The verification of seasonal rapid evolution in a habitat is delicate and therefore requires elaborate research methods.

Seasonal environmental heterogeneity presents a rapid, cyclic, persistent, and geographically widespread variation over time. Generally, in organisms with a life span of more than one year, responding evolutionarily to seasonal change is expected to be difficult. In contrast, in species having multiple generations within a year, environmental changes across seasonal time may create different selection regimes, possibly leading to seasonal evolution. For instance, in the fruit flies *Drosophila melanogaster* and *D. simulans*, strains established from females collected at four

different seasonal timepoints exhibited remarkably different phenotypes with respect to several life-history traits under laboratory conditions (Behrman et al., 2015). Another study observed the evolutionary tracking of various phenotypes in response to seasonal environmental change in a field experiment (Rudman et al., 2022). These suggested rapid evolutionary responses to seasonal changes in the environment. However, most previous studies that demonstrated seasonal changes in phenotypes are not able to fully reject the possibility that environmental factors, such as conditions in which the experiments were conducted, accidentally generated an apparent seasonal variation. It is unknown whether the response of individuals across generations is derived from genetic differences or plastic differences because previous experiments examined the phenotypes of each seasonal generation within each season. Thus, it is imperative to measure the phenotypes in an accurate common garden experiment to remove environmental factors that could potentially affect the phenotype. Here it should be noted that differences in unknown and/or uncontrollable environmental variables among seasons in which the phenotypes are measured may unknowingly affect the phenotypes expressed (Stone, Erickson and Bergland, 2020). Even under laboratory conditions, I might erroneously find evidence of evolution between seasons if I measure the phenotype of each strain derived from a different season in the seasons corresponding to the derivations. Therefore, simultaneous measurements of phenotype in strains derived from different seasons are needed to detect direct evidence of seasonal evolution.

Seasonal changes/oscillations in allele frequency are another form of evidence of seasonal evolution. In *D. melanogaster,* genome-wide population genetic analysis revealed that hundreds of single nucleotide polymorphisms (SNPs) oscillated between spring and fall over multiple years (Bergland et al., 2014). This suggests that

evolutionary changes may occur along seasons. However, it remains unclear whether oscillating SNPs are reflected in the seasonal variation in selection regime and directly associated with phenotypes, such as thermal tolerance and body size. In addition, differences in phenotype among seasons could be detected without seasonal evolution if the populations observed in different seasons are independent or they are differentially affected by seasonal migration. Thus, to verify seasonal evolution in phenotypes, I must examine whether seasonal populations are genetically identical.

In the present study, I explored direct evidence for rapid phenotypic evolution in *D. lutescens* in response to seasonal environmental fluctuations. First, I established two sets of multiple isofemale lines of *D. lutescens* derived from two seasons in a single location. I then estimated the genetic structure and calculated the genetic differentiation to reveal that the two periods were almost identical across seasons. Second, I simultaneously measured and compared the thermal tolerance and body size of the strains derived from the two different seasons.

Material and Methods

Field collections of study species

Drosophila lutescens is a species of the *takahashii* subgroup that is distributed in Korea and Japan (Markow and O'Grady, 2006). Adults of *D. lutescens* are observed throughout the year in Japan (Beppu, 2014). For this study, adults were collected at the Nishi-Chiba campus of Chiba University, Japan (35° 37′ 34″ N, 140° 6′ 8″ E), using baited traps during two seasons in 2020: from mid-February to early March (i.e., spring generation) and from early October to early November (i.e., autumn generation). Each female collected was isolated to establish isofemale lines. These siblings were

maintained on media contained in plastic vials (φ30 mm × 100 mm) (KFB-1M, Chiyoda Science). The media used was the one described in the study by Fitzpatrick et al. (2007) (500 ml H_2O, 50 g sucrose, 25 g active yeast, 8 g agar, 5.36 g $KNaC_4H_4O_6 \cdot 4H_2O$, 0.5 g KH_2PO_4, 0.25 g NaCl, 0.25 g $MgCl_2$, 0.25 g $CaCl_2$, 0.35 g $Fe_2(SO_4) \cdot 6.9H_2O$). The flies were reared under a 12L:12D cycle at 25°C, which is known to be a standard condition in *D. melanogaster* (MacKay et al., 2012; Bergland et al., 2014; Pallares et al., 2023). In total, 49 and 23 isofemale lines were established for the spring and autumn generations, respectively. All isofemale lines were reared for at least three generations to reduce genetic variation within a line, and to remove environmental and maternal effects, before being used in all experiments described in detail below. All experiments throughout our study used adult females during December 24th, 2020, and February 3rd, 2021. In the assay of heat tolerance and chill coma recovery, mature females with undamaged wings and swollen abdomen were selected from rearing vials. Selected individuals were anesthetized with CO_2 and transferred into new vials containing a piece of filter paper soaked in 10% sucrose. Individual flies were allowed to recover for more than two hours in an incubator (12L:12D, 25°C) before initiating the assays.

Molecular analyses

To infer the population genetic structure of spring and autumn periods, double-digest restriction site-associated DNA sequencing (ddRAD-seq) was conducted. To achieve a high concentration of DNA, genomic DNA was extracted from approximately 10 adult females for each isofemale line using a Maxwell® 16 LEV Plant DNA Kit (cat. #AS1420, Promega), and subsequently fragmented by restriction enzyme digestion using *PstI* and *MspI*. All isofemale lines were sequenced using ddRAD-seq of 100-bp

paired-end reads and a DNBSEQ-G400 (MGI Tech. Co., Ltd.). Raw sequence reads were cleaned using Trimmomatic (ver. 0.39). Quality-filtered reads were processed using denovo_map.pl script with the -M 3 option in Stacks v2.53 (Rochette et al., 2019) to reduce sequencing artifacts within data and allow for SNPs. Only the first SNP per RAD tag was used for population genetic analyses to avoid the strong linkage between SNPs. The minor allele frequency threshold was set at 3% and the missingness by line filter at 1%. Based on the filtered SNPs, the population genetic structure was estimated using principal component analysis (PCA) in PLINK v1.90. In addition, Wright's F_{ST} between the two periods was calculated using all filtered SNPs and the SambaR package in R version 4.03 (R Core Team, 2020).

Heat tolerance assay

Heat tolerance was measured in terms of the heat knockdown time using 5–10 recovered females per isofemale line placed individually in a small plastic tube (12 × 12 × 45 mm) that was sealed with an air-penetrable plug. The containers were tethered in a stainless-steel tube rack placed in a 37°C water bath. Following the procedure of Sgrò et al. (2010), the heat knockdown time was scored as the time taken by an individual fly to be knocked down and remain immobilized even after the containers were shaken.

Chill coma recovery assay

Cold tolerance was measured in terms of chill coma recovery time using 5–10 recovered females per isofemale line. Each Female was placed in a small plastic vial (φ15 mm × 95 mm) that was sealed with an air-penetrable plug. Following the procedure of MacMillan et al. (2015), the vials were immersed directly into a 1 L mixture of ice and

water in a container (24.5 × 18.5 cm, 10.2 cm in height) at 0°C, where they remained for 10 min. Individual flies were then quickly transferred into a petri dish preheated to 25°C and filmed for 15 min under LED lighting in an incubator (25°C). The time until the flies started walking was recorded. The recovery time of the individuals that were not walking within 15 min was recorded as 900 s.

Body size

As proxies for overall body size, thorax length, and wing length were quantified in 4 or 5 adult females per isofemale line. Following the procedure of Lack et al. (2016), the length of a wing removed from a female was measured as a straight line drawn from the intersection of the L2 and L3 longitudinal veins to where the L3 longitudinal vein intersects the wing margin. Thorax length was measured from the base of the anterior humeral bristle to the posterior tip of the scutellum. In addition, as an index of dispersal ability, the wing to thorax ratio was calculated as wing length/thorax length.

Statistical analyses

All analyses were conducted in R version 4.03. Differences between seasons were analyzed by linear mixed models (LMMs) for body size and generalized linear mixed models (GLMMs) for thermal tolerance (Gamma distribution) in the *lme4* package. In both mixed models, isofemale lines and experimental assay dates were used as random effects. The *P*-values of the fixed effect in the LMM and GLMM were calculated using the χ^2 test in the *car* package.

Results

Information on the number of raw reads in each isofemale line was summarized in Table 3-S1. I identified a total of 1,826 SNP loci that were shared among the isofemale lines. Using these loci, PCA was performed to identify the relationship between the two seasons. Two-dimensional plots of PC1 and PC2 showed that isofemale lines derived from the two seasons overlapped (Fig. 3-1). Using all SNPs, the F_{ST} value between spring and autumn periods was estimated to be 0.007.

There was no significant difference in the thorax length of the adult females between spring and autumn periods (Fig. 3-2a; $F = 0.20$, $P = 0.66$). Wing length of the autumn period was slightly longer than that of the spring period, but the difference was not statistically significant (Fig. 3-2b; $F = 2.83$, $P = 0.097$). However, the autumn period had a significantly greater wing to thorax ratio than the spring period (Fig 3-2c; $F = 4.33$, $P = 0.04$).

In the heat tolerance assay, females often remained on the tube wall or stood on the tube bottom prior to heating. Upon transfer to a preheated water bath, the females dropped to the bottom of the tube and were immobilized within an average of 876 s (minimum: 335 s, maximum: 1,441 s). The heat knockdown time in the autumn period was significantly longer than that in the spring period (Fig. 3-3a; $\chi^2 = 27.0$, $P < 0.001$). On the other hand, in the chill coma recovery assay, females immersed in a cold ice bath started to walk in ca. 220 s on average after being transferred to a 25°C incubator. Although the time taken by the flies to start walking tended to be shorter in the spring period than in the autumn period, the difference in the recovery time was not significant between the two collection time periods (Fig. 3-3b; $\chi^2 = 0.18$, $P = 0.67$).

Discussion

Differences in traits across seasons are known to occur in various organisms (Brakefield, 1985; Carvalho, 1987; Hendry, Letcher and Gries, 2003; Danks, 2004). This phenomenon is suggested to be a piece of evidence supporting rapid evolution. However, it is insufficient to merely measure phenotypes in each season and compare them across seasons as proof of rapid evolution among seasons. In many cases where seasonal changes were observed, the effects of plasticity and season-specific migration of populations with different genotypes could also explain such changes. The present study demonstrated that the F_{ST} value between the spring and autumn periods of *D. lutescens* were very low; that is, two periods exhibited little genetic differentiation, and therefore season-specific migration is not likely to occur in the population that I observed. I did, however, detect a phenotypic difference between the two collection time periods by simultaneously measuring phenotypic traits under constant conditions. Removing environmental effects that could potentially affect phenotypic traits allows for strong confirmation of the presence of genetic changes across seasons. Thus, my results provide strong evidence indicating that seasonal environmental heterogeneity induces rapid evolution at a phenotypic level. I also note that flies collected in the spring period were kept in my lab for a much longer time than flies collected in the autumn period. I could not remove genetic variability by the effects of different inbreeding or drift that occurred during rearing (David et al., 2005). To confirm that these effects do not influence slightly, I should rear my lines for a longer time or experiment in reverse sampling order.

Genetic variation is suggested to be one of the critical factors that enhances evolution and speciation. Recent studies revealed that balancing selection and selection associated with spatiotemporal environmental heterogeneity in a habitat maintains

genetic variation throughout the year within a population (Ehrman, 1970; Ravigné, Olivieri and Dieckmann, 2004). The present study found adaptive replacement across seasonal periods with respect to heat tolerance. My population genetic analysis also showed that such variation was not due to external sources. Thus, my results suggest that the frequencies of genotypes associated with heat tolerance oscillate among seasons and that seasonal oscillations in the environment contribute to the maintenance of multiple genotypes within a population.

A higher heat tolerance after summer (i.e., autumn period) suggests that selection associated with seasonal climate drives the adaptive evolutionary response. The prevalence of individuals with higher heat tolerance might increase during summer and decrease during winter in population levels. Many previous studies have focused on families of heat shock proteins (Hsps) as one of the most important genetic candidates related to high-temperature responses. Although inducible Hsps protect organisms against temperature high enough to kill them in several minutes to hours, its Hsps also have a variety of deleterious consequences such as low fecundity and retarded development. For example, in *D. buzzatii*, the expression of inducible Hsp70 was reduced in a population maintained under a high- vs. a low-temperature environment (Sørensen, Dahlgaard and Loeschcke, 2001). This interpretation was that the costs of Hsp70 expression in the high-temperature population, rather than its benefits. Evolutionary adaptation to temperatures high enough to induce Hsp70 expression, but insufficient to kill rapidly, is suggested to lead to a reduced sensitivity of the heat stress response owing to the higher baseline tolerance in populations of warmer regions (Sørensen, Kristensen and Loeschcke, 2003), and maybe even under summer conditions. In addition, other genes may contribute to the variation in the thermal

response, including the Turandot (Ekengren and Hultmark, 2001) and Methuselah genes (Lin, Seroude and Benzer, 1998). A genome-wide association analysis study on *D. melanogaster* identified a genetic basis associated with tolerance to high temperatures (Rolandi et al., 2018). This study found 12 SNPs associated with heat tolerance, including some genes, such as *atet*, *mbl*, *nemy*, *Nhe2*, and *ome*. Further research requires more genomic analysis to verify candidate genes contributing to the seasonal evolutionary response to heat tolerance.

Insects often show latitudinal variation in flight morphology, such as wing size and the ratio of wing size to body size, which directly affects foraging, mating, dispersal, and thus reproductive success. Along the environmental gradient on a continental scale, the relative wing size to body size of *Drosophila* spp. is known to be larger in cold regions than in warm regions. A relatively larger wing is advantageous in the cold because ectotherms generate less energy per wingbeat (Gilchrist and Huey, 2004). However, I found the opposite pattern in the wing to thorax ratio in the context of seasonal environmental changes; a larger ratio of wing to thorax length was observed in the autumn period, which had just experienced summer. Such an opposite pattern could be explained by the seasonal variation in the density of the population. Previous theoretical studies demonstrated that dispersal strategy could evolve density-dependently (Travis, Murrell and Dytham, 1999). The wing to thorax ratio could reflect the ability to access resources; that is, a larger wing to thorax ratio could increase dispersal ability (Hoffmann et al., 2007). Since the population density of *Drosophila* spp. could be higher during a warm season than during a cold season, genotypes expressing a higher dispersal ability (i.e., larger wing to thorax ratio) may be favored during summer, when densities and competition increase.

Although cold tolerance was slightly higher after winter and wing size tended to be longer after summer, I showed non-significant evidence for seasonal variation in cold tolerance and body size. Cold tolerance has been shown to often evolve adaptively in *Drosophila* (Hoffmann, Sørensen and Loeschcke, 2003; Pool, Braun and Lack, 2017). Even in *D. lutescens*, a previous study showed latitudinal variation in cold tolerance through the evaluation of adult survival (Kimura et al., 1994). One reason that I failed to detect differences in cold tolerance between periods might be due to the lower resolution of the experiment. Since I measured the chill coma recovery time at 25°C, the variation in time to recover was not large. Measuring the chill coma recovery time at a temperature less than 25°C could help detect more subtle differences. I should also examine cold tolerance using various indices to determine whether *D. lutescens* evolves adaptively at low temperatures. A recent study suggests that interspecific competition changes rapid evolutionary trajectories across seasons (Grainger and Levine, 2022). More specifically, when *D. melanogaster* was exposed to interspecific competition with an invasive competitor over the summer, the flies evolved during autumn to be larger and have lower cold fecundity and faster development than flies not exposed to the competitor. Thus, my results suggest that other *Drosophila* species might affect the rapid evolutionary responses of cold tolerance and body size observed in *D. lutescens*.

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Figures

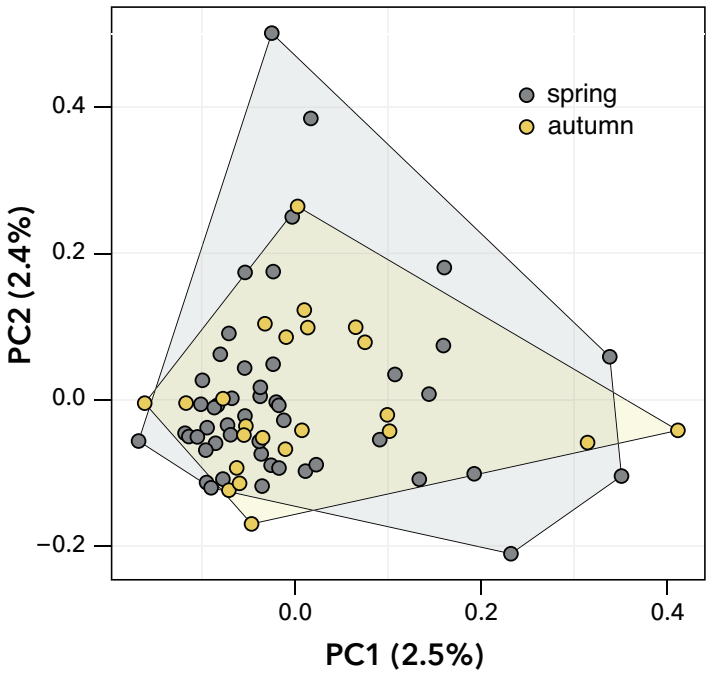

Figure 3-1. Representation of *Drosophila lutescens* periods between spring and autumn via principal component analysis (PCA). Convex polygons in each period are shown in the two-dimensional space. Plots represent isofemale lines ($N = 72$; spring: 49, autumn: 23).

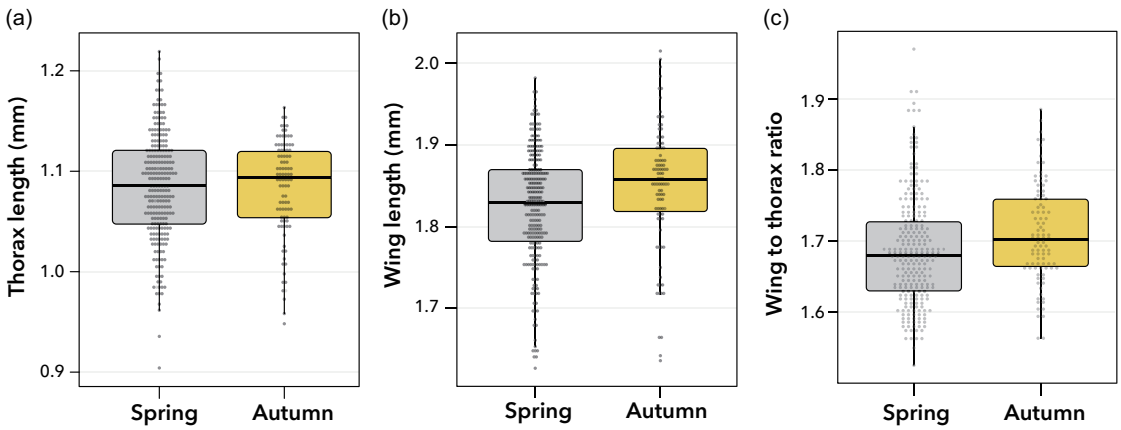

Figure 3-2. Body size of the spring and autumn periods of *Drosophila lutescens*. Thorax length (a), wing length (b), and wing to thorax ratio (c). In all boxplots, boxes and thick black lines represent the 25th (lower) to 75th (upper) percentile and median, respectively. The upper and lower whiskers represent scores outside the middle 50%. The plots are all sample data.

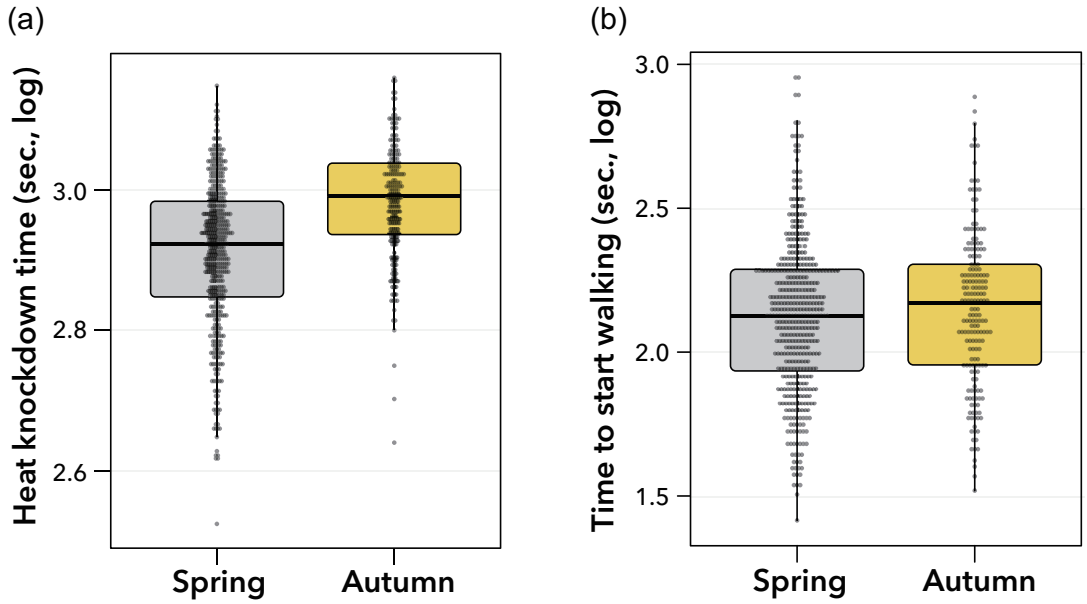

Figure 3-3. Thermal tolerance of spring and autumn periods of *Drosophila lutescens* to heat (a) and cold (b). The meaning of boxes, thick black lines, and plots was described in Figure 2.

Supplemental material

Table 3-S1. The number of raw reads in each isofemale line.

Isofemale line No.	Seasonal period	Reads
15	Spring	3338867
23	Spring	3733218
24	Spring	3424709
25	Spring	2896408
26	Spring	3516919
27	Spring	3857630
28	Spring	3707780
29	Spring	3133594
31	Spring	3203799
39	Spring	4135463
40	Spring	2984048
42	Spring	3454823
44	Spring	2845660
49	Spring	3348005
50	Spring	3829729
51	Spring	3108543
53	Spring	3285473
54	Spring	3767425
55	Spring	3776354
58	Spring	2915372
59	Spring	3130028
66	Spring	3503538
75	Spring	4049027
77	Spring	3335159
78	Spring	3005131
79	Spring	3461525
81	Spring	2981003
82	Spring	3722754
83	Spring	3584832
84	Spring	4486455

85	Spring	3231009
88	Spring	3132774
91	Spring	4174406
97	Spring	4706774
98	Spring	3585392
99	Spring	3915297
101	Spring	4021596
102	Spring	4724621
104	Spring	2744121
106	Spring	2660050
110	Spring	3038443
113	Spring	3507002
114	Spring	2545051
120	Spring	3193415
122	Spring	3115692
130	Spring	3303736
131	Spring	4074485
135	Spring	2640265
137	Spring	2714557
355	Autumn	3945930
384	Autumn	3141066
385	Autumn	3745097
387	Autumn	3974438
389	Autumn	2909073
500	Autumn	2687524
508	Autumn	3018026
510	Autumn	3434852
531	Autumn	3729816
533	Autumn	3230959
535	Autumn	2896025
538	Autumn	3278880
539	Autumn	4400868
541	Autumn	2901996
543	Autumn	3382202

544	Autumn	3372686
547	Autumn	3623843
551	Autumn	3254899
552	Autumn	3415532
555	Autumn	3373317
556	Autumn	3475196
581	Autumn	3559574
591	Autumn	3232658

Multi-trait association analysis of genetic diversity effects on population performance in a wild *Drosophila*

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Abstract

Over a quarter century of ecological research, species diversity and intraspecific genetic diversity non-additively affected ecological properties, such as productivity and stability in ecosystems, communities, and populations—an effect known as the diversity effect. Most previous studies examined the contribution of species number and genotype number. However, few studies focused on the contribution of dissimilarity among species and genotypes. Although phenotypic dissimilarity is one of the indices of functional differentiation, so far, there has been no effort to predict the extent of such effects by phenotypic dissimilarity. Here, I explored which phenotypic variations among various traits are responsible for improving productivity and stability in a population. I focused on natural genetic variation in inbred lines of *Drosophila immigrans* and measured the phenotypic dissimilarity of 21 traits in three representative trait groups: morphology, behavior, and life history. Population performance was evaluated in experimental populations consisting of one or two inbred lines. Using an implementation of association analysis at the phenotypic level, I identified key variations associated with population performance. My experiment showed that population performance in mixtures was greater than in monocultures. More importantly, behavioral variations contributed to diversity effects more than those in morphological and life-history traits. My approach might lead to fundamental new insights into the traits and genetic underpinnings of diversity effects in natural systems.

Keywords: intrapopulation genetic diversity, productivity, stability, *Drosophila immigrans*

Introduction

Ecologists have recently focused on the ecological effects of genetic diversity. Some pioneering studies have gradually unveiled the ecological importance of intraspecific diversity. Increasing genetic richness (e.g., the number of genotypes and phenotypes) in a population non-additively improves population performance in pygmy grasshoppers (Caesar, Karlsson and Forsman, 2010), ecological performance in diatom (Sjöqvist and Kremp, 2016), and ecosystem functioning in eelgrass, tall goldenrod, and crayfish (Reusch et al., 2005; Crutsinger et al., 2006; Raffard et al., 2017). Functional differentiation, or dissimilarity among genotypes, was also a major driver of enhancing population performance and ecosystem functioning (Jousset et al., 2011; Ellers et al., 2011). These measurable components at various levels of the ecological organization are often called ecological properties (Hughes et al., 2008). The emergent, non-additive effects driven by diversity on the ecological properties described above have been well known as the diversity effect—which was extensively investigated in the context of community ecology (e.g., Naeem et al., 1995).

Phenotypic diversity is helpful for evaluating genetic richness and dissimilarity among individuals and species. It is typically measured as the number of phenotypes, variability in trait values, and trait distance between pairs of individuals and species (Laliberte and Legendre, 2010; Ellers et al., 2011; Abbott et al., 2017). Phenotypic variances among species were revealed to be related to ecosystem functioning or the stability of the community and ecosystem processes (Loreau and Behera, 1999; Norberg et al., 2001). Also, previous studies indicated that the degree of trait distinctiveness linearly increased productivity and stability in ecosystems (Flynn et al., 2011; Tilman et al., 2014). These findings suggest that functional differences among species reflect

different ecological niches occupied by each species and result in niche complementarity in the community and ecosystem. Analogously, inter-individual phenotypic diversity in personalities, body colors, and life history often affected population performance, such as productivity, survival rate, and collective behavior (Dyer et al., 2009; Caesar et al., 2010; Ellers et al., 2011; Takahashi et al., 2018). Previous studies have proposed that genetic diversity effects occurred through improved use of resources in fish (Dyer et al., 2009), reduced predation, and reduced sexual harassment in insects (Ahnesjö and Forsman, 2006; Takahashi et al., 2014). However, phenotypic diversity was mainly taken to be the number of phenotypes (i.e., phenotypic richness) because most of the previous studies have focused on qualitative phenotypic variation or categorized quantitative phenotypic variation into some discrete phenotypes. Therefore, few studies have handled a quantitative phenotypic variation in each trait, which affects diversity effects.

Genetic variation in traits is a widespread phenomenon that is maintained by evolutionary processes, including balancing selection or migration-selection balance, mutation-selection balance, and diversifying selection (Slatkin, 1973; Mallet and Barton, 1989). Although such variation in traits contributes to the difference in survival and reproductive success among individuals within a population, it does not always affect ecological properties positively (Bolton, Rollins and Griffith, 2015, 2016). For example, color variation was expected to enhance the survival and the expansion of geographical distribution in insects (Caesar et al., 2010; Takahashi and Noriyuki, 2019), whereas reducing resource use in two reptile species (Broennimann et al., 2014). Variations in behavioral traits that occurred between the sexes facilitated faster detection of novel food patches in guppies (Snijders et al., 2019, 2021) while decreasing female

survival probability due to sexual harassment by male aggression in lizards (Le Galliard et al., 2005). Thus, the relationships between genetic diversity and diversity effects lack consistency even in the same type of traits. This means that experimental research is necessary to consider phenotypic diversity in various traits of multiple trait groups. Such research would lead to understanding the strength and direction of diversity effects that might depend on phenotypic variations in traits.

Here, I investigated "which phenotypic diversity" out of various traits non-additively affected population performance for *Drosophila immigrans*, related to the model organism *D. melanogaster*. *Drosophila* is traditionally advantageous for measuring phenotypic variation in various trait groups, such as morphogenesis, behavior, sociality, and life history, to elucidate evolutionary and ecological dynamics (Ray et al., 2016; Anreiter and Sokolowski, 2019; Flatt, 2020). Hence, phenotypic values in various traits, comprising three trait groups (morphology, behavior, and life history), were quantified on each isofemale line (i.e., full-sib mating lines). Because isofemale lines are largely homozygous, I assumed that the phenotypic variation among these lines mainly comes from genetic differentiation. For each trait, the pairwise phenotypic distance between isofemale lines was calculated as indices of phenotypic dissimilarity. Then, I grew fruit flies within a single generation in two treatments of experimental populations: monocultures for each isofemale line and pairwise mixtures. The pairwise approach is fundamental and amenable to predicting ecological dynamics based on trait differences between paired species/individuals (Veech, 2014). After growing them, I assessed five properties of populations, as indices of population performance, and stability of population performance. Because of my experimental design, I did not consider any effects derived from sexual reproduction, such as

heterosis. Finally, I explored which phenotypic variations contribute to improving population performance by using multi-trait association analysis.

Material and Methods

Study species

Drosophila immigrans is a globally distributed generalist which can oviposit on a wide array of substrates such as fungi, fruits, sap fluxes, and flowers. Previously, I established 21 isofemale lines of *D. immigrans* from the Sapporo population in Japan (Ueno and Takahashi, 2021). For this study, twelve lines out of them were used and reared with agar media in plastic vials (100 mm in height, φ27 mm; KFB-1M, Chiyoda Science) under a 12L:12D cycle at 23°C. The composition of the agar medium followed the methodology described by Fitzpatrick et al. (2007), with the exception of the quantity of dry yeast, which was double the amount used in their study. To prevent the growth of bacteria and mold, I added 2 g potassium sorbate and 50 mg ampicillin to the medium. This nutrient condition is sufficient for this species to grow healthy (data not shown). Prior to measuring variation in various traits in each isofemale line, flies were reared for at least ten generations to reduce genetic variation within a line and to remove environmental effects.

Wing morphology

To measure the wing size in each line, I collected 10 males and 10 females reared under the standard condition described above. The left wings were dissected from the body and mounted on a sheet of paper to measure four indices of the wing size with ImageJ (ver. 2.0.0-rc-69/1.52p). Following Robertson and Reeve (1952) and Gilchrist et al.

(2001), I measured wing length in two segments along vein IV, *L1* (from the base of the fourth longitudinal vein to the posterior cross vein), *L2* (from the posterior cross vein to the distal extreme of the fourth longitudinal vein), and the sum of *L1* and *L2*. I also measured wing width, *W* (the distance from the distal extreme of the fifth longitudinal vein on the trailing edge of the wing to the leading edge in a line perpendicular to vein III).

Behavior

I used and reanalyzed the variations in larval and adult activities reported by Ueno and Takahashi (2021). The data for activities in each line consisted of the larval locomoting distance during 10 minutes and the movement of adults over a period of 24 hours. The larval activity in each line was evaluated by determining the average value of the locomotion speed. For the daily activity of adults, the number of activity levels per hour was measured under the 12L:12D cycle at 23°C (Ueno and Takahashi, 2021). Based on the 24-hour data, I calculated four indices to determine the adult activity in males and females of each line, namely: the level of total activity, daytime activity, nighttime activity, and the ratio of daytime and nighttime activity.

Life-history traits and population performance

To evaluate the dry weights of an individual male and female, egg size, and 50% pupation rates as indices of life-history traits in each line, I placed approximately 50 adult males and females into bottles containing Petri dishes (φ90 mm) filled with grape agar (50 ml grape juice, 50 ml water, 2 g agar, 2 ml ethanol, and 2 ml acetic acid) and collected fertilized eggs laid on the agar. Ten randomly chosen eggs from the collected

eggs of each line were used to measure egg lengths (Markow, Beall and Matzkin, 2009). Then, 24 randomly chosen eggs were transferred onto 10 ml low-nutrient media (50% reduction of the sucrose and dry yeast content relative to the method in Fitzpatrick et al. (2007)) in an experimental vial (100 mm in height, φ27 mm). These monocultures of each line were maintained until emergence in an incubator with a 12L:12D cycle at 23°C with illumination from white LEDs. In each monoculture treatment, I scored the number of pupae, adult males, and adult females once within four days during the rearing and measured the dry weights of an individual male and female after the end. Then, by fitting logistic curves to all growth data of each line, I identified the day to reach the 50% pupation rate. I also built mixture treatments of randomized pairwise combinations consisting of the same number of individuals on each line. The same experimental procedures were conducted in each combination. Fig. 4-S1 describes the IDs of isofemale lines used and an incomplete mixed design (randomly 49 of the 66 combinations in this study).

Population performance in monoculture and mixture treatments was evaluated by five population properties: the dry weights of an individual male and female, the day to reach the 50% pupation rate (an indicator of growth speed), survival rate, and population biomass at adult emergence. The former three properties are the same as life-history traits. Survival rate was calculated by the proportion of the survived adults. Population biomass (B_p) was estimated by the following equation:

$$B_p = (S \times R_M \times W_M) + (S \times R_F \times W_F)$$

where S, R, and W are the number of survived adults, the rates of adults, and the dry weights of an individual, respectively. Subscripts of M and F in the equation represented males and females, respectively. The stability of a population performance in

monocultures and mixtures was assessed with the inverse of the coefficient of variation (CV) of population biomass, calculated from 3 to 10 replicates.

Diversity effects and multi-trait association analysis

Prior to conducting an analysis, all variables of population properties, stability, and phenotypes were standardized. The non-additive changes in the population performance and stability in mixtures compared to monocultures were denoted as diversity effects in this study. To determine the diversity effects, I calculated the deviation of the observed value of a mixture from the average value of two monocultures in each of the 49 combinations. To determine phenotypic diversity in mixtures, I used phenotypic variables from each isofemale line and calculated the Euclidean distance between each of the 49 pairs of isofemale lines for 21 morphological, behavioral, and life-history traits. Subsequently, I conducted an analysis of the association between the phenotypic diversity of each trait and the diversity effects in mixtures.

Statistical analyses

All statistical analyses were performed in R ver. 4.2.2 (R Core Team, 2022). Pearson's correlation test was used to analyze the correlations of diversity effects between population properties and stability and the correlations of phenotypic values between traits in isofemale lines. I tested whether genetic diversity changed each population property non-additively in two ways. First, the difference in each population property and stability between the pooled mixtures and the pooled monocultures was tested using the linear mixed model (LMM) with genetic diversity (monocultures or mixtures) as a fixed factor and the combinations and the assay month as a random factor in the *lme4*

package. The *p*-value of the independent variable (monoculture or mixture) in the LMM was calculated from the χ^2-tests in the *Anova* function of the *car* package. Second, a one-sample *t*-test was conducted to determine whether the diversity effects in each population property and stability were significantly different from zero.

For multi-trait association analysis, I used phenotypic distances as an explanatory variable and diversity effects as a response variable and performed simple linear regression in every trait and population property. The *p*-value of the independent variable was calculated from the *F* tests in the *Anova* function of the *car* package.

Multiple regression analysis was also used to directly evaluate the relative importance of phenotypic diversity in morphological, behavioral, and life-history traits for diversity effects. I initially applied principal component analysis (PCA) on phenotypic data separately for phenotypic data of morphological, behavioral, and life-history traits. The Kaiser criterion was applied to retain principal components (PCs) in each trait group with an eigenvalue greater than 1 (Fig. 4-S2). Multicollinearity between PCs of each trait was assessed using the variance inflation factor (VIF), and since five selected PCs (namely, $PC1_{morphology}$, $PC2_{morphology}$, $PC1_{behavior}$, $PC2_{behavior}$, and $PC1_{life\text{-}history}$) showed VIF < 5, none were excluded. The multiple linear regression models included the diversity effect of each property as a response variable and the Euclidean distance of each PC between pairs of isofemale lines as explanatory variables. Selection of multiple regression models was performed based on Akaike information criteria (AIC) to establish a confidence set of models with $\Delta AIC < 2$ using the *dredge* function in the *MuMIn* package.

Results

Standardized phenotypic values in each trait varied among 12 isofemale lines (Fig. 4-S3). Phenotypic correlations tended to be found between traits belonging to the same trait group, such as wing morphology and activity (Fig. 4-S4). That is, correlations among wing morphologies and among activity levels were significantly positive, while a part of the correlations of phenotypic values between wing morphology and individual weights was significantly negative. Then, the PCAs separately performed for each of the three trait groups (morphology, behavior, and life history), identifying the highest variance for each trait group. For wing morphology, the variation was summarized in $PC1_{morphology}$, in which the wing size of isofemale lines showing high scores were greater than those with low scores (accounting for 73% of the total variance; Fig. 4-S5a). For behavior, the variation was summarized in $PC1_{behavior}$, in which isofemale lines with high scores were more active compared to those with low scores throughout the day (accounting for 56% of the total variance; Fig. 4-S5b). For life-history traits, the variation was summarized in $PC1_{life\text{-}history}$, in which isofemale lines with high scores were heavier and grew more rapidly than those with low scores (accounting for 57% of the total variance; Fig. 4-S5c).

Population properties in monocultures and mixtures are shown in Fig. 4-1. On average, all population properties and stability in mixtures exhibited higher values than those in monocultures (see Table 4-S1). Notably, population biomass, male weights, and stability in mixtures were significantly greater than those in monocultures. Hence, diversity effects also tended to be overall positive (Fig. 4-2a). Positive effects in population biomass, male weights, and stability were significantly different from zero (see Table 4-S1). Among population properties and stability, diversity effects on the population biomass were positively correlated with those of the survival rate and female

weights but not the male weights (Fig. 4-2b and 4-S6).

The results of the phenotypic variation contributing to diversity effects are summarized in Fig. 4-3 and Table 4-1. In multi-trait association analysis, each simple linear regression indicated that variation in total activity levels of males and nighttime activity levels of females non-additively improved the dry weights of an individual male ($F = 4.56$, $p = 0.04$) and survival rate ($F = 6.04$, $p = 0.02$), while variation in the rate of activity of males and females non-additively decreased the dry weights of an individual female (males: $F = 5.69$, $p = 0.02$; females: $F = 4.44$, $p = 0.04$). In multiple linear regressions, various combinations of five explanatory variables were adopted to provide models with $\Delta AIC < 2$ to explain diversity effects on each population property and stability. Among all selected models in each population property, except for the null model, variations in $PC1_{behavior}$ (which incorporates total activity levels and nighttime activity levels) mainly had a positive impact on the diversity effects on the dry weights of an individual male and the survival rate of adults (Table 4-1b, d). In contrast, variations in $PC2_{behavior}$ (which incorporates the rate of activity levels of males and females) had a negative impact on the dry weights of an individual female (Table 4-1c). However, the best model was a null model in diversity effects on population biomass, growth speed, and stability (Table 4-1a, e, f). Note that other models included some PCs for them.

Discussion

In this study, I revealed that genotype richness in *D. immigrans* significantly improved population performance and stability under my experimental conditions. This finding is consistent with previous studies in various organisms (Reusch et al., 2005; Caesar et al.,

2010). I used the range of phenotypic variation within a wild-derived population of this species, which is assumed to co-occur under natural conditions (i.e., standing genetic variation). Variations in traits maintained by balancing selection are suggested to occur positive diversity effects (Takahashi et al., 2018). Thus, diversity effects in some population properties and stability might be totally positive by various combinations of such variations. On the other hand, there were no significant positive diversity effects in some population properties and negative effects in some mixtures. This is consistent with previous studies, which showed positive and negative diversity effects, depending on genotype/phenotype numbers and abiotic/biotic environmental conditions (Caesar et al., 2010; Drummond and Vellend, 2012; Turner et al., 2020).

I also found a significant positive correlation between the diversity effects on population biomass and those on female weights, but not those on male weights. This implies that the non-additive increase in individual weights of surviving females was directly connected to the non-additive improvement of population biomass. Conversely, such a relationship was not observed for the non-additive increase in individual weights of surviving males. This could be attributed to the fact that the body size of females was generally larger than that of males in *D. immigrans* (Fartyal et al., 2017), and the trend was consistent in this study. Thus, increasing population productivity may rely on the survival of larger females. However, since few studies have addressed the correlations among diversity effects on population properties, I cannot ascertain whether this pattern was ubiquitous under other conditions or in other species. To confirm this, further research needs to test diversity effects of as many combinations as possible in various experimental systems or predict the relationships among population properties with sophisticated statistical methods.

To identify the key traits responsible for the non-additive changes in population performance, some previous studies have focused on only a few specific traits and tested whether variation in these traits affected population performance and stability. Consequently, some candidates for key traits, including morphology, behavior, and life history, have been identified across different studies (Caesar et al., 2010; Ellers et al., 2011; Takahashi et al., 2018). However, there have been inconsistencies in identifying key traits among some previous studies (Bolton et al., 2015; Forsman, 2016). Here, the present study revealed the variation in traits that were responsible for diversity effects and those that were not. Multi-trait association analysis and models selected by AIC indicate that behavioral variations contribute to diversity effects more than those in morphological and life-history traits. Also, phenotypic variation in behavior affected the increase or decrease in diversity effects on some population properties. The direction of diversity effects would depend on variations in traits, that is, it differs even in the same type of traits.

The diversity effects on male weights were significantly increased by behavioral variation in this study. Previous studies also suggested that phenotypic variation in behavior improved population performance by altering ecological, social, and sexual interactions among individuals. For example, behavioral variation influenced by the *foraging* gene increased population biomass in *D. melanogaster* (Takahashi et al., 2018). Besides, personality differences and sex-dependent social behavior enhanced the faster detection of novel food patches in guppies (Dyer et al., 2009; Snijders et al., 2019). However, my experiments did not measure larval behavioral phenotypes, such as social clustering (Dombrovski et al., 2017), foraging behavior (Anreiter and Sokolowski, 2019), and locomotion rhythm (Malpel et al. 2004), in which inter-

individual interactions could be more active than in the adult stage. This is because it was technically challenging for us to quantify the rhythm of larval daily activity at the current moment. I need to test whether the various variations in larval phenotypes directly affect the diversity effect in *D. immigrans*, although phenotypes in adults could be proxies of those in larvae. In addition, abiotic variables, such as humidity, temperature, and photoperiod, were kept constant in my experiments, whereas these would show strong fluctuations under more natural situations. It may be important to consider that the interactions between individuals of different isofemale lines (genotypes) in natural conditions cause different directions of diversity effects (Ellers et al., 2011; Wennersten and Forsman, 2012).

This study investigated variation in 21 traits across trait groups to explore phenotypic diversity responsible for diversity effects. On the other hand, my investigation and analysis were not sufficient because the number of traits is considered to exist infinitely. However, analyses at the phenotypic level are limited to investigating all possible candidates of traits for diversity effects. Therefore, further research is required to focus on genomic levels to understand traits and genes responsible for diversity effects and the mechanisms driving them. Although previous studies identified candidate diversity-effect genes to enhance population productivity in *Arabidopsis thaliana* (Wuest and Niklaus, 2018; Turner et al., 2020), there is a limited utilizing genetic variation, and the problem of false positives raised from genetic architecture population. To confirm the understanding of the genetic basis of diversity effects on ecological dynamics, a genomic approach should be adopted to survey genes contributing to diversity effects exhaustively using natural genetic variation within a population.

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Tables and Figures

Table 4-1. Ranking of models with ΔAIC < 2 for explaining diversity effects on each population property and stability with all possible combinations of $PC1_{morphology}$, $PC2_{morphology}$, $PC1_{behavior}$, $PC2_{behavior}$, and $PC1_{life\text{-}history}$. Models were ranked by the Akaike model weight (W_i) after Burnham and Anderson (2002). Models are arranged in order of increasing Akaike information criterion (AIC). ΔAIC is the difference in AIC from that of the best model.

Model	**AIC**	**ΔAIC**	**W_i**
(a) Population biomass			
null	59.779	0.000	0.132
$0.03 \times PC1_{behavior}$	60.139	0.360	0.111
$-0.04 \times PC2_{behavior}$	61.057	1.279	0.070
$0.03 \times PC1_{behavior} - 0.04 \times PC2_{behavior}$	61.386	1.608	0.059
$0.02 \times PC1_{life\text{-}history}$	61.646	1.867	0.052
$0.04 \times PC1_{behavior} + 0.03 \times PC1_{life\text{-}history}$	61.727	1.948	0.050
$0.01 \times PC1_{morphology}$	61.744	1.965	0.050
$-0.003 \times PC2_{morphology}$	61.776	1.997	0.049
(b) Male weights			
$0.05 \times PC1_{behavior}$	45.065	0.000	0.204
$0.05 \times PC1_{behavior} + 0.03 \times PC2_{behavior}$	46.572	1.507	0.096
$0.05 \times PC1_{behavior} + 0.02 \times PC1_{life\text{-}history}$	46.933	1.868	0.080
$0.05 \times PC1_{behavior} - 0.002 \times PC1_{morphology}$	47.058	1.993	0.075
$0.05 \times PC1_{behavior} - 0.003 \times PC2_{morphology}$	47.061	1.996	0.075

(c) Female weights			
$-0.12 \times PC2_{behavior}$	66.989	0.000	0.170
$-0.11 \times PC2_{behavior} + 0.05 \times PC1_{life\text{-}history}$	68.209	1.220	0.093
$-0.02 \times PC1_{behavior} - 0.12 \times PC2_{behavior}$	68.252	1.262	0.091
$-0.12 \times PC2_{behavior} - 0.02 \times PC1_{morphology}$	68.557	1.567	0.078
$-0.12 \times PC2_{behavior} + 0.02 \times PC2_{morphology}$	68.890	1.901	0.066
(d) Survival rate			
$0.05 \times PC1_{behavior}$	72.249	0.000	0.152
$0.06 \times PC1_{behavior} + 0.07 \times PC2_{morphology}$	73.200	0.951	0.095
$0.05 \times PC1_{behavior} - 0.04 \times PC1_{life\text{-}history}$	73.802	1.553	0.070
null	73.856	1.607	0.068
$0.06 \times PC1_{behavior} - 0.02 \times PC2_{behavior}$	74.038	1.789	0.062
$0.06 \times PC1_{behavior} + 0.01 \times PC1_{morphology}$	74.155	1.905	0.059
(e) Growth speed			
null	172.758	0.000	0.129
$0.12 \times PC1_{morphology}$	173.284	0.526	0.099
$-0.13 \times PC2_{behavior}$	173.949	1.191	0.071
$-0.15 \times PC2_{behavior} + 0.13 \times PC1_{morphology}$	174.217	1.459	0.062
$0.05 \times PC1_{behavior}$	174.478	1.719	0.055
$0.05 \times PC2_{morphology}$	174.701	1.943	0.049
$-0.02 \times PC1_{life\text{-}history}$	174.739	1.981	0.048
(f) Stability			
null	174.094	0.000	0.121
$0.21 \times PC2_{morphology}$	174.895	0.801	0.081

$-0.10 \times PC1_{morphology}$	175.240	1.146	0.068
$0.12 \times PC2_{behavior}$	175.446	1.351	0.061
$0.17 \times PC2_{behavior} + 0.27 \times PC2_{morphology}$	175.653	1.559	0.055
$-0.04 \times PC1_{behavior}$	175.900	1.806	0.049
$0.05 \times PC1_{life\text{-}history}$	176.023	1.929	0.046

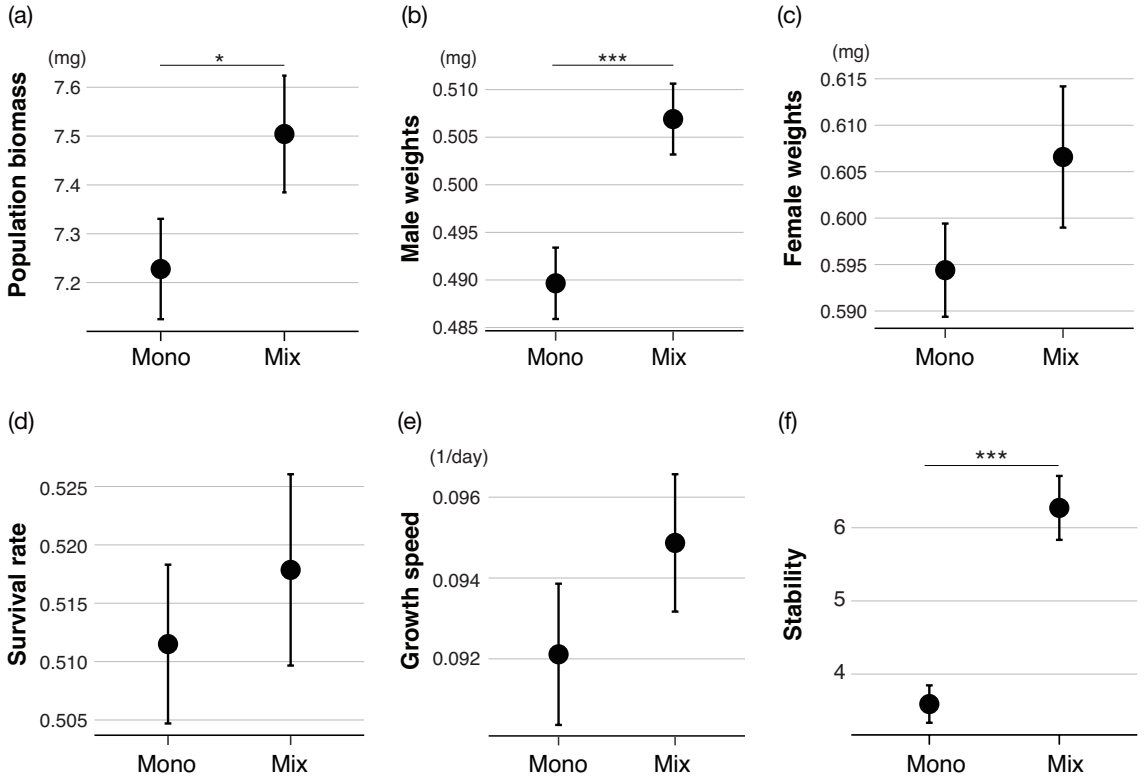

Figure 4-1. Population performance and stability on population type (Mono = isofemale line monoculture; Mix = mixture of two isofemale lines), showing (a) population biomass calculated by the dry weights of individuals, (b) the dry weights of an individual male, (c) the dry weights of an individual female, (d) survival rate of adults, (e) the growth speed of larvae, and (f) the inverse of the coefficient of variation (CV) of population biomass in monocultures and mixtures. Plots and error bars represent average population properties and standard error of the mean, respectively. Asterisks indicate a significant difference in a population property between mono and mix (*$0.01 < p < 0.05$; ***$p < 0.001$). See Table 4-S1 for details.

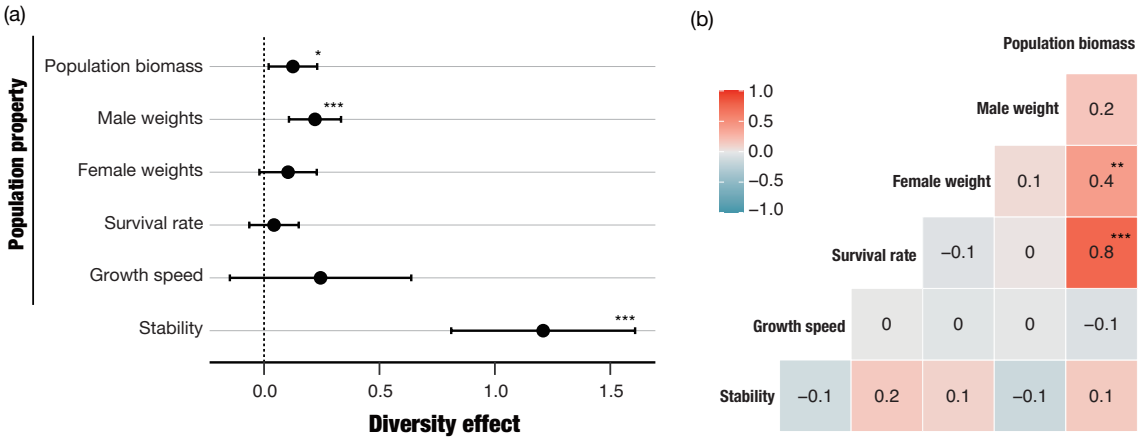

Figure 4-2. (a) Diversity effects on each population property and stability. Plots and error bars represent average population properties and confidence intervals (95%), respectively. (b) Correlation between pairwise combinations of diversity effects on population properties and stability. The number and continuous color scale represent the Pearson correlation coefficient (r) in each cell. Asterisks indicate (a) a significant difference from zero (see Table 4-S1 for details) and (b) a significant correlation between the two traits by Pearson's correlation test (*$0.01 < p < 0.05$; **$0.001 < p < 0.01$; ***$p < 0.001$).

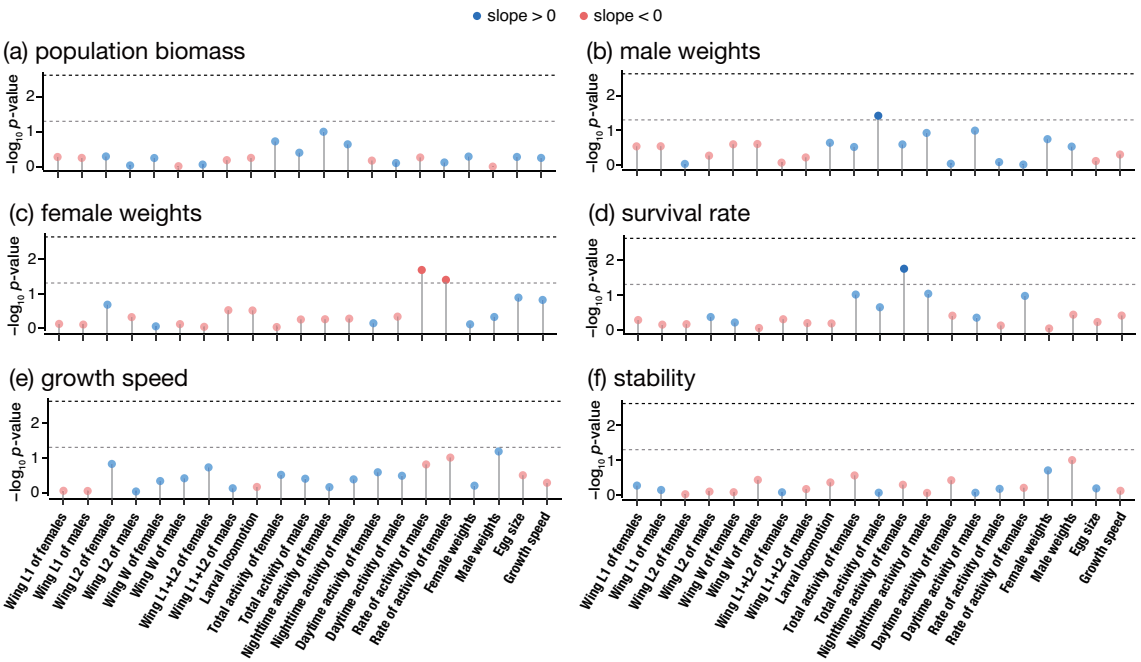

Figure 4-3. Manhattan plots showing variation in traits responsible for diversity effects in muti-trait association analysis. This analyzed the association between the Euclidean distances of each phenotype and diversity effects on (a) population biomass calculated by the dry weights of individuals, (b) the dry weights of an individual male, (c) the dry weights of an individual female, (d) survival rate of adults, (e) the growth speed of larvae, and (f) the inverse of the coefficient of variation (CV) of population biomass. Horizontal dashed grey and dark grey lines indicate p-value < 0.05 and criteria from Bonferroni corrections, respectively.

Supplemental material

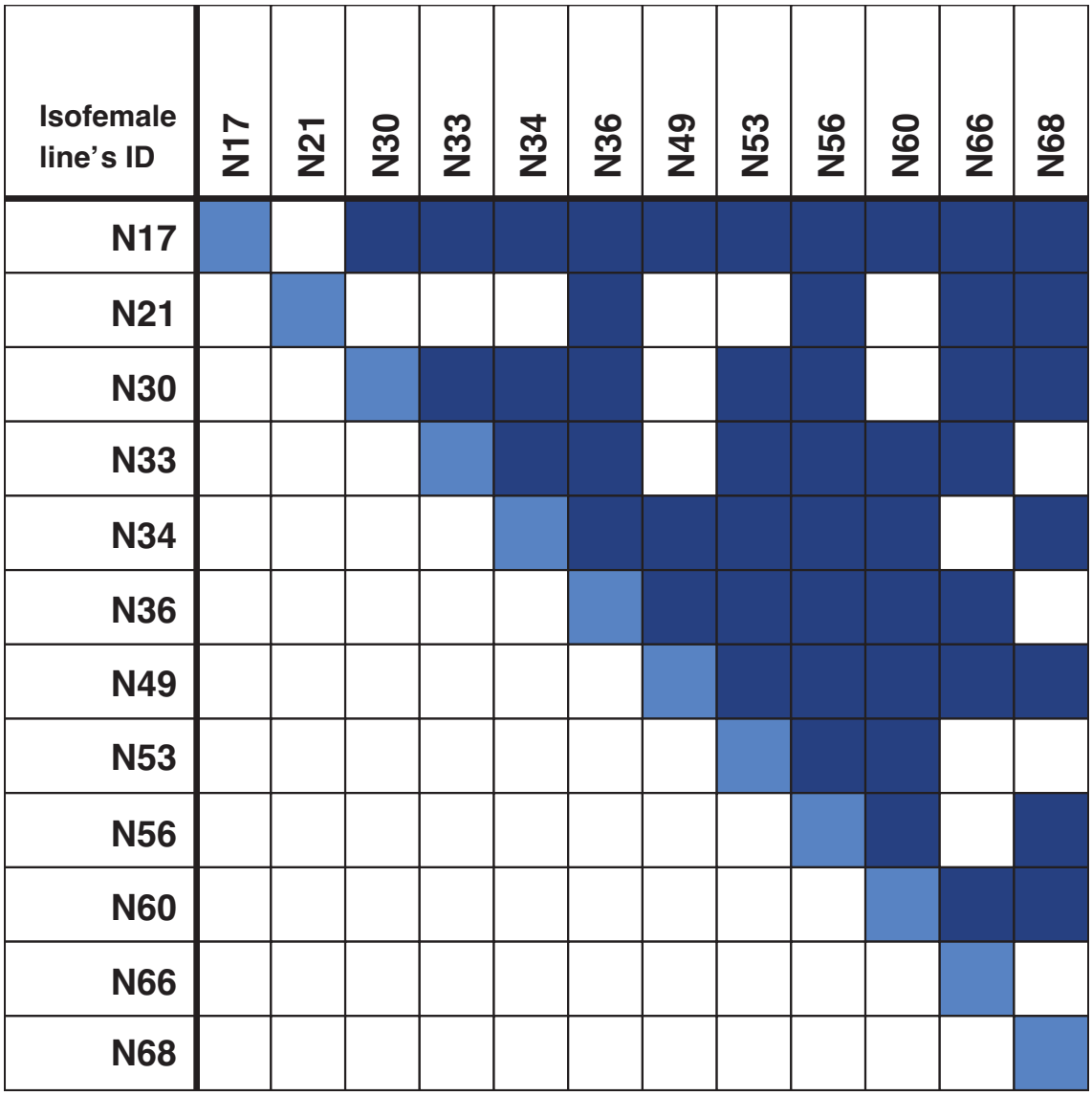

Figure 4-S1. Mixture designs of measuring diversity effects on population performance. The 12 isofemale line's ID of *Drosophila immigrans* are presented in rows and columns. The dark squares are the mixtures of the two isofemale lines, and the light squares on the diagonal represent the monocultures.

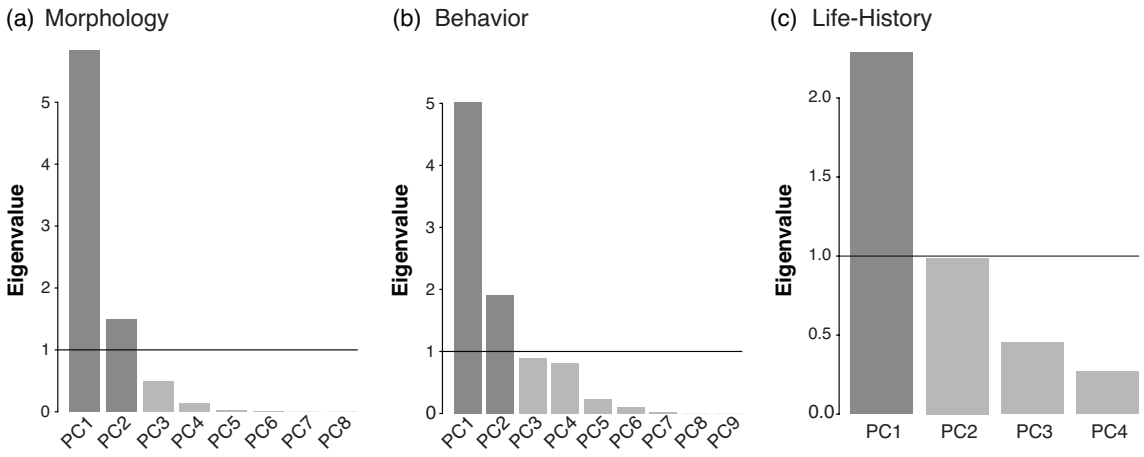

Figure 4-S2. Eigenvalues are represented separately for principal component analysis of morphological (a), behavioral (b), and life-history (c) traits. A horizontal line in each panel is a threshold where a principal component is used or rejected.

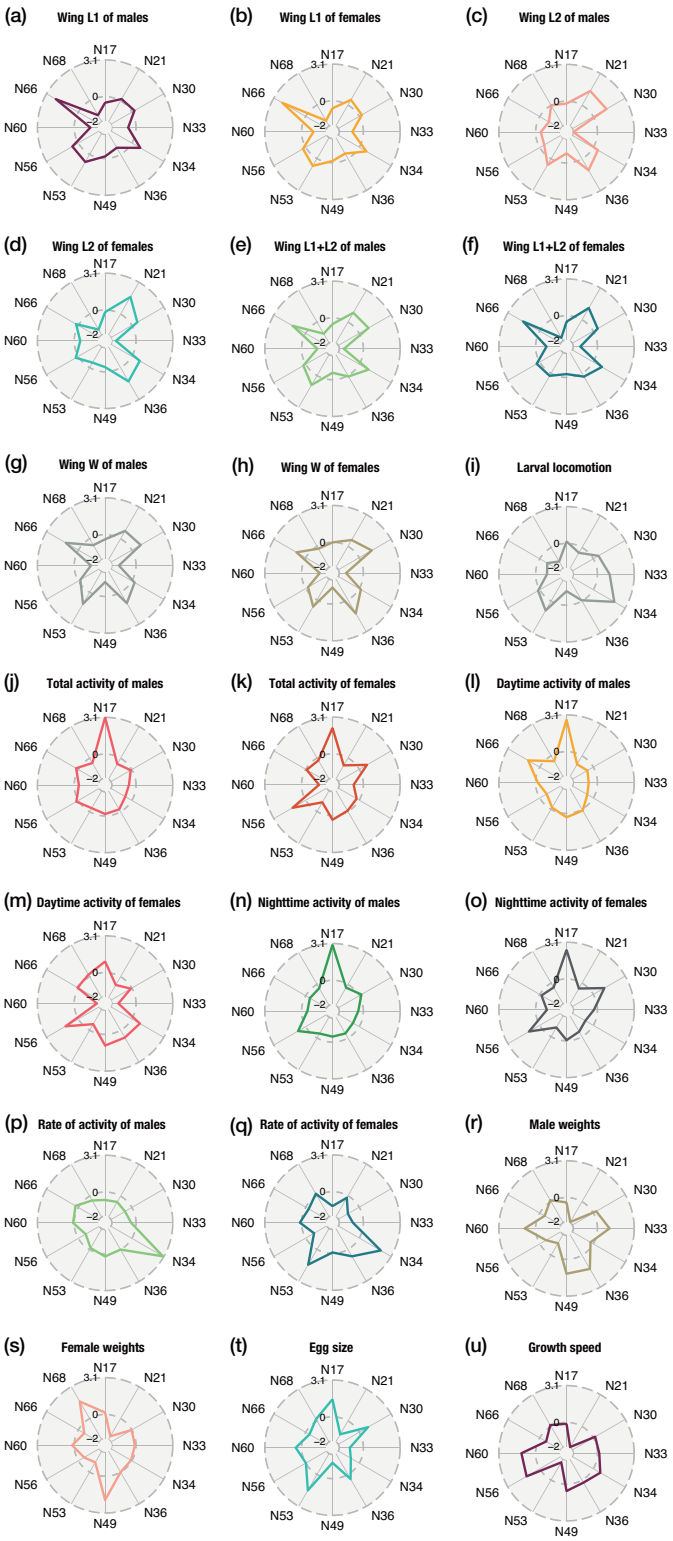

Figure 4-S3. Phenotypic diversity among 12 isofemale lines represented by standardized average values.

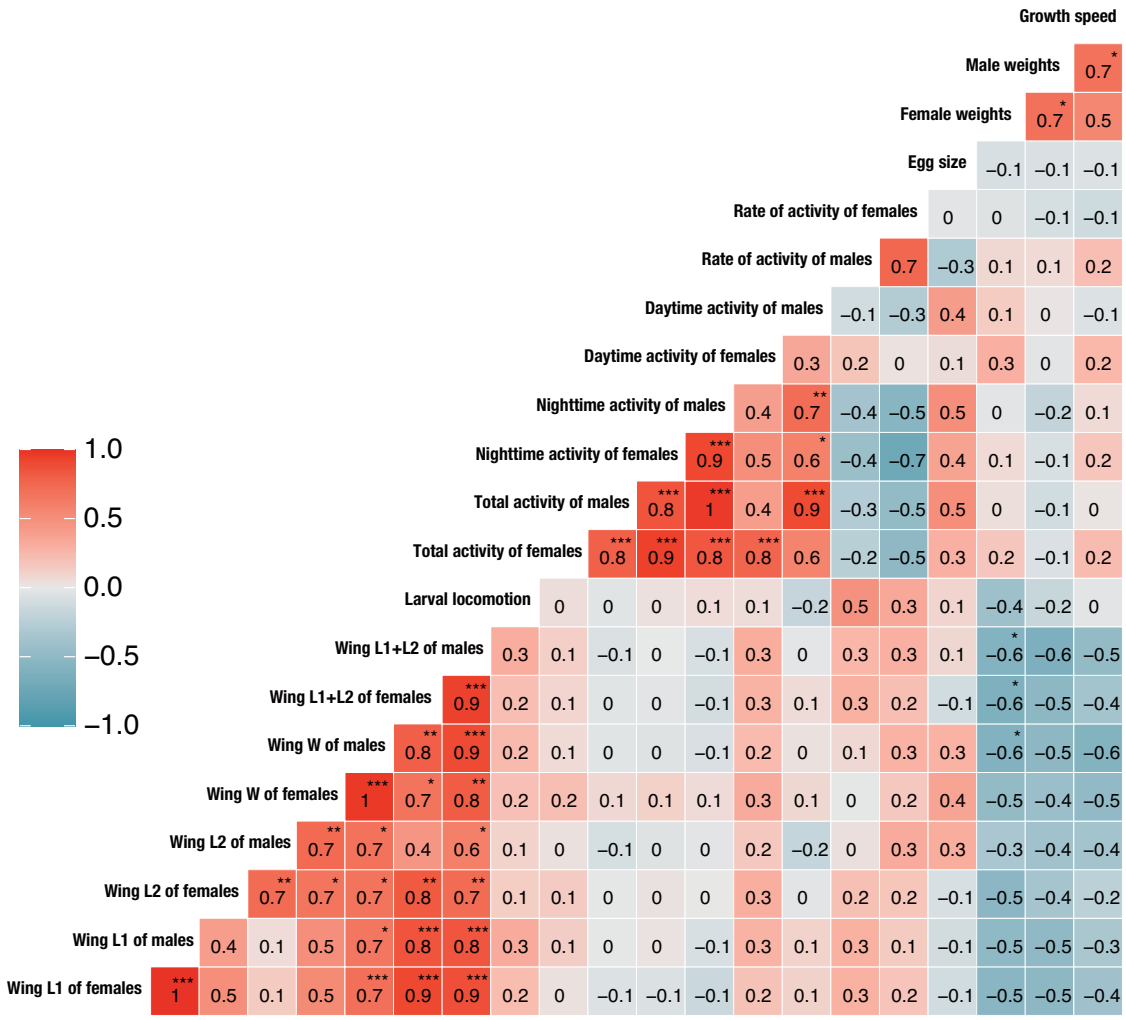

Figure 4-S4. Correlation between pairwise combinations of 21 standardized phenotypic values in 12 isofemale lines. The number and continuous color scale represent the Pearson correlation coefficient (r) in each cell. Asterisks indicate a significant correlation between the two traits by Pearson's correlation test (*$0.01 < p < 0.05$; **$0.001 < p < 0.01$; ***$p < 0.001$).

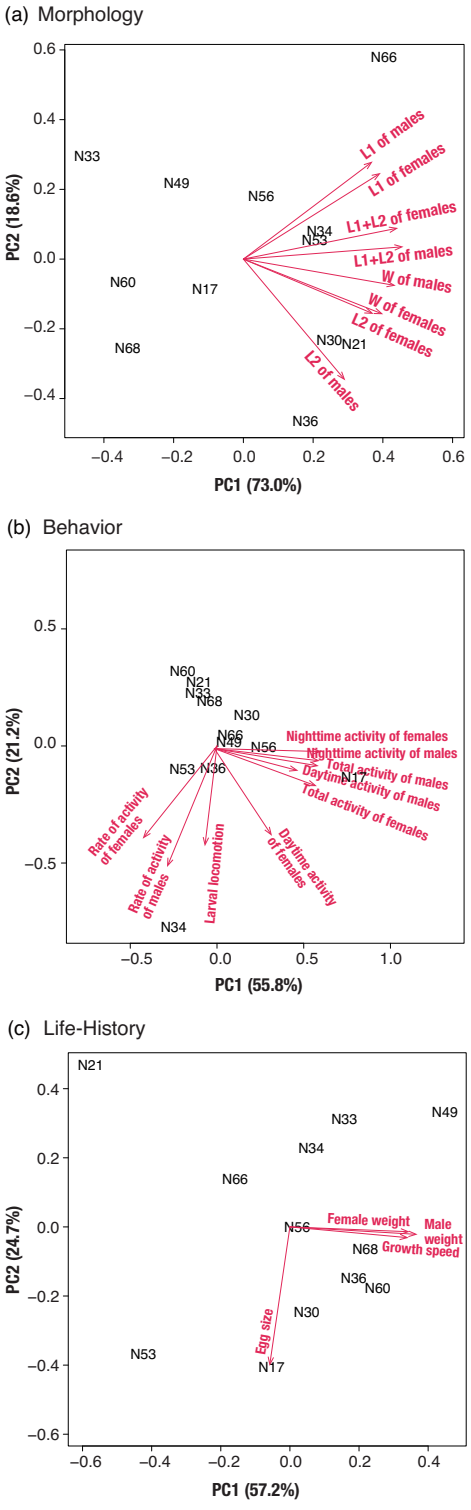

Figure 4-S5. The first and second principal components on morphological (a), behavioral (b), and life-history (c) traits. Each number represents the isofemale line's ID. Red arrows indicate factor loadings of each PCA for traits.

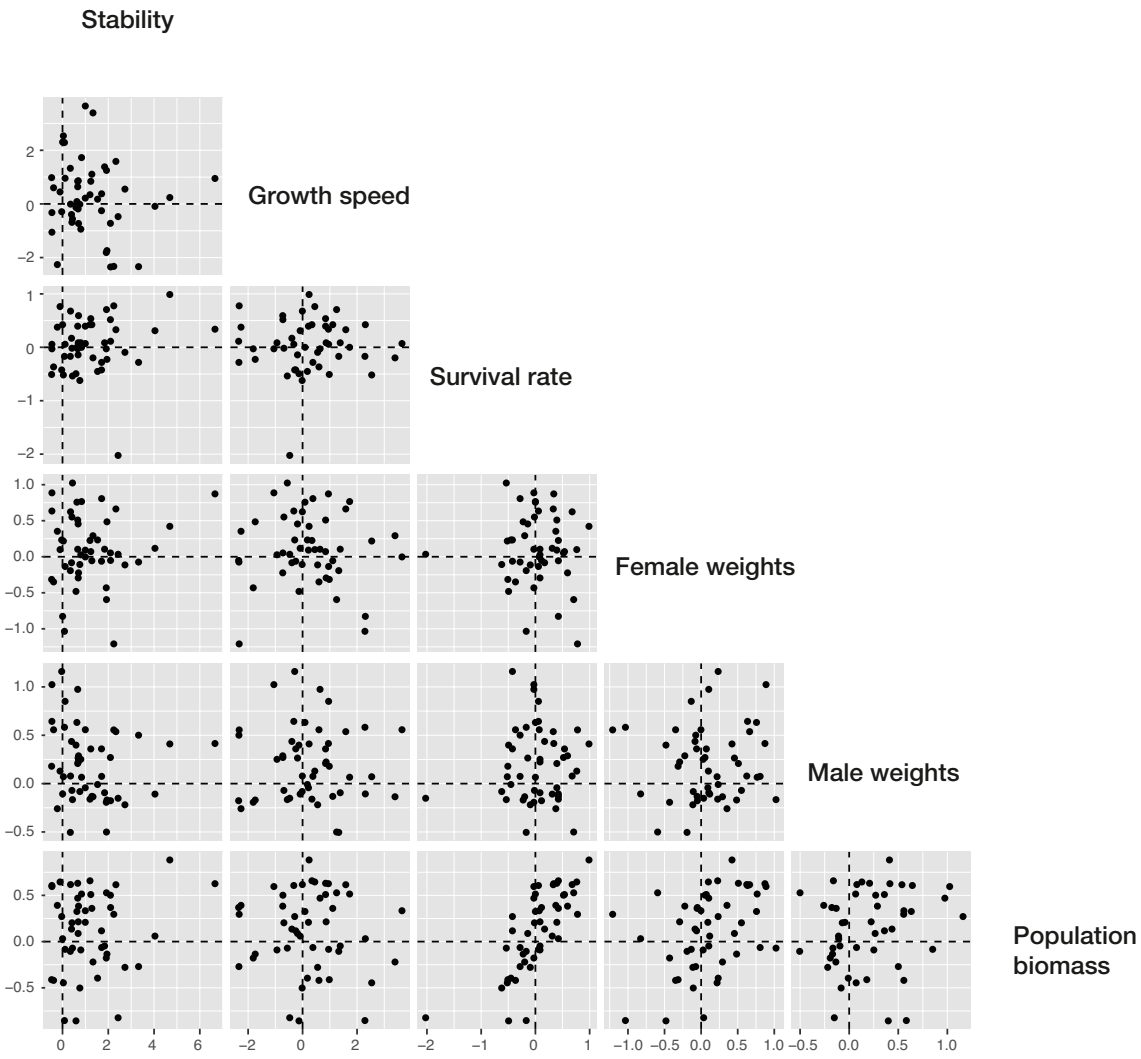

Figure 4-S6. Correlations between pairwise combinations of five population properties and stability. Each dot in all panels represents a combination of two isofemale lines. The horizontal and vertical dashed lines in each panel show the intercepts of the x-y coordinates.

Table 4-S1. Deviations of the population properties of the mixtures from the averaged properties of the two monocultures by a one-sample *t*-test. The difference in population properties between monocultures and mixtures, using the χ^2-test in a linear mixed model. Boldface rows are considered significant.

Population property	***t***	***p***	χ^2	***p***
Population biomass	**2.34**	**0.02**	**4.97**	**0.03**
Male weights	**3.85**	**<0.001**	**14.80**	**< 0.001**
Female weights	1.65	0.10	2.59	0.11
Survival rate	0.79	0.43	0.59	0.44
Growth speed	1.25	0.22	1.57	0.21
Stability	**6.10**	**<0.001**	**37.25**	**< 0.001**

The genomic basis of ecological emergent effects induced by genetic diversity in *Drosophila melanogaster*

Manuscript.

Abstract

Ecological dynamics are always complex and nonlinear. Ecological studies have demonstrated non-additively positive and negative relationships between biological diversity and ecological properties (e.g., productivity). For the relationships, trait differences are assumed to cause niche complementarity among species and conspecific individuals. However, it is currently unclear which "diversity of genes" is responsible to determine ecological dynamics. Here, I used inbred lines of *Drosophila melanogaster* to demonstrate the new attempt to investigate how genomic structures within populations are associated with ecological properties. I then identified genes whose diversity is responsible for the observed deviations between genetically identical and heterogeneous populations, well-known as diversity effects. My results showed multiple genes responsible for positive and negative diversity effects in high- and low-nutrient conditions. Surprisingly, the genes identified were not shared among the directions of correlations and the nutrient conditions, suggesting that different population processes and inter-individual interactions govern positive and negative diversity effects. Taken together, my study did find lots of candidate genes responsible for the ecological diversity effect, an emergent property, led by complex interactions among individuals with different traits. This study is the first genome-wide study to address the genomic basis of population dynamics which is a higher level of biological organization.

Keywords: genetic diversity, diversity effect, *Drosophila* Genetic Reference Panel, association study

Introduction

Ecological systems always exhibit complex and nonlinear dynamics. After the 1990s, studies in ecology have shown positive relationships between biological diversity and ecological dynamics and stability in various levels of ecological organizations, such as populations, communities, and ecosystems (Tilman et al., 1996; Reusch et al., 2005), while negative relationships have been reported (Creed et al., 2009; Bongers et al., 2020; Hagan, Vanschoenwinkel and Gamfeldt, 2021). Even in some systems, the direction of relationships is known to depend on environmental conditions and indices of ecological dynamics (Jousset et al., 2011; Ellers et al., 2011; Pennekamp et al., 2018; Takahashi et al., 2018). These pioneering studies typically investigated ecological properties, defined as any measurable component of each ecological organization, such as productivity, survival rate, and so on. The emergent effects on ecological properties and their stability driven by biological diversity have been well known as the "diversity effect" in ecology. Biological diversity is typically quantified as the richness (i.e., the number) of genotypes and species (reviewed by Wennersten and Forsman, 2012; Tilman, Isbell and Cowles, 2014). Trait differences, which often arise from the richness of genotypes and species, are suggested to facilitate niche differentiation (e.g., niche complementarity) among species or conspecific individuals and consequently determine the direction and strength of diversity effects. However, it is currently unclear which "diversity of traits/genes" is responsible for diversity effects. Several recent studies have expanded the traditional genomic approach, which associates genotypes with phenotypes among genetically diverse individuals, to examine the relationship between the genomic structure of populations and population biomass across genetically heterogeneous populations (Wuest and Niklaus, 2018; Turner et al., 2020; Wuest et al., 2022, 2023). The results of these studies showed different genes responsible for diversity effects on

productivity. This fact indicates that diversity effects on certain ecological properties (e.g., productivity) can be generated by the diversity of independent genes (so-called here as genetic heterogeneity).

Although some previous studies have successfully identified genes that can lead to diversity effects, these studies relied on a limited number of single nucleotide polymorphisms (SNPs) derived from only two genotypes (Wuest and Niklaus, 2018; Wuest et al., 2022, 2023). Moreover, the diversity of genes responsible for diversity effects is expected to vary among the kinds of ecological properties. In order to facilitate our understanding of the comprehensive mechanisms driving diversity effects, an experimental analysis is needed based on a wider range of variation by considering that diversity effects on an ecological property arise from various ecological processes.

In the present study, I used inbred lines of the *Drosophila* Genetic Reference Panel (DGRP; MacKay et al., 2012) to demonstrate another attempt to investigate how genomic structures within populations (i.e., genetic diversity) are associated with ecological properties in a population (i.e., population properties). *Drosophila melanogaster* is one of the best-established model organisms with a well-identified genome and sufficient tools for genetics. DGRP consists of a fully sequenced population of more than 200 genetically diverse inbred lines derived from Raleigh, USA. This is advantageous for collecting many natural genetic variations and their whole genome information. I experimentally reared both genetically identical and heterogeneous populations and measured several population properties potentially affecting population productivity under two nutrient conditions to infer the cause of the change in population productivity. Then, I calculated diversity effects by comparing the population properties between genetically identical and heterogeneous populations. Finally, I identified genes whose diversity is responsible for the observed deviations between

genetically identical and heterogeneous populations (i.e., diversity effects).

Material and methods

Fly strain

I randomly selected 48 lines of *D. melanogaster* in the DGRP obtained from the Bloomington Stock Center (DGRP_208, DGRP_287, DGRP_309, DGRP_324, DGRP_335, DGRP_357, DGRP_358, DGRP_360, DGRP_362, DGRP_365, DGRP_370, DGRP_375, DGRP_382, DGRP_399, DGRP_437, DGRP_441, DGRP_443, DGRP_461, DGRP_517, DGRP_535, DGRP_555, DGRP_703, DGRP_707, DGRP_712, DGRP_716, DGRP_774, DGRP_799, DGRP_801, DGRP_808, DGRP_820, DGRP_832, DGRP_837, DGRP_843, DGRP_849, DGRP_852, DGRP_855, DGRP_857, DGRP_859, DGRP_879, DGRP_882, DGRP_884, DGRP_890, DGRP_892, DGRP_897, DGRP_900, DGRP_907, DGRP_911, DGRP_913). The files of each line were kept in plastic vials (100 mm in height, φ27 mm; KFB-1M, Chiyoda Science) with standard fly medium (Bloomington Formulation, Nutri-Fly, cat. no. 66-113, Genesee Scientific) under a 12L:12D cycles at 23°C. To prevent the growth of bacteria and mold, I added 4 mL of propionic acid to the 1 L medium. Before my experiments, all lines were reared for at least three generations under the conditions described above to remove environmental and maternal effects.

Population properties in rearing experiments

To assess population properties in genetically identical (monocultures) and heterogeneous populations (mixtures), I reared fruit flies from eggs to adults (i.e., within a single generation) under experimental environmental conditions (12L:12D cycle, 23°C, and illumination from white LEDs) and two nutrient levels. The higher and lower nutrient media were formulated

with 50% and 85% reduction of the sucrose and dry yeast content relative to the method in Fitzpatrick et al. (2007). In my rearing experiments, I did not have to consider any effects derived from sexual reproduction, such as heterosis, because I measured population properties within a single generation.

Approximately 80 adult males and females were placed into bottles containing the Petri dishes (φ90 mm) filled with grape agar (50 ml grape juice, 50 ml water, 2 g agar, 2 ml ethanol, and 2 ml acetic acid) to collect fertilized eggs from them. Then, 32 randomly chosen eggs were transferred onto 10 ml media in an experimental vial (100 mm in height, φ27 mm). The rearing experiment was designed as a half-diallel containing incomplete pairwise combinations of mixtures and monocultures of 48 DGRP lines, which were divided into four blocks consisting of 12 lines (Fig. 5-S1). Each mixture consisted of the same number of individuals derived from each inbred line (i.e., 16 individuals per inbred line). I thus established 48 monocultures and 144 different mixtures in five replicates.

Population properties in monocultures and mixtures were evaluated by five indices: the dry weights of an individual male and female, the day to reach the 50% pupation rate (an indicator of growth speed), survival rate, and population biomass at adult emergence. In the rearing experiment, I scored the number of pupae, adult males, and adult females every two days during the rearing and measured the dry weights of an individual male and female after the end. Then, by fitting logistic curves to all growth data of each line, I identified the day to reach the 50% pupation rate. Survival rate was calculated by the proportion of the survived adults. Population biomass (B_p) was estimated by the following equation:

$$B_p = (S \times R_M \times W_M) + (S \times R_F \times W_F),$$

where S, R_M, R_F, W_M, and W_F are the number of survived adults, the adult male ratio, the adult female ratio, the dry weights of a male, and the dry weights of a female, respectively. The

stability of a population property in monocultures and mixtures was assessed with the inverse of the coefficient of variation (CV) of population biomass.

Nucleotide diversity and diversity effects

To measure the genomic diversity of mixtures, I used published single nucleotide polymorphisms (SNP) from resequencing data (Huang et al., 2014). The SNP data were downloaded from the DGRP2 website (http://dgrp2.gnets.ncsu.edu). The pairwise nucleotide diversity (π) between two DGRP lines that constituted a mixture was calculated along all four chromosomes using overlapping sliding windows methodology (1,000 bp window with 500 bp overlapping). An available Perl script for this calculation was produced by Wang et al. (2016) (https://github.com/wl13/BioScripts/blob/master/calc_vcf_diversity.pl). The extent of genetic diversity in 144 mixtures out of all combinations was analyzed using principal component analysis (PCA) on the pairwise π data to confirm that mixtures were not biased in genetic diversity.

The non-additive changes in a population property and stability of mixtures compared to monocultures were denoted as diversity effects in the present study. Diversity effects (*D*) were calculated by the following equation:

$$D = \log_{10} \frac{M_{A+B}}{\frac{M_A + M_B}{2}},$$

where *M* is a measurement of focal population property and stability, and subscripts of *A* and *B* represent lines A and B mixed. I calculated *D* in each of the 144 combinations.

Ecological genome-wide association study (eGWAS)

Ecological genome-wide association analyses (eGWAS) were conducted separately for the

results of nutrients (high or low) and signs of coefficients (plus or minus), using multiple linear regressions. I used the diversity effects (*D*) on population biomass as a response variable because population biomass is assumed to be one of the most important final outputs of a population process. Because outliers of mixtures (i.e., population structure) can inflate estimates, PC1 and PC2 calculated by PCA on the pairwise π data in 144 mixtures were used as the covariates. The model was written as follows:

$$D_{population\ biomass} \sim \pi_i + PC1 + PC2,$$

where π_i is a variable of nucleotide diversity at a 1000 bp window *i*. The *p*-value of the independent variable π_i was calculated from the *F* statistics.

Gene ontology (GO)

GO analysis was performed on the genes associated with the genomic windows that met the significance threshold of $p = 10^{-5}$ separately for the results of nutrients (high or low) and signs of coefficients (plus or minus). The data for gene annotation, including gene names, FlyBase gene IDs, and site classes (e.g., exon and intron), was downloaded from the DGRP2 website. GO analysis was carried out using *enrichGO* function of the *clusterProfiler* package in R. Only the "biological process" enrich terms were used. GO terms with $p = 0.05$ by the Benjamini-Hochberg method were considered significant.

Statistical analyses

All statistical analyses were performed in R ver. 4.2.2 (R Core Team, 2022). The averages of dry weights of an individual male and female, survival rate, and population biomass in each monoculture and mixture were obtained by estimated marginal means for linear mixed models with assay month as a random factor in the *lme4* and *emmeans* packages. The correlations of

diversity effects between population properties and stability were analyzed using Pearson's correlation test. The effects of nutrients and diversity (monoculture or mixture) on each population property were analyzed using the linear model in the *lme4* package. Note that the effect of sex was also included in the model of the dry weights of an individual. The *p*-value of the independent variable (monoculture or mixture) in the linear model was calculated from the *F*-tests in the *Anova* function of the *car* package. Moreover, a one-sample *t*-test was conducted to determine whether the diversity effects in each population property and stability under each nutrient were significantly different from zero.

Results

Nutrient conditions significantly affected each population property, except for stability (Fig. 5-1). Genetically heterogeneous populations showed slightly higher growth speed and lower stability (Fig. 5-1d, e). Female weights were significantly higher than male weights under both nutrient conditions (Fig. 5-1b). The differences in diversity effect between high and low nutrients were significant for growth speed ($F_{1, 286} = 8.05$, $p < 0.01$) and marginally significant for male and female weights (male weights: $F_{1, 286} = 2.93$, $p = 0.09$; female weights: $F_{1, 286} = 2.90$, $p = 0.09$), but not for others (population biomass: $F_{1, 286} = 1.02$, $p = 0.31$; survival rate: $F_{1, 286} = 1.98$, $p = 0.16$; stability: $F_{1, 286} = 2.46$, $p = 0.12$,; Fig. 5-2; Fig. 5-S2). Mean diversity effects on female weights and growth speed under the high nutrient were positive and significantly different from zero (see Table 5-S1 in detail). Note that, indices of population properties and stability were not independent of each other (Fig. 5-3). Basically, positive correlations among combinations of diversity effects on population properties and stability were found under both nutrient conditions. The correlations between survival rate and male, female weights were positive only under the high-nutrient condition.

Genetic diversity in 144 used mixtures, calculated by nucleotide diversity (π) at the whole genome between two DGRP lines, was varied (Fig. 5-S3) and not biased compared to all the possible combinations (Fig. 5-4). The results of eGWAS were summarized in Fig. 5-5 (see also Fig. 5-S4). I found some significant genomic windows, which were responsible for increasing or decreasing diversity effects under high- and low-nutrient conditions. The significant windows contained various genes responsible for increasing and decreasing diversity effects under each nutrient condition (Table 5-1, see also Table 5-S2). A part of SNPs in the identified genes contains nonsynonymous SNPs (Table 5-S3). Under the high nutrient, the ratio of the significant windows in chromosome 2 was higher in the positive correlation, whereas the ratio of the significant windows in chromosome 3 was higher in the negative correlation. On the other hand, this relationship was reversal under the low nutrient. I also found few genes sharing among the directions of correlation and nutrients (Fig. 5-6). GO terms in the top 5 were different among the directions of correlation and nutrients, although no GO term was identified in the positive correlations and high-nutrient conditions (Fig. 5-7). A GO term related to feeding behavior was included in the negative correlation and the high nutrient conditions. On the other hand, under the low nutrient condition, GO terms related to the movements of a cell or organism, such as “locomotion” and “taxis,” were seen in a positive correlation, while GO terms related to the size or mass of a cell or organism were included in a negative correlation (see also Table 5-S4).

Discussion

I here compared five population properties and stability of 48 monocultures with those of 144 mixtures. I found that diversity effects in a part of the mixtures were positive. On average, mixtures did not enhance population biomass and stability under both nutrient conditions,

while the mean diversity effects on female weights and growth speed were significantly positive only under the high nutrient condition. These findings suggest that diversity effects on population properties were positive on average when there were a lot of combinations, which observed large functional differences. For population properties and stability on which the mean diversity effects were zero, the richness of genotypes may not be enough to lead to positive diversity effects on average because many previous studies that showed population properties were improved with increasing genotype richness (Wennersten and Forsman, 2012). Notably, my results showed a clear relationship between genetic diversity and female weights or growth speed within a population. Previous research on diversity effects generally focused on population biomass as an important output of population productivity. However, my results indicate the importance of other population properties in understanding the mechanisms underlying diversity effects. Moreover, diversity effects on population properties and stability were found to correlate with each other. The assessment of multiple population properties may be needed to reveal the nonlinear population process because few studies verified the correlations among population properties. Also, considering environmental conditions is also important to detect diversity effects because my results showed that the detection of diversity effects depended on the nutrient conditions.

Identifying the genes whose diversity is important to determine ecological properties in ecological organizations may be useful to understand the complex and nonlinear dynamics in ecology (Barbour et al., 2022; Wuest et al., 2023). My results show multiple genes responsible for positive and negative diversity effects in each nutrient condition. I also found that the functions of genes (GO terms) varied among directions of diversity effects and nutrient conditions. This finding suggests that the diversity of these genes led to diversity effects via niche differentiation and reduced resource competition. Surprisingly, the genes

identified were not shared among the directions of correlations and the nutrient conditions. This result indicates that different population processes and inter-individual interactions govern positive and negative diversity effects. My experimental approach did not focus on a specific diversity of a gene and a specific population process as in the cases of previous studies(Caesar et al., 2010; Takahashi et al., 2014, 2018). The results in the present study included the various diversity of the independent genes and many population processes. However, I could at least distinguish genomic regions responsible for diversity effects from whole genomes and also divide such genomic regions into those contributing to two directions (positive or negative) of the diversity effects. Thus, my approach might help us explore new genes responsible for diversity effects, which could not be conducted in a candidate gene approach.

Note that, importantly, the outcomes of my current study, as in the cases of other genome-wide association studies, include a lot of false positive genes. Further research requires to narrow down the list of candidate genes and find true positive genes responsible for diversity effects. One of the most effective techniques is a genome editing approach, which can generate modifications of an individual gene. Nonsynonymous SNPs identified in the present study may be useful in deciding genes object to genome editing. If the diversity of a single gene modified by genome editing causes a diversity effect, I can decide that the variation in the genes is important to determine ecological dynamics.

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Tables and Figures

Table 5-1. Summary of the number of genes identified in each condition.

	Positive correlation	Negative correlation
High nutrient	28	75
Low nutrient	98	220

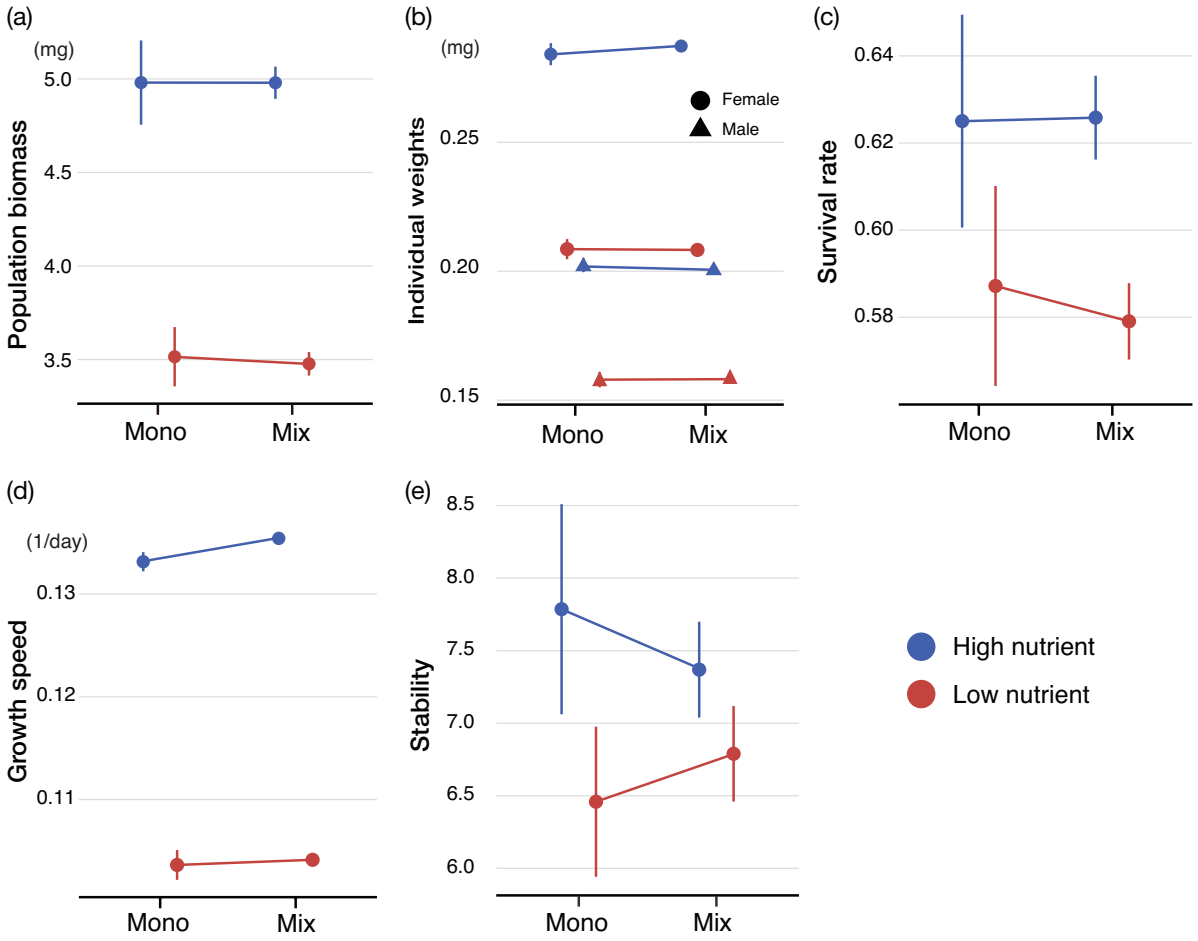

Figure 5-1. Population properties and stability on population type (Mono = inbred line monoculture; Mix = mixture of inbred lines), showing (a) population biomass calculated by the dry weights of individuals (nutrient: $F_{1, 380} = 200.3$, $p < 0.001$; diversity: $F_{1, 380} = 0.03$, $p = 0.87$; interaction: $F_{1, 380} = 0.02$, $p = 0.88$), (b) the dry weights of an individual (nutrient: $F_{1, 760} = 1717.7$, $p < 0.001$; diversity: $F_{1, 760} = 0.21$, $p = 0.65$; sex: $F_{1, 760} = 2128.0$, $p < 0.001$), (c) survival rate of adults (nutrient: $F_{1, 380} = 12.0$, $p < 0.001$; diversity: $F_{1, 380} = 0.05$, $p = 0.81$; interaction: $F_{1, 380} = 0.09$, $p = 0.76$), (d) the growth speed of larvae (nutrient: $F_{1, 380} = 1994.9$, $p < 0.001$; diversity: $F_{1, 380} = 3.03$, $p = 0.08$; interaction: $F_{1, 380} = 1.23$, $p = 0.27$), and (e) the inverse of the coefficient of variation (CV) of population biomass (nutrient: $F_{1, 380} = 3.42$, $p = 0.07$; diversity: $F_{1, 380} = 0.008$, $p = 0.93$; interaction: $F_{1, 380} = 0.61$, $p = 0.44$). Plots and error bars represent average population properties and standard error of the mean, respectively.

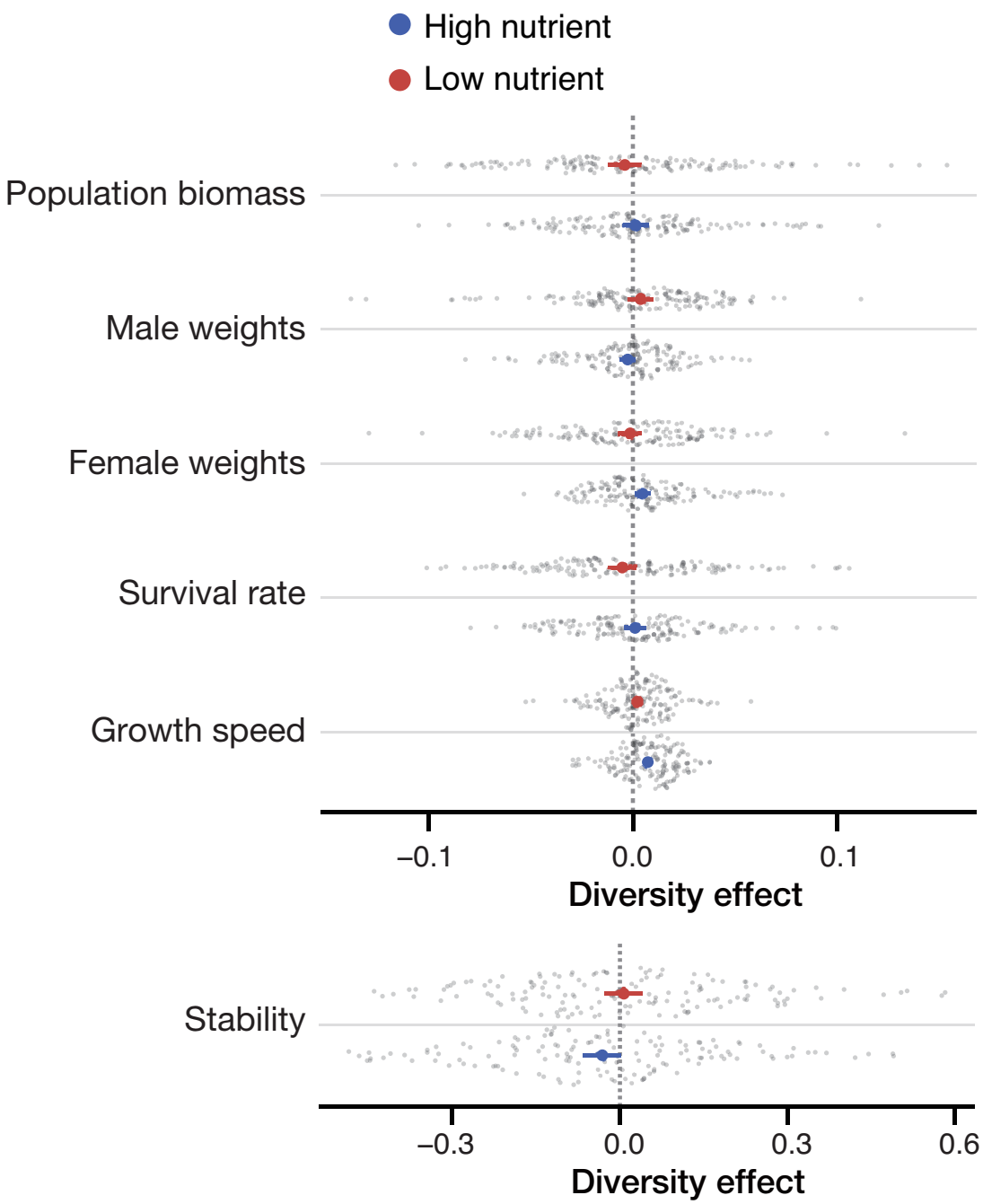

Figure 5-2. Diversity effects on each population property and stability with all the raw data. Red and blue plots and error bars represent average population properties and confidence intervals (95%), respectively. Black plots indicate 144 mixtures consisting of two DGRP lines.

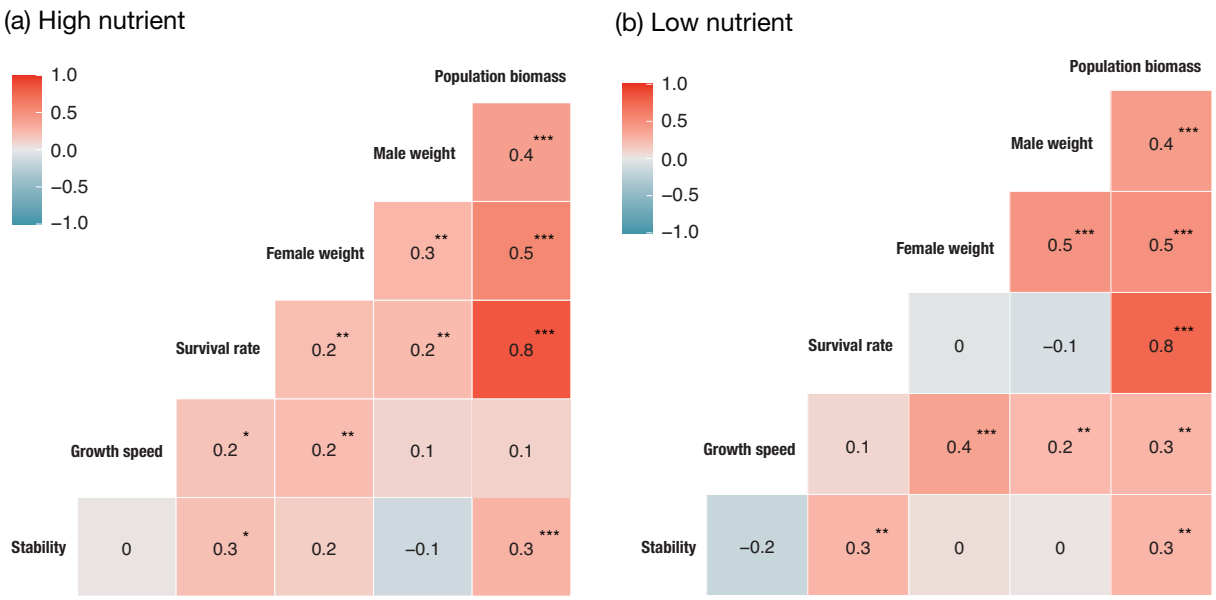

Figure 5-3. Correlation between pairwise combinations of diversity effects on population properties and stability in high-nutrient (a) and low-nutrient (b) conditions. The number and continuous color scale represent the Pearson correlation coefficient (r) in each cell. Asterisks indicate a significant correlation between the two traits (*$0.01 < p < 0.05$; **$0.001 < p < 0.01$; ***$p < 0.001$).

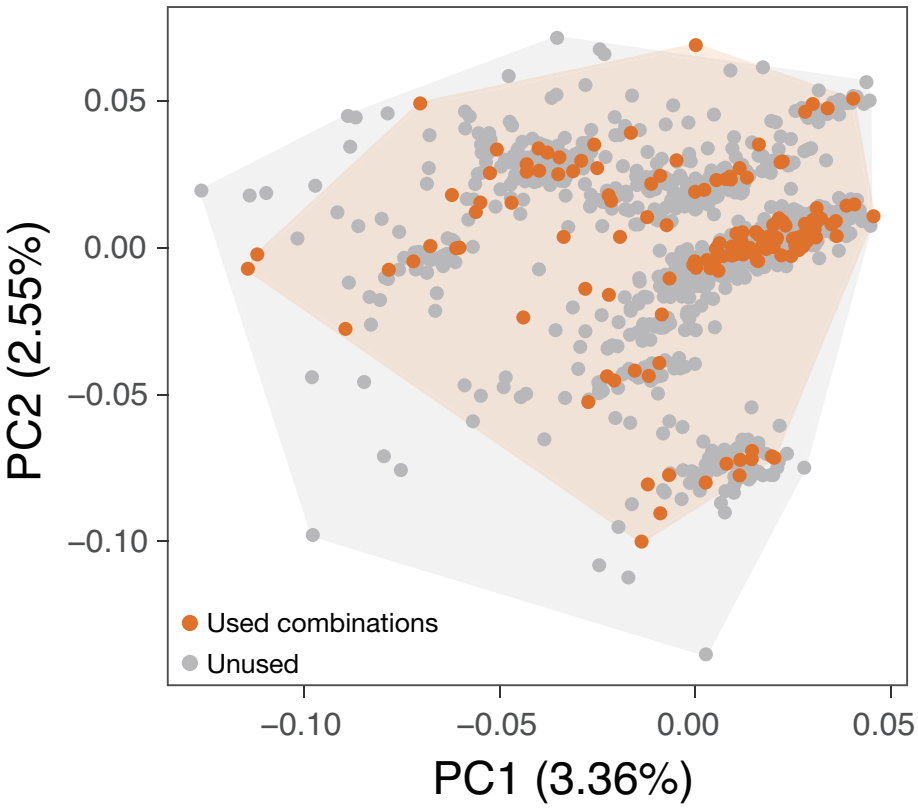

Figure 5-4. Nucleotide diversity in used and unused combinations. Plots represent combinations (144 used combinations and 948 unused combinations). Convex polygons in two categories are shown in the two-dimensional space.

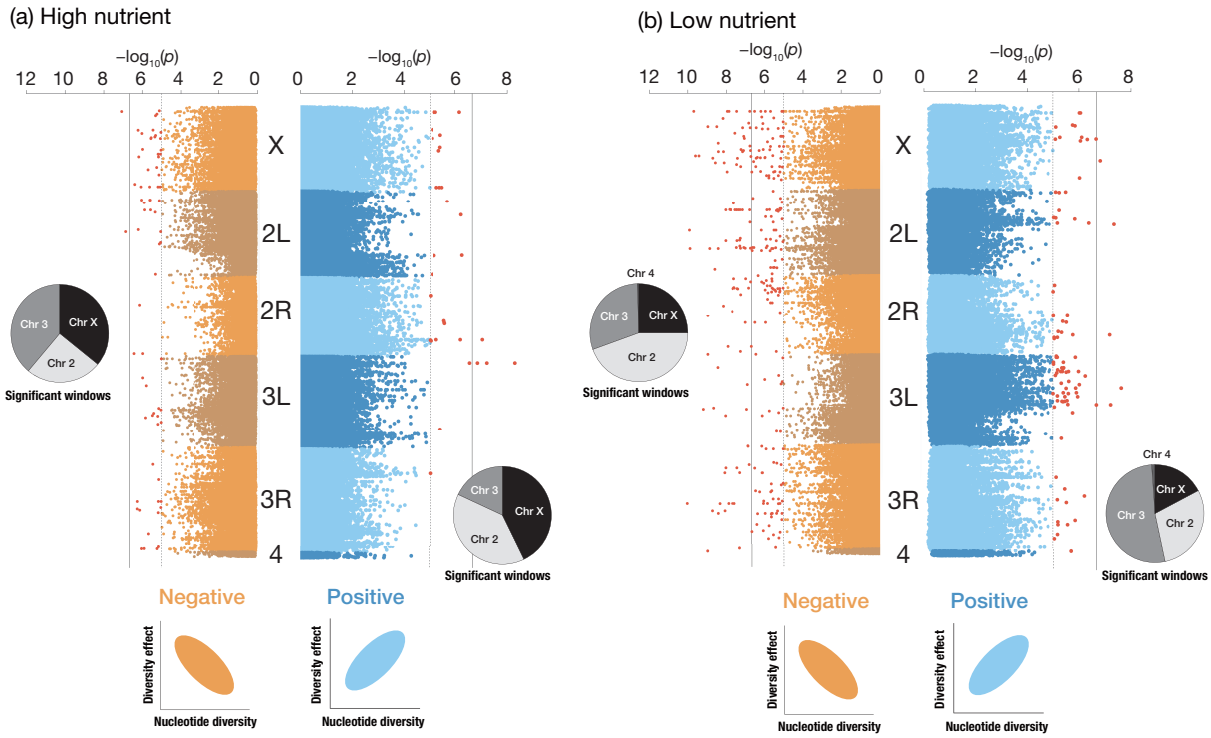

Figure 5-5. Ecological genome-wide association mappings. Multiple linear models analyzed associations between nucleotide diversity (π) in mixtures and diversity effects on population biomass in the high-nutrient (a) and low-nutrient (b) conditions. 230,431 windows were used across the whole genome. The blue and orange manhattan plots represent positive associations and negative associations, respectively. Black dotted lines and grey dotted lines in each plot mark criteria from Bonferroni corrections and the p-value $< 10^{-5}$, respectively. Windows significant with $p < 10^{-5}$ are shown in red. Pie chart diagrams in plots show the ratio of chromosomes in which the significant windows are present.

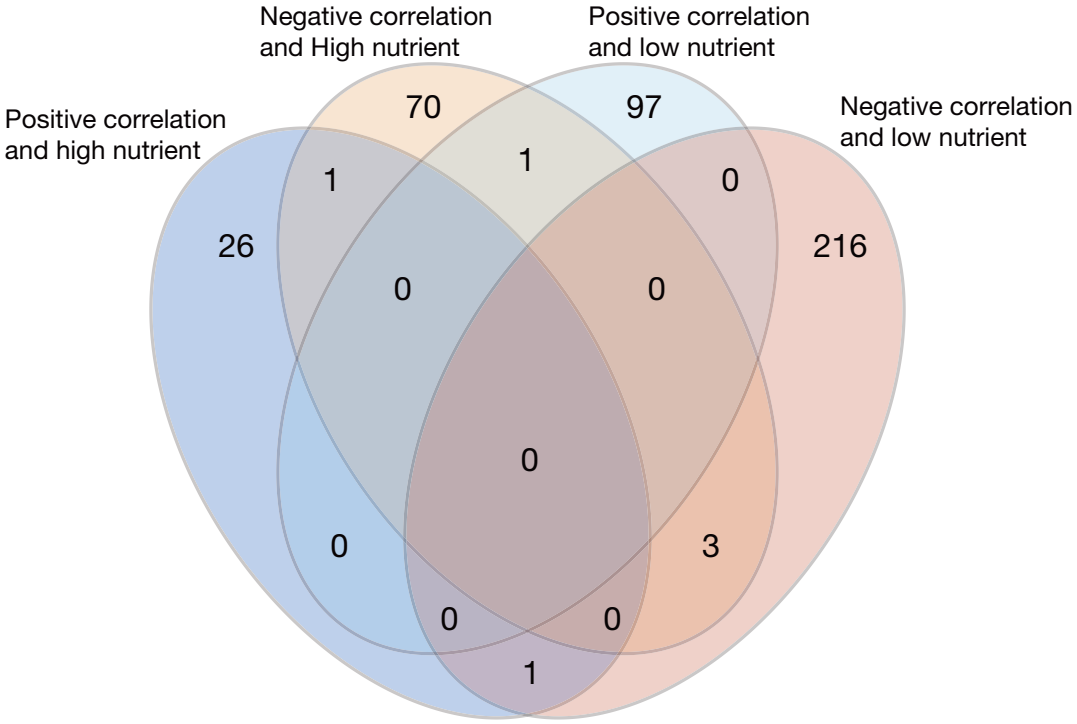

Figure 5-6. Venn diagrams detailing the number of genes identified in each direction of correlation and nutrient condition.

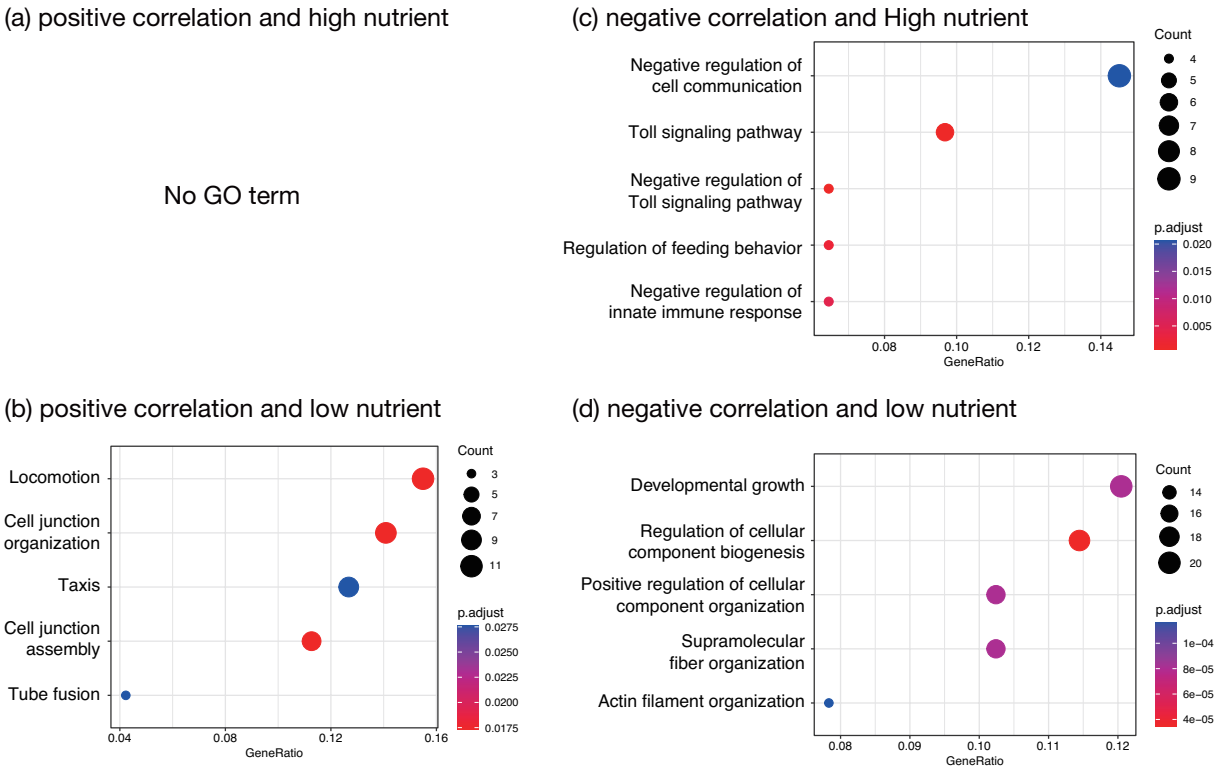

Figure 5-7. Top 5 of the significant GO terms in each direction of correlations and each nutrient condition. Gene ratio is referred to the ratio of the number of genes with the focused GO term to the total number of the identified genes with a GO term. The size of the circles represents the count of the genes with the focused GO term. The color of the circles was based on the adjusted *p*-value.

Supplemental material

Table 5-S1. Summary of one-sample t-test.

	Nutrient	***t***	***df***	***p***
Population biomass	High	0.42	143	0.68
Population biomass	Low	−0.96	143	0.34
Male weights	High	−1.29	143	0.20
Male weights	Low	−1.21	143	0.23
Female weights	High	2.39	143	0.02
Female weights	Low	−0.45	143	0.66
Survival rate	High	0.39	143	0.69
Survival rate	Low	−1.47	143	0.15
Growth speed	High	6.06	143	<0.001
Growth speed	Low	1.59	143	0.11
Stability	High	−1.82	143	0.07
Stability	Low	0.40	143	0.69

Table 5-S2. Genes identified by eGWAS.

	Chromosome	FlyBase gene ID	Gene name
Positive correlation and high nutrient			
	2L	FBgn0264944	CR44113
	2L	FBgn0085409	CG34380
	2L	FBgn0265543	CR44393
	2L	FBgn0032625	CG15136
	2R	FBgn0050089	CG30089
	2R	FBgn0261612	CngA
	2R	FBgn0265876	CR44665
	2R	FBgn0015524	otp
	2R	FBgn0262690	CR43160
	2R	FBgn0003748	Treh
	2R	FBgn0034585	Rbpn-5
	3L	FBgn0035260	CG7991
	3L	FBgn0036860	CG14086
	3R	FBgn0037834	Art1
	3R	FBgn0260944	Rbp1
	3R	FBgn0017577	Mcm5
	X	FBgn0024985	CG11448
	X	FBgn0030316	CG11695
	X	FBgn0030314	CG11696
	X	FBgn0031171	CG1801
	X	FBgn0064116	CG33713
	X	FBgn0064117	CG33714
	X	FBgn0265693	CR44500
	X	FBgn0029067	Dd
	X	FBgn0000618	e_y_2
	X	FBgn0015519	nAChRalpha3
	X	FBgn0020369	Rpt6
	X	FBgn0052677	X11Lbeta
Positive correlation and low nutrient			
	2L	FBgn0031952	cdc14
	2L	FBgn0085427	CG34398

2L	FBgn0031912	CG5261
2L	FBgn0031913	CG5958
2L	FBgn0031268	cold
2L	FBgn0266323	CR44988
2L	FBgn0266825	CR45287
2L	FBgn0000497	ds
2L	FBgn0023489	Pph13
2L	FBgn0013323	Ptth
2L	FBgn0264895	RapGAP1
2L	FBgn0041092	tai
2R	FBgn0004168	5-HT1A
2R	FBgn0040505	Alk
2R	FBgn0034570	CG10543
2R	FBgn0034122	CG15711
2R	FBgn0050291	CG30291
2R	FBgn0050479	CG30479
2R	FBgn0050480	CG30480
2R	FBgn0033095	CG3409
2R	FBgn0266525	CG45092
2R	FBgn0034145	CG5065
2R	FBgn0034121	CG6262
2R	FBgn0034479	CG8654
2R	FBgn0263586	CR43611
2R	FBgn0033292	Cyp4ad1
2R	FBgn0001133	grau
2R	FBgn0020269	mspo
2R	FBgn0264753	Rgk1
3L	FBgn0260941	app
3L	FBgn0262870	axo
3L	FBgn0023095	caps
3L	FBgn0036290	CG10638
3L	FBgn0036289	CG10657
3L	FBgn0035555	CG13720
3L	FBgn0036146	CG14141
3L	FBgn0035581	CG17150

3L	FBgn0052103	CG32103
3L	FBgn0035802	CG33275
3L	FBgn0085295	CG34266
3L	FBgn0035948	CG5644
3L	FBgn0036145	CG7607
3L	FBgn0264460	CR43868
3L	FBgn0265415	CR44327
3L	FBgn0267309	CR45745
3L	FBgn0040823	dpr6
3L	FBgn0005640	Eip63E
3L	FBgn0016013	Faa
3L	FBgn0053556	form3
3L	FBgn0036144	GlcAT-P
3L	FBgn0035610	Lkr
3L	FBgn0266757	mfr
3L	FBgn0011817	nmo
3L	FBgn0261526	NT1
3L	FBgn0263232	Nxf3
3L	FBgn0036147	Plod
3L	FBgn0085447	sif
3L	FBgn0086906	sls
3L	FBgn0036282	Smyd4
3L	FBgn0010905	Spn
3L	FBgn0035947	Srp68
3L	FBgn0036389	ssp2
3L	FBgn0024179	wit
3R	FBgn0038499	Brf
3R	FBgn0039538	CG12883
3R	FBgn0085319	CG34290
3R	FBgn0038679	CG6040
3R	FBgn0039178	CG6356
3R	FBgn0038610	CG7675
3R	FBgn0262825	CR43196
3R	FBgn0266251	CR44946
3R	FBgn0042206	GstD10

	3R	FBgn0038020	GstD9
	3R	FBgn0262871	lute
	3R	FBgn0016754	sba
	3R	FBgn0003330	Sce
	3R	FBgn0038018	Tim17a1
	3R	FBgn0266719	Unc-13-4B
	3R	FBgn0039269	veli
	3R	FBgn0024273	WASp
	4	FBgn0085432	pan
	X	FBgn0015010	Ag5r
	X	FBgn0020508	Ag5r2
	X	FBgn0029771	CG12730
	X	FBgn0029814	CG15765
	X	FBgn0267253	CG32700
	X	FBgn0259734	CG42388
	X	FBgn0262945	CR43264
	X	FBgn0264384	CR43836
	X	FBgn0000382	csw
	X	FBgn0011761	dhd
	X	FBgn0030090	fend
	X	FBgn0086778	nAChRalpha7
	X	FBgn0264975	Nrg
	X	FBgn0261549	rdgA
	X	FBgn0003449	snf
	X	FBgn0052761	tRNA:CR32761:Psi
	X	FBgn0029752	TrxT
Negative correlation and high nutrient			
	2L	FBgn0000097	aop
	2L	FBgn0028931	CG16863
	2L	FBgn0051646	CG31646
	2L	FBgn0032222	CG5037
	2L	FBgn0032221	CG5375
	2L	FBgn0031413	CG9967
	2L	FBgn0264368	CR43820
	2L	FBgn0266829	CR45291

2L	FBgn0016930	Dyrk2
2L	FBgn0031414	eys
2L	FBgn0262256	mir-959
2L	FBgn0262321	mir-960
2L	FBgn0262239	mir-961
2L	FBgn0262200	mir-962
2L	FBgn0028572	qtc
2L	FBgn0028538	Sec71
2L	FBgn0015600	toc
2R	FBgn0035049	Mmp1
2R	FBgn0033636	tou
3L	FBgn0087007	bbg
3L	FBgn0005592	btl
3L	FBgn0036549	CG10516
3L	FBgn0052163	CG32163
3L	FBgn0052191	CG32191
3L	FBgn0052409	CG32409
3L	FBgn0250814	CG4169
3L	FBgn0036576	CG5151
3L	FBgn0036756	cln3
3L	FBgn0263599	l_3_72Ab
3L	FBgn0002283	l_3_73Ah
3L	FBgn0011817	nmo
3L	FBgn0013563	Pex1
3L	FBgn0052412	QC
3L	FBgn0036643	Syx8
3R	FBgn0038084	beat-Vc
3R	FBgn0038629	CG14304
3R	FBgn0051191	CG31191
3R	FBgn0038811	CG4159
3R	FBgn0265163	CR44232
3R	FBgn0265164	CR44233
3R	FBgn0001180	hb
3R	FBgn0013984	InR
3R	FBgn0083975	Nlg4

	3R	FBgn0010441	pll
	3R	FBgn0263974	qin
	3R	FBgn0051005	qless
	3R	FBgn0038810	Srp72
	3R	FBgn0011289	TfIIA-L
	X	FBgn0264386	Ca-alpha1T
	X	FBgn0026143	CDC45L
	X	FBgn0029980	CG10778
	X	FBgn0029930	CG12541
	X	FBgn0029895	CG14441
	X	FBgn0031163	CG14579
	X	FBgn0014903	CG14630
	X	FBgn0030012	CG18262
	X	FBgn0029896	CG3168
	X	FBgn0052719	CG32719
	X	FBgn0053082	CG33082
	X	FBgn0261444	CG3638
	X	FBgn0259143	CG42258
	X	FBgn0260869	CG42579
	X	FBgn0030720	CG8939
	X	FBgn0030763	CG9782
	X	FBgn0023509	mip130
	X	FBgn0015519	nAChRalpha3
	X	FBgn0030298	Or10a
	X	FBgn0003079	phl
	X	FBgn0023510	Rbcn-3B
	X	FBgn0004403	RpS14a
	X	FBgn0003380	Sh
	X	FBgn0265630	sno
	X	FBgn0003638	su_wa_
	X	FBgn0265464	Traf6
	X	FBgn0003969	vap
Negative correlation and low nutrient			
	2L	FBgn0010100	Acon
	2L	FBgn0000053	ade3

2L	FBgn0023407	B4
2L	FBgn0001991	Ca-alpha1D
2L	FBgn0032717	CG10600
2L	FBgn0031505	CG12400
2L	FBgn0040496	CG17104
2L	FBgn0032873	CG2614
2L	FBgn0051627	CG31627
2L	FBgn0051646	CG31646
2L	FBgn0051678	CG31678
2L	FBgn0051752	CG31752
2L	FBgn0085423	CG34394
2L	FBgn0259213	CG42313
2L	FBgn0263354	CG42784
2L	FBgn0031526	CG8838
2L	FBgn0028540	CG9008
2L	FBgn0032507	CG9377
2L	FBgn0053194	CheA29a
2L	FBgn0264392	CR43840
2L	FBgn0266154	CR44860
2L	FBgn0266913	CR45373
2L	FBgn0266914	CR45374
2L	FBgn0031689	Cyp28d1
2L	FBgn0031688	Cyp28d2
2L	FBgn0262029	d
2L	FBgn0011202	dia
2L	FBgn0014859	Hr38
2L	FBgn0011648	Mad
2L	FBgn0261836	Msp300
2L	FBgn0028946	Or35a
2L	FBgn0024846	p38b
2L	FBgn0003046	Pcp
2L	FBgn0051660	pog
2L	FBgn0264087	Slob
2L	FBgn0065104	snmRNA:158
2L	FBgn0083028	snoRNA:Psi18S-525k

2L	FBgn0016977	spen
2L	FBgn0031390	tho2
2L	FBgn0015600	toc
2R	FBgn0020766	Aats-phe
2R	FBgn0040780	Atg10
2R	FBgn0033149	CG11060
2R	FBgn0033174	CG11125
2R	FBgn0033259	CG11210
2R	FBgn0033028	CG11665
2R	FBgn0033229	CG12822
2R	FBgn0033608	CG13220
2R	FBgn0033089	CG17266
2R	FBgn0050161	CG30161
2R	FBgn0050416	CG30416
2R	FBgn0050503	CG30503
2R	FBgn0053140	CG33140
2R	FBgn0261373	CG33228
2R	FBgn0085243	CG34214
2R	FBgn0085265	CG34236
2R	FBgn0261698	CG42732
2R	FBgn0260456	CG4806
2R	FBgn0033271	CG8708
2R	FBgn0033258	CG8712
2R	FBgn0033605	CG9067
2R	FBgn0033092	CG9422
2R	FBgn0034844	CG9861
2R	FBgn0033313	Cirl
2R	FBgn0033192	Corin
2R	FBgn0013770	Cp1
2R	FBgn0086519	Cpr47Eg
2R	FBgn0050374	CR30374
2R	FBgn0264877	CR44068
2R	FBgn0265660	CR44467
2R	FBgn0266859	CR45320
2R	FBgn0267028	CR45472

2R	FBgn0033397	Cyp4p3
2R	FBgn0033015	d4
2R	FBgn0050502	fa2h
2R	FBgn0033609	fbl6
2R	FBgn0029082	hbs
2R	FBgn0010114	hig
2R	FBgn0033087	Hsepi
2R	FBgn0025830	IntS8
2R	FBgn0040513	kappaB-Ras
2R	FBgn0259247	laccase2
2R	FBgn0262394	mir-281-1
2R	FBgn0262423	mir-281-2
2R	FBgn0033379	Mys45A
2R	FBgn0035046	NDUFA8
2R	FBgn0033029	Not3
2R	FBgn0014184	Oda
2R	FBgn0261588	pdm3
2R	FBgn0033088	PGAP3
2R	FBgn0033380	Phax
2R	FBgn0003090	pk
2R	FBgn0035047	Pof
2R	FBgn0003317	sax
2R	FBgn0023167	SmD3
2R	FBgn0003460	so
2R	FBgn0264959	Src42A
2R	FBgn0050297	tRNA:CR30297
3L	FBgn0025525	bab2
3L	FBgn0260960	Baldspot
3L	FBgn0036536	CG12713
3L	FBgn0040809	CG13465
3L	FBgn0036537	CG18081
3L	FBgn0052264	CG32264
3L	FBgn0052432	CG32432
3L	FBgn0037026	CG3634
3L	FBgn0037027	CG3680

3L	FBgn0259099	DCX-EMAP
3L	FBgn0263219	Dscam4
3L	FBgn0262579	Ect4
3L	FBgn0035101	p130CAS
3L	FBgn0035106	rno
3L	FBgn0003984	vn
3R	FBgn0266717	Bruce
3R	FBgn0020556	bxd
3R	FBgn0013759	CASK
3R	FBgn0038737	CG11447
3R	FBgn0039633	CG11873
3R	FBgn0038246	CG14853
3R	FBgn0037698	CG16779
3R	FBgn0051224	CG31224
3R	FBgn0063261	CG31275
3R	FBgn0051525	CG31525
3R	FBgn0085386	CG34357
3R	FBgn0085454	CG34425
3R	FBgn0038268	CG3631
3R	FBgn0250754	CG42232
3R	FBgn0260234	CG42508
3R	FBgn0039078	CG4374
3R	FBgn0038750	CG4465
3R	FBgn0266418	CG45058
3R	FBgn0038745	CG4538
3R	FBgn0038740	CG4562
3R	FBgn0038744	CG4733
3R	FBgn0039492	CG6051
3R	FBgn0039165	CG6204
3R	FBgn0259212	cno
3R	FBgn0054046	CR34046
3R	FBgn0266254	CR44949
3R	FBgn0267192	CR45632
3R	FBgn0262081	Csk
3R	FBgn0086901	cv-c

3R	FBgn0259938	cwo
3R	FBgn0051158	Efa6
3R	FBgn0015229	glec
3R	FBgn0046886	Gr98c
3R	FBgn0046885	Gr98d
3R	FBgn0037697	GstZ2
3R	FBgn0011224	heph
3R	FBgn0261984	Ire1
3R	FBgn0266282	jnj
3R	FBgn0051363	Jupiter
3R	FBgn0038980	Octbeta1R
3R	FBgn0044826	Pak3
3R	FBgn0037296	Prosbeta2R2
3R	FBgn0003165	pum
3R	FBgn0262614	pyd
3R	FBgn0263974	qin
3R	FBgn0038747	RhoGAP92B
3R	FBgn0038269	Rrp6
3R	FBgn0016061	side
3R	FBgn0037248	srl
3R	FBgn0039338	XNP
3R	FBgn0038749	Xport
X	FBgn0022710	Ac13E
X	FBgn0052626	AMPdeam
X	FBgn0000179	bi
X	FBgn0024366	CG11409
X	FBgn0030048	CG12112
X	FBgn0030317	CG1561
X	FBgn0030478	CG1640
X	FBgn0052645	CG32645
X	FBgn0052698	CG32698
X	FBgn0029791	CG4096
X	FBgn0259699	CG42353
X	FBgn0259700	CG42354
X	FBgn0030455	CG4318

X	FBgn0263706	CG43658
X	FBgn0029939	CG9650
X	FBgn0030514	CG9941
X	FBgn0000259	CkIIbeta
X	FBgn0040859	CR32658
X	FBgn0052773	CR32773
X	FBgn0264675	CR43960
X	FBgn0265457	CR44357
X	FBgn0266277	CR44962
X	FBgn0267228	CR45668
X	FBgn0025641	DAAM
X	FBgn0000479	dnc
X	FBgn0052666	Drak
X	FBgn0087008	e_y_3
X	FBgn0000635	Fas2
X	FBgn0004650	fs_1_N
X	FBgn0001087	g
X	FBgn0066114	GlcAT-I
X	FBgn0026679	IntS4
X	FBgn0030530	jub
X	FBgn0030334	Karl
X	FBgn0028369	kirre
X	FBgn0267033	mamo
X	FBgn0004456	mew
X	FBgn0261260	mgl
X	FBgn0052580	Muc14A
X	FBgn0015774	NetB
X	FBgn0030505	NFAT
X	FBgn0002948	nod
X	FBgn0030613	Rab3-GEF
X	FBgn0030931	Rad51D
X	FBgn0030479	Rbp1-like
X	FBgn0031006	rictor
X	FBgn0003447	sn
X	FBgn0263987	spoon

	X	FBgn0085443	spri
	X	FBgn0010329	Tbh
	X	FBgn0267001	Ten-a
	X	FBgn0028397	Tob
	X	FBgn0030049	Trf4-1
	X	FBgn0010194	Wnt5
	X	FBgn0052677	X11Lbeta
	4	FBgn0026869	Thd1

Table 5-S3. The number of SNPs in each site class.

Site class	(a) positive correlation and high nutrient	(b) positive correlation and low nutrient	(c) negative correlation and high nutrient	(d) negative correlation and low nutrient
Upstream	127	498	461	1,400
Downstream	263	769	401	1,405
Exon	32	35	28	132
Intron	477	2,176	1,487	3,399
Synonymous cording	40	326	130	727
Nonsynonymous cording	19	199	52	433
Splice site region	–	6	4	5
Start Gained	2	40	8	16
Start lost	–	1	1	–
Stop gained	–	–	–	5
Synonymous stop	–	–	1	1
UTR 3 prime	58	98	80	294
UTR 5 prime	2	116	35	94

Table 5-S4. GO terms enriched in each direction of correlation and nutrient condition.

	Gene ontology ID	Term	Adjusted *p*-value
Positive correlation and high nutrient			
	_	_	_
Positive correlation and low nutrient			
	GO:0040011	locomotion	0.01728492
	GO:0034330	cell junction organization	0.01728492
	GO:0034329	cell junction assembly	0.01728492
	GO:0042330	taxis	0.02761742
	GO:0035146	tube fusion	0.02761742
	GO:0035147	branch fusion, open tracheal system	0.02761742
	GO:0040007	growth	0.02761742
	GO:0050808	synapse organization	0.03128592
	GO:0007268	chemical synaptic transmission	0.03128592
	GO:0098916	anterograde trans-synaptic signaling	0.03128592
	GO:0099537	trans-synaptic signaling	0.03128592
	GO:0031175	neuron projection development	0.03128592
	GO:0007424	open tracheal system development	0.03128592
	GO:0099536	synaptic signaling	0.03128592
	GO:0007416	synapse assembly	0.03233241
	GO:0060541	respiratory system development	0.03235191
	GO:0051963	regulation of synapse assembly	0.03529517
	GO:0008045	motor neuron axon guidance	0.03529517
	GO:1901888	regulation of cell junction assembly	0.03529517
	GO:0050803	regulation of synapse structure or	0.03529517
	GO:0048812	neuron projection morphogenesis	0.03529517
	GO:0048858	cell projection morphogenesis	0.03529517
	GO:0120039	plasma membrane bounded cell projection morphogenesis	0.03529517
	GO:0000768	syncytium formation by plasma membrane fusion	0.03529517
	GO:0006949	syncytium formation	0.03529517
	GO:0007520	myoblast fusion	0.03529517
	GO:0014902	myotube differentiation	0.03529517

	GO:0140253	cell-cell fusion	0.03529517
	GO:0032990	cell part morphogenesis	0.03618981
	GO:0007338	single fertilization	0.0392039
	GO:0030708	germarium-derived female germ-line cyst encapsulation	0.0392039
	GO:0048139	female germ-line cyst encapsulation	0.0392039
	GO:0048589	developmental growth	0.0392039
	GO:0000904	cell morphogenesis involved in differentiation	0.04612304
	GO:0060562	epithelial tube morphogenesis	0.04612304
	GO:0009566	fertilization	0.04612304
	GO:0035335	peptidyl-tyrosine dephosphorylation	0.04941994
Negative correlation and high nutrient			
	GO:0008063	Toll signaling pathway	0.00070782
	GO:0045751	negative regulation of Toll signaling pathway	0.00070782
	GO:0060259	regulation of feeding behavior	0.00250017
	GO:0008592	regulation of Toll signaling pathway	0.00250017
	GO:0045824	negative regulation of innate immune response	0.00454904
	GO:0009968	negative regulation of signal transduction	0.01793609
	GO:0010648	negative regulation of cell communication	0.02059236
	GO:0050795	regulation of behavior	0.02059236
	GO:0023057	negative regulation of signaling	0.02059236
	GO:0050777	negative regulation of immune response	0.02059236
	GO:0048585	negative regulation of response to stimulus	0.02175397
	GO:0007631	feeding behavior	0.02175397
	GO:0006885	regulation of pH	0.02175397
	GO:0030641	regulation of cellular pH	0.02175397
	GO:0002832	negative regulation of response to biotic stimulus	0.02175397

	GO:0002831	regulation of response to biotic stimulus	0.02175397
	GO:0031348	negative regulation of defense response	0.02175397
	GO:0045087	innate immune response	0.02175397
	GO:0031347	regulation of defense response	0.02231298
	GO:0050776	regulation of immune response	0.0223333
	GO:0032102	negative regulation of response to external stimulus	0.02742609
	GO:0030004	cellular monovalent inorganic cation homeostasis	0.02742609
	GO:0002683	negative regulation of immune system process	0.02742609
	GO:0008543	fibroblast growth factor receptor signaling pathway	0.02742609
	GO:0044344	cellular response to fibroblast growth factor stimulus	0.02742609
	GO:0071774	response to fibroblast growth factor	0.02742609
	GO:0046777	protein autophosphorylation	0.03084583
	GO:0055067	monovalent inorganic cation homeostasis	0.03697138
	GO:0045088	regulation of innate immune response	0.0397322
	GO:0080134	regulation of response to stress	0.04455468
	GO:0001508	action potential	0.04455468
	GO:0002682	regulation of immune system process	0.04627588
Negative correlation and low nutrient			
	GO:0044087	regulation of cellular component biogenesis	3.45×10^{-5}
	GO:0051130	positive regulation of cellular component organization	8.06×10^{-5}
	GO:0048589	developmental growth	8.06×10^{-5}
	GO:0097435	supramolecular fiber organization	8.06×10^{-5}
	GO:0044089	positive regulation of cellular component biogenesis	8.06×10^{-5}
	GO:0007015	actin filament organization	0.00011654
	GO:0040007	growth	0.00011654
	GO:0048638	regulation of developmental growth	0.0001613

GO:1902531	regulation of intracellular signal transduction	0.00023642
GO:0031334	positive regulation of protein-containing complex assembly	0.00023642
GO:0050807	regulation of synapse organization	0.00023642
GO:0031175	neuron projection development	0.00023642
GO:0000904	cell morphogenesis involved in differentiation	0.00023642
GO:0048812	neuron projection morphogenesis	0.00023642
GO:0048858	cell projection morphogenesis	0.00023642
GO:0120039	plasma membrane bounded cell projection morphogenesis	0.00023642
GO:0032990	cell part morphogenesis	0.00028953
GO:0032271	regulation of protein polymerization	0.00028953
GO:1902903	regulation of supramolecular fiber organization	0.00029414
GO:0040008	regulation of growth	0.0003644
GO:0043254	regulation of protein-containing complex assembly	0.00039248
GO:0051963	regulation of synapse assembly	0.0004586
GO:0048667	cell morphogenesis involved in neuron differentiation	0.00050057
GO:0045927	positive regulation of growth	0.00055217
GO:1901888	regulation of cell junction assembly	0.00055217
GO:0040011	locomotion	0.00055217
GO:0048639	positive regulation of developmental growth	0.00064893
GO:0007560	imaginal disc morphogenesis	0.00064893
GO:0048563	post-embryonic animal organ morphogenesis	0.00064893
GO:0050803	regulation of synapse structure or activity	0.00071893
GO:0007167	enzyme-linked receptor protein signaling pathway	0.00072307
GO:0035120	post-embryonic appendage morphogenesis	0.00073606
GO:0061564	axon development	0.00075616

GO:0034329	cell junction assembly	0.00075616
GO:0098609	cell-cell adhesion	0.00082891
GO:0032273	positive regulation of protein polymerization	0.00086614
GO:0035114	imaginal disc-derived appendage morphogenesis	0.0008912
GO:0030036	actin cytoskeleton organization	0.0008912
GO:1902905	positive regulation of supramolecular fiber organization	0.0008935
GO:0035107	appendage morphogenesis	0.0008935
GO:0007416	synapse assembly	0.00097868
GO:0048737	imaginal disc-derived appendage development	0.00097868
GO:0008582	regulation of synaptic assembly at neuromuscular junction	0.00097868
GO:0016477	cell migration	0.00097868
GO:0051124	synaptic assembly at neuromuscular junction	0.00097868
GO:0060562	epithelial tube morphogenesis	0.00098321
GO:0048736	appendage development	0.00099373
GO:0051495	positive regulation of cytoskeleton organization	0.00108909
GO:0030029	actin filament-based process	0.00113764
GO:0007409	axonogenesis	0.00126063
GO:1904396	regulation of neuromuscular junction development	0.00126063
GO:0034330	cell junction organization	0.00140791
GO:0007476	imaginal disc-derived wing morphogenesis	0.00149383
GO:0048707	instar larval or pupal morphogenesis	0.00149383
GO:0007411	axon guidance	0.00149383
GO:0007173	epidermal growth factor receptor signaling pathway	0.00159356
GO:0038127	ERBB signaling pathway	0.00159356
GO:0051258	protein polymerization	0.00162379
GO:0007163	establishment or maintenance of cell polarity	0.00162379

GO:0097485	neuron projection guidance	0.00185342
GO:0009886	post-embryonic animal morphogenesis	0.00197931
GO:0007472	wing disc morphogenesis	0.00203844
GO:0051493	regulation of cytoskeleton organization	0.00203844
GO:0051094	positive regulation of developmental process	0.00235984
GO:0006935	chemotaxis	0.00237197
GO:0042059	negative regulation of epidermal growth factor receptor signaling pathway	0.00239654
GO:1901185	negative regulation of ERBB signaling pathway	0.00239654
GO:0007552	metamorphosis	0.00241959
GO:2000026	regulation of multicellular organismal development	0.00248386
GO:0040012	regulation of locomotion	0.00494945
GO:0007528	neuromuscular junction development	0.00494945
GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	0.00504393
GO:0050808	synapse organization	0.0052009
GO:0048870	cell motility	0.00527418
GO:0031113	regulation of microtubule polymerization	0.00527418
GO:0035218	leg disc development	0.00606453
GO:0051965	positive regulation of synapse assembly	0.00754526
GO:1901890	positive regulation of cell junction assembly	0.00822654
GO:0048082	regulation of adult chitin-containing cuticle pigmentation	0.00900225
GO:0035220	wing disc development	0.00918037
GO:0008586	imaginal disc-derived wing vein morphogenesis	0.01027251
GO:0042058	regulation of epidermal growth factor receptor signaling pathway	0.01027251
GO:1901184	regulation of ERBB signaling pathway	0.01027251
GO:0048675	axon extension	0.01184594
GO:0001654	eye development	0.01184594

GO:0048880	sensory system development	0.01184594
GO:0150063	visual system development	0.01184594
GO:0051960	regulation of nervous system development	0.01210529
GO:0110053	regulation of actin filament organization	0.01210529
GO:0032956	regulation of actin cytoskeleton organization	0.01210529
GO:0051962	positive regulation of nervous system development	0.01210529
GO:0048085	adult chitin-containing cuticle pigmentation	0.01254298
GO:0007155	cell adhesion	0.01283018
GO:0032970	regulation of actin filament-based process	0.01302007
GO:0042330	taxis	0.01304229
GO:0048592	eye morphogenesis	0.01304229
GO:0090596	sensory organ morphogenesis	0.01304229
GO:0008038	neuron recognition	0.01314655
GO:0048149	behavioral response to ethanol	0.01314655
GO:1990138	neuron projection extension	0.01314655
GO:0048066	developmental pigmentation	0.01393638
GO:0048079	regulation of cuticle pigmentation	0.01400451
GO:0030833	regulation of actin filament polymerization	0.01498895
GO:0007268	chemical synaptic transmission	0.01503595
GO:0098916	anterograde trans-synaptic signaling	0.01503595
GO:0048749	compound eye development	0.01505854
GO:0099537	trans-synaptic signaling	0.01516214
GO:0008037	cell recognition	0.01519554
GO:0010638	positive regulation of organelle organization	0.01519554
GO:0034332	adherens junction organization	0.01586708
GO:0007613	memory	0.01643636
GO:0008064	regulation of actin polymerization or depolymerization	0.01719894
GO:0051017	actin filament bundle assembly	0.01719894

GO:0099536	synaptic signaling	0.01761709
GO:0030832	regulation of actin filament length	0.01804906
GO:0030838	positive regulation of actin filament polymerization	0.01804906
GO:0010648	negative regulation of cell communication	0.01827787
GO:0072553	terminal button organization	0.01827787
GO:0023057	negative regulation of signaling	0.01849563
GO:0043473	pigmentation	0.01980739
GO:0007623	circadian rhythm	0.02021861
GO:0007593	chitin-based cuticle sclerotization	0.02039443
GO:0048511	rhythmic process	0.02049947
GO:0051656	establishment of organelle localization	0.02049947
GO:0030041	actin filament polymerization	0.02059577
GO:0001745	compound eye morphogenesis	0.02059577
GO:0007611	learning or memory	0.02158859
GO:0050890	cognition	0.02223604
GO:0061572	actin filament bundle organization	0.02243401
GO:0007432	salivary gland boundary specification	0.02250825
GO:0051240	positive regulation of multicellular organismal process	0.02408018
GO:0032535	regulation of cellular component size	0.02408018
GO:0032272	negative regulation of protein polymerization	0.02563287
GO:0007189	adenylate cyclase-activating G protein-coupled receptor signaling pathway	0.02591408
GO:0045887	positive regulation of synaptic assembly at neuromuscular junction	0.02807123
GO:0045471	response to ethanol	0.02810385
GO:0048067	cuticle pigmentation	0.028667
GO:0009952	anterior/posterior pattern specification	0.028667
GO:0099172	presynapse organization	0.02968423
GO:0009798	axis specification	0.0300346
GO:0008154	actin polymerization or depolymerization	0.03061758

GO:1904398	positive regulation of neuromuscular junction development	0.03136719
GO:0110020	regulation of actomyosin structure organization	0.03136719
GO:1902667	regulation of axon guidance	0.03136719
GO:0098742	cell-cell adhesion via plasma-membrane adhesion molecules	0.03159464
GO:0007478	leg disc morphogenesis	0.03326611
GO:0050804	modulation of chemical synaptic transmission	0.0356061
GO:0099177	regulation of trans-synaptic signaling	0.0356061
GO:0035330	regulation of hippo signaling	0.03653481
GO:1902533	positive regulation of intracellular signal transduction	0.03667262
GO:0030855	epithelial cell differentiation	0.03748296
GO:0048070	regulation of developmental pigmentation	0.03805578
GO:0120305	regulation of pigmentation	0.03805578
GO:0048588	developmental cell growth	0.03947934
GO:0090066	regulation of anatomical structure size	0.03961044
GO:0046578	regulation of Ras protein signal transduction	0.04105959
GO:0048732	gland development	0.04105959
GO:2000331	regulation of terminal button organization	0.04147328
GO:0021700	developmental maturation	0.04391976
GO:0045595	regulation of cell differentiation	0.04391976
GO:0051051	negative regulation of transport	0.04391976
GO:0031333	negative regulation of protein-containing complex assembly	0.04500721
GO:0099174	regulation of presynapse organization	0.04500721
GO:0035329	hippo signaling	0.04817331
GO:0007448	anterior/posterior pattern specification, imaginal disc	0.04953815

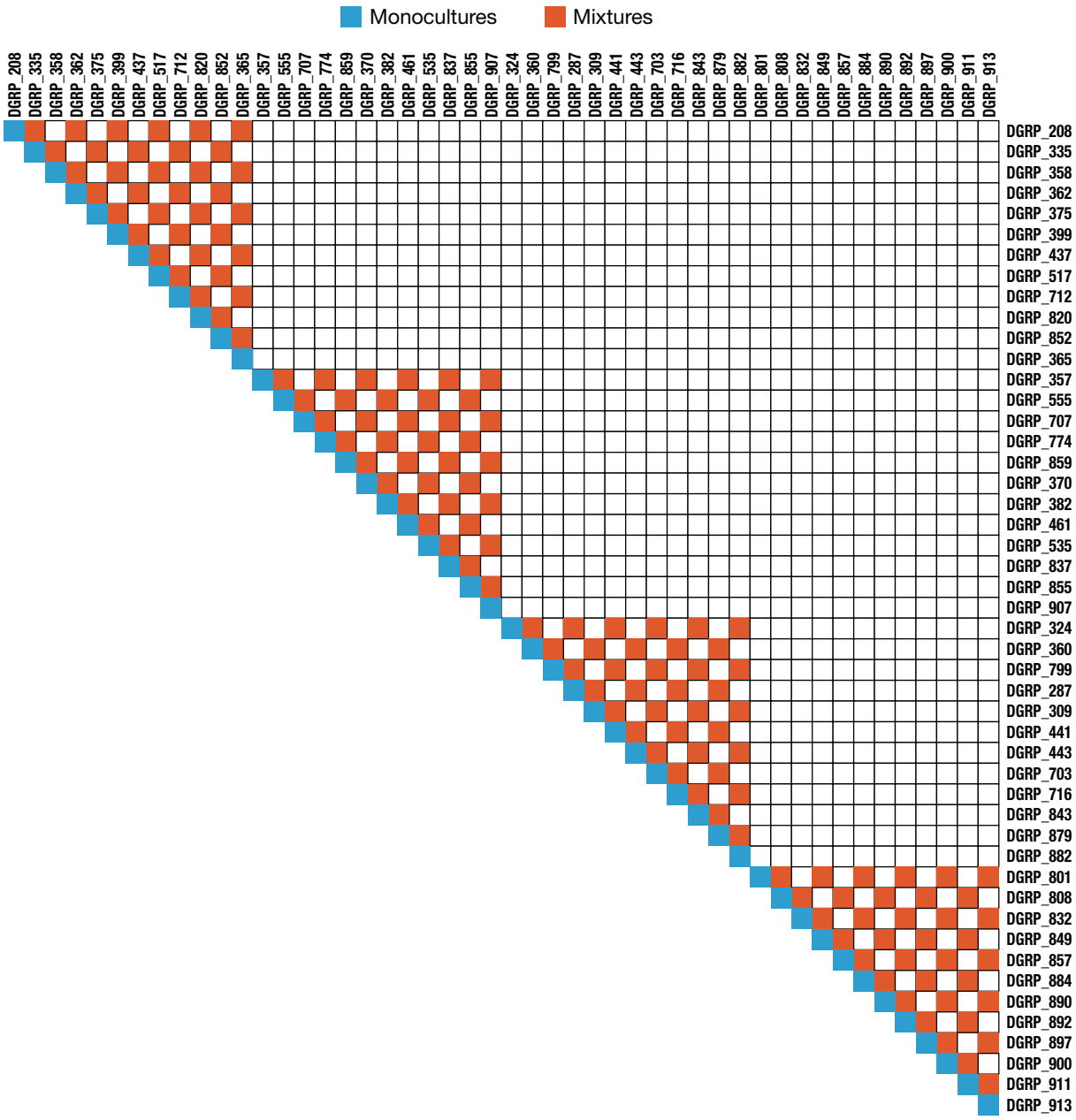

Figure 5-S1. A half-diallel containing incomplete pairwise combinations of mixtures and monocultures of 48 DGRP lines in the rearing experiment.

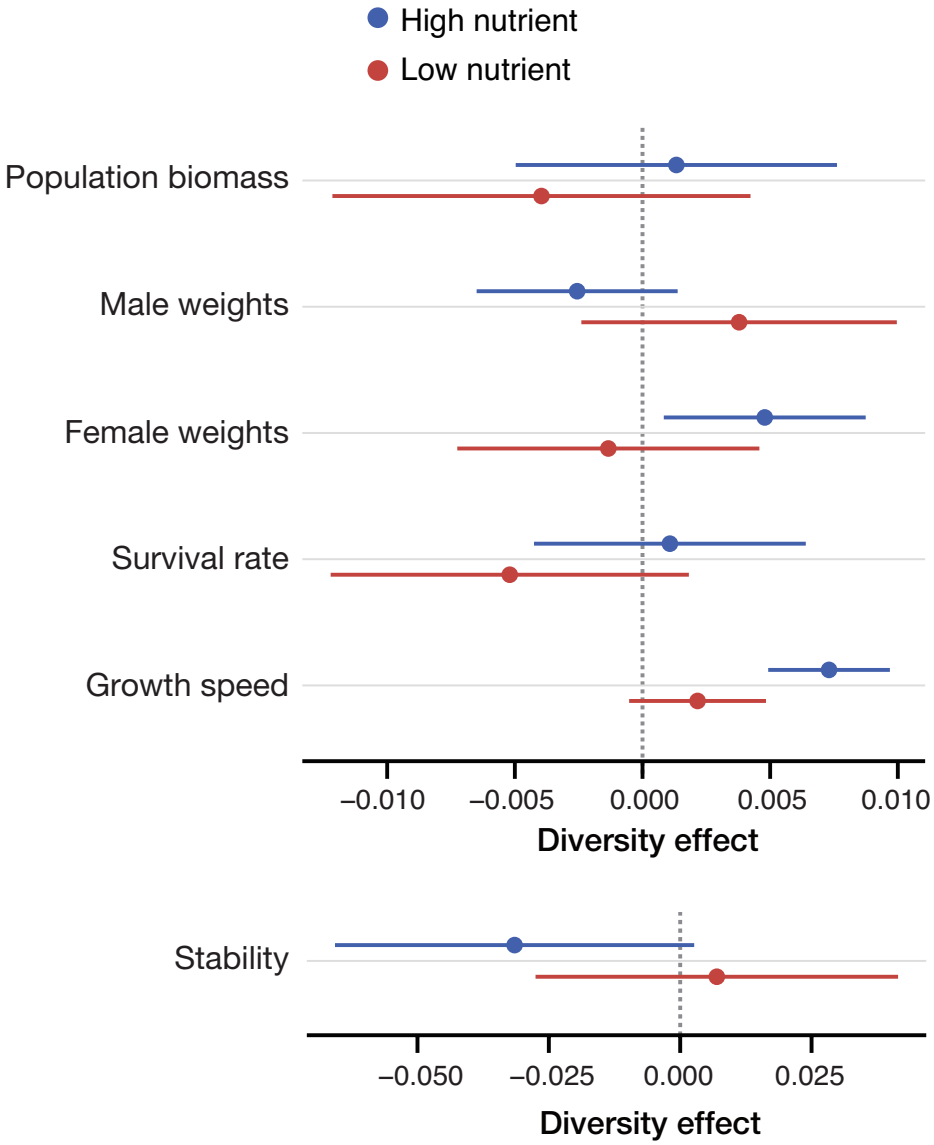

Figure 5-S2. Diversity effects on each population property and stability. Plots and error bars represent average population properties and confidence intervals (95%), respectively.

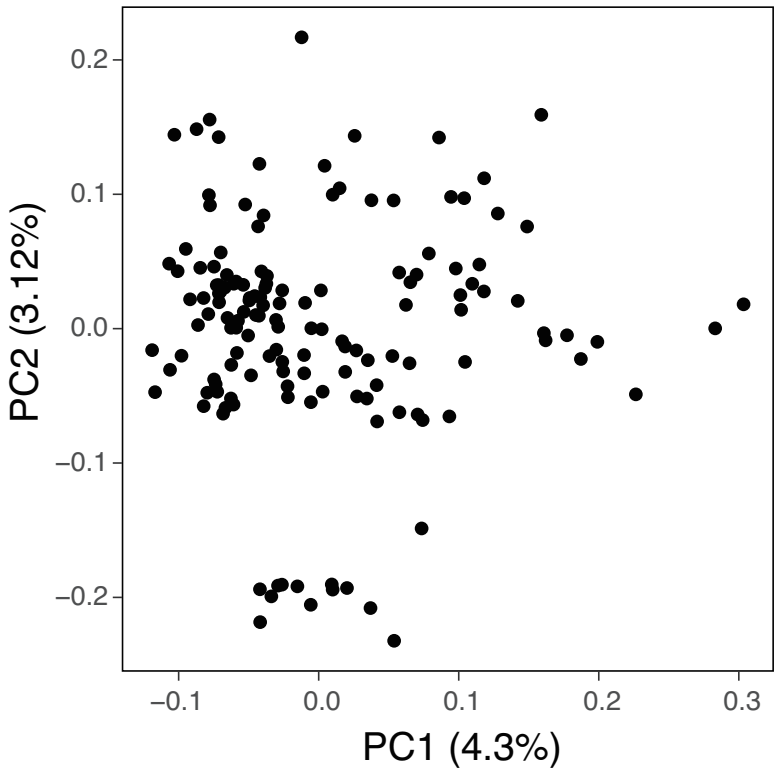

Figure 5-S3. Nucleotide diversity in only used combinations. Plots represent 144 used combinations.

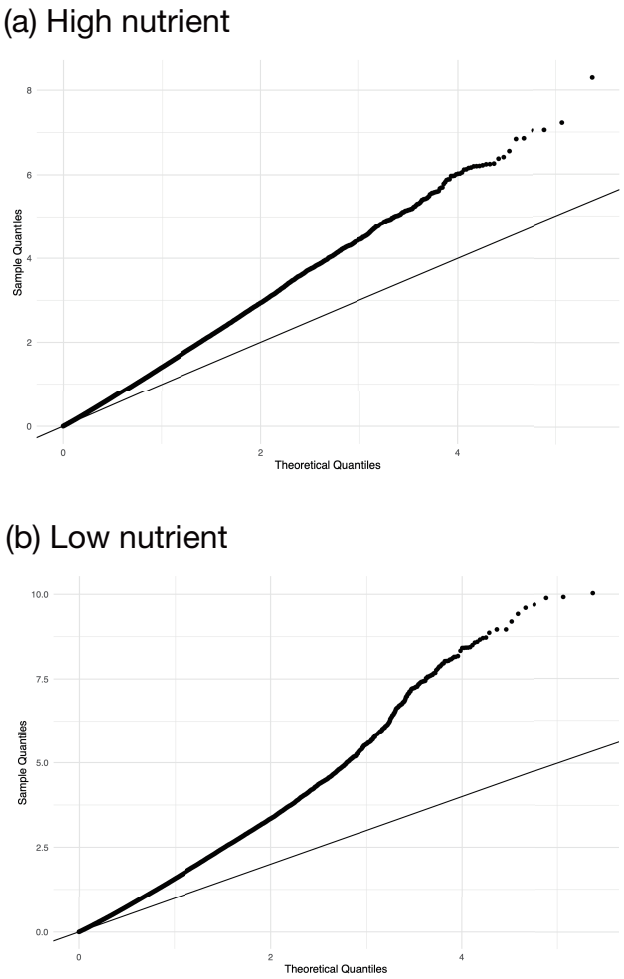

Figure 5-S4. Q-Qplots for eGWAS results in high (a) and low (b) nutrients.

General Discussion

Genetic diversity is always present in all species. The present thesis has focused on the evolutionary processes and ecological consequences of genetic diversity within a population. Previously, the research of genetic diversity has primarily fallen within the realm of evolutionary biologists since Darwin (Wright, 1920; Fisher, 1930). The widespread evidence supporting evolution by natural selection and gene flow confirms the important role of genetic diversity through evolutionary processes. However, there is a growing awareness of the ecological consequences of genetic diversity in recent years. Ironically, one of the opportunities for focusing on genetic diversity is the rapid changes in biodiversity caused by human activity. Notably, recent biodiversity loss by human activity is expected to alter the ecological functioning of the ecological organizations, such as populations, communities, and ecosystems, and its effects on planetary health, such as the quality of ecosystem services and the risk of infectious diseases (Cardinale et al., 2012). In the age of biodiversity crisis, conservation biology suggests that maintaining genetic diversity can be one of the essential tools for managing threatened populations (Kardos et al., 2021). Therefore, unraveling the ecological consequences of genetic diversity is necessary not only to understand the ecological dynamics of ecological organizations but also to apply their knowledge to conservation and human society (e.g., agriculture).

To what extent does genetic variation within a population exist? In Chapter 1, I demonstrated genetic variation in behavioral traits of larvae and adults. In *Drosophila*, the adults generally show a bimodal distribution in activity (Ferguson et al., 2015; Helfrich-Förster et al., 2020). Even my results showed that adult *Drosophila immigrans* showed a bimodal distribution in the average activity, indicating that the daily rhythm of

this species is consistent with known adult activity patterns in *Drosophila*. On the other hand, few studies have examined intra-population variation in their behavior, such as larval locomotion and adult daily activity. I here revealed the presence of intra-population genetic variation in the level of activity in both larvae and adults. I also found the intrapopulation genetic variation in the daily pattern of activity in the adult stage. This finding indicates that genetic and phenotypic variation in activity rhythms themselves may maintain in natural populations.

How does the diversity of the mito-nuclear genome affect phenotypic variation? Recent studies suggest that phenotypic variation in behavior may not be fully captured in the nuclear genome, as it does not account for mitochondrial genomes (Arnqvist and Rowe, 2023). In Chapter 2, I examined the effect of mitochondrial haplogroups on intrapopulation differences in larval and adult activity in two natural populations of *D. immigrans*. My results showed that mitochondrial genetic variation can explain the variation in activity levels between haplogroups within a population. I also noted that the change in activity level by mitochondrial haplotypes was different between populations. This finding suggests an interaction between the mitochondrial and nuclear genomes during different developmental stages. Still, further studies are needed to fully test the epistasis between nuclear and mitochondrial genes and the age of individuals in each developmental stage.

Differences in genetic variation over time, such as across seasons, have been observed in various organisms (Brakefield, 1985; Carvalho, 1987; Hendry et al., 2003; Danks, 2004). This phenomenon is suggested to be a piece of evidence supporting rapid evolution. However, comparing phenotypes in each seasons alone is insufficient to prove rapid evolution among seasons. The observed seasonal changes can also be

explained by the effects of plasticity and season-specific migration of populations with different genotypes (Stone et al., 2020). To detect rapid evolution, one of the most important steps is to reconsider the approach or methodology being used. In Chapter 3, I conducted simultaneous measurements of phenotypic traits under constant conditions and found that heat tolerance and wing to thorax ratio, but not cold tolerance, differed between the two periods of *D. lutescens.* Additionally, I demonstrated that the spring and autumn periods exhibited little genetic differentiation. My results suggest that traits showing significant differences between the two periods were more influenced by seasonal factors rather than plasticity. Conversely, traits with no significance may be more indicative of plasticity rather than seasonal selection.

Genetic variation maintained within a population can non-additively influence ecological consequences for population dynamics, also known as diversity effects. Understanding diversity effects on ecological dynamics is thought to be necessary to predict ecological organization in the future (Nosil and Gompert, 2022). Previously, diversity has been quantified as the richness of genotypes because genotype number is assumed to lead to niche differences (dissimilarity) (Wennersten and Forsman, 2012; Forsman and Wennersten, 2016). However, the dissimilarity among genotypes may be more important in determining ecological dynamics rather than genotype numbers (Heemsbergen et al., 2004; Jousset et al., 2011; Ellers et al., 2011). In Chapter 4, I assessed phenotypic dissimilarity to investigate which "phenotypic diversity" non-additively affected population properties for *D. immigrans*. As a result of the rearing experiment, genotype richness significantly improved five population properties and stability under my experimental conditions. Multi-trait association analysis and models selected by AIC indicate that behavioral variations contribute to positive and negative

diversity effects more than those in morphological and life-history traits. Notably, the diversity effects on male weights were significantly increased by behavioral variation. This finding indicates that genetic diversity of behavioral traits led to diversity effects via niche differentiation and reduced resource competition, rather than the diversity of other traits.

Analyses at the phenotypic level are limited to investigating all possible candidates of traits for diversity effects because the number of traits is considered to exist infinitely. Therefore, further research is required to focus on genomic levels to understand traits and genes responsible for diversity effects and the mechanisms driving them. In Chapter 5, I conducted the first genome-wide study to address the genomic basis of population dynamics which is a higher level of biological organization. Inbred lines of *Drosophila melanogaster* to demonstrate the new attempt to investigate how genomic structures within populations are associated with ecological properties. My results showed multiple genes responsible for positive and negative diversity effects in high- and low-nutrient conditions. Surprisingly, the genes identified were not shared among the directions of correlations and the nutrient conditions, suggesting that different population processes and inter-individual interactions govern positive and negative diversity effects.

Identifying the genes whose diversity is important to determine ecological properties in ecological organizations may be useful to understand the complex and nonlinear dynamics in ecology (Barbour et al., 2022; Wuest et al., 2023). Taking inspiration from “keystone species,” a recent study proposed a plant “keystone gene” in a plant whose existence determined the persistence of a plant-insect food web (Barbour et al., 2022). A single allele at a single gene in a plant is suggested to promote the

coexistence or extinction of species in an experimental food web. My approach will explain which diversity of genes within a population contributes to the change in population dynamics (i.e., diversity effects); that is, I could propose “keystone diversity” in the future. However, the research on exploring genes responsible for ecological dynamics has just begun. One of the most important further experiments requires narrowing down the list of candidate genes and finding true positive genes responsible for diversity effects within various species. Moreover, a comprehensive understanding of diversity effects across ecological organizations is also interesting. Currently, the relative ecological importance of genetic diversity effects compared to species diversity effects remains unclear. Previous meta-analyses suggested that genetic diversity can have a more pronounced impact on regulating ecological dynamics than species diversity in some cases (Des Roches et al., 2018; Raffard et al., 2019). Notably, there is no examination to investigate the relative importance of two types of diversity effects by shared ecological properties and shared diversity index. Nucleotide diversity on homologous genes among and within species may be useful to assess diversity. Further studies are expected to explore which diversity (genetic diversity or species diversity) in the homologous genes contributes to ecological properties. The results of Such studies will be able to be applied to the development of conservation which mainly focuses on species units in the present.

Pursuing negative diversity effects also deserves attention. Diversity is disadvantageous for ecological dynamics in some cases. For group-living animals, relatedness among conspecific individuals was more important to reduce cannibalism rate at the population level, rather than genetic diversity in *D. melanogaster* (Fisher et al., 2021). Considering the genomic mechanisms underlying positive and negative

diversity effects simultaneously is important to lead to reducing the bias of knowledge about biodiversity.

The ability to control the diversity of such genes responsible for diversity effects will have essential applications in not only conservation but also agriculture. Historically, high-yielding crop varieties were selected and bred in monoculture, which is well known as the “Green Revolution.” However, Breeding mixtures in such varieties are suggested to provide a range of benefits for yielding (Renard and Tilman, 2019; Wuest et al., 2021). Further studies are required to explore a keystone diversity of a gene responsible for positive diversity effects in the situation of increasing global food demand and climate change that threaten the stability of the present food systems.

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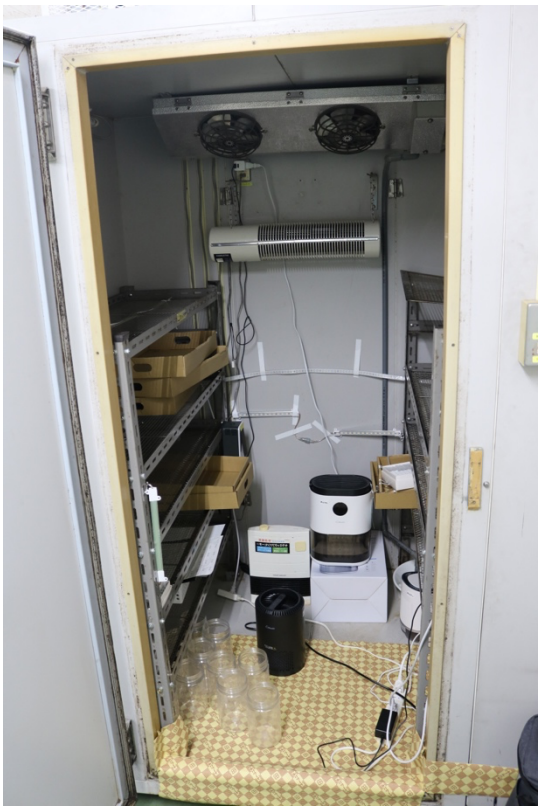
My temperature-controlled room malfunctioned on August 8th, 2023, after faithfully serving me since my days as a graduate student in the master's program. This room was handed down from Prof. Shimada in University of Tokyo. I greatly appreciate Prof. Shimada for readily agreeing to provide me with this room. I am profoundly grateful to my temperature-controlled room for its unwavering dedication in preserving my samples over an extended period.



My temperature-controlled room shut down its power supply on August 8th, 2023.