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Rapid evolutionary change, constraints and the maintenance of polymorphism in natural populations of *Drosophila melanogaster*

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Abstract

Allele frequencies can shift rapidly within natural populations. Under certain conditions, repeated rapid allele frequency shifts can lead to the long-term maintenance of polymorphism. In recent years, studies of the model insect *Drosophila melanogaster* have suggested that this phenomenon is more common than previously believed and is often driven by some form of balancing selection, such as temporally fluctuating or sexually antagonistic selection. Here we discuss some of the general insights into rapid evolutionary change revealed by large-scale population genomic studies, as well as the functional and mechanistic causes of rapid adaptation uncovered by single-gene studies. As an example of the latter, we consider a regulatory polymorphism of the *D. melanogaster fezzik* gene. Polymorphism at this site has been maintained at intermediate frequency over an extended period of time. Regular observations from a single population over a period of 7 years revealed significant differences in the frequency of the derived allele and its variance across collections between the sexes. These patterns are highly unlikely to arise from genetic drift alone or from the action of sexually antagonistic or temporally fluctuating selection individually. Instead, the joint action of sexually antagonistic and temporally fluctuating selection can best explain the observed rapid and repeated allele frequency shifts. Temporal studies such as those reviewed here further our understanding of how rapid changes in selection can lead to the long-term maintenance of polymorphism as well as improve our knowledge of the forces driving and limiting adaptation in nature.

KEYWORDS
balancing selection, gene regulation, genetic variation., rapid evolution, sexual antagonism, temporally varying selection

1 | ALLELE FREQUENCY SHIFTS AND THE LONG-TERM MAINTENANCE OF GENETIC VARIATION

In natural populations, the frequencies of genetic variants change over time, sometimes quite rapidly (i.e. over the course of only a few generations). These allele frequency shifts can be driven by neutral forces, such as genetic drift, or by non-neutral forces, such as natural selection. However, the extent of changes in allele frequency may also be constrained by factors such as variation in environmental conditions or genomic conflict. In such cases, two (or more) alleles may be maintained at a locus by balancing selection.

1.1 | Mechanisms that can maintain genetic variation within a species

Overdominant selection is perhaps the best-known type of balancing selection. Also referred to as heterosis or heterozygote advantage as it is characterized by higher fitness in heterozygotes than in both homozygotes, it leads to deviations from Hardy–Weinberg equilibrium (HWE) due to an excess of heterozygotes. A textbook example of overdominant selection can be seen in humans at the β-haemoglobin locus, where the sickle cell allele (HbS) is associated with sickle cell disease but confers increased malaria resistance; thus, in areas with a high incidence of malaria, heterozygotes carrying one wild-type and one HbS allele have the highest fitness (reviewed in Carter & Mendis, 2002; Hedrick, 2011). However, some studies have predicted that HbC, another non-synonymous variant at this locus, may eventually replace HbS or go to fixation in some populations (Modiano et al., 2001; Hedrick, 2004; Hedrick, 2011; but see Modiano et al., 2008). It has been predicted that in fast-changing environments, where adaptation is expected to be both rapid and frequent, adaptive mutations may often display heterozygote advantage as adaptive variants with large effects may meet the fitness optimum in their heterozygous state but overshoot it when homozygous (Sellis et al., 2011). However, evidence from allozyme frequencies, genome-wide selection scans and gene expression data suggests that the phenomenon may be relatively rare in *Drosophila melanogaster* (Houle, 1989; Gibson et al., 2004; reviewed in Croze et al., 2016). Indeed, balancing selection driven by heterozygote advantage, coevolution with parasites and negative frequency-dependent selection (see section 1.2 below) in general appears to be relatively rare in *D. melanogaster* (reviewed in Croze et al., 2016), although multiple studies have identified genes thought to be under balancing selection in this species (Chapman et al., 2019; Comeron, 2014; Croze et al., 2016; Croze et al., 2017; Ferreira & Amos, 2006; Fitzpatrick et al., 2007; Hudson et al., 1987; Unckless & Lazzaro, 2016).

Genomic conflict, such as sexual antagonism, is another mechanism that can maintain polymorphism in a population over time. It occurs when genetic variants have conflicting fitness effects between the sexes, resulting in balancing selection and increased genetic variation until the sexual conflict is fully resolved (reviewed in Mank, 2017). Thus, sexual antagonism is thought to help drive genetic and phenotypic divergence between species and populations (Lund-Hansen et al., 2021; Payseur et al., 2018) as well as play an important role in maintaining polymorphism in natural populations (Connallon & Clark, 2014a; Connallon & Clark, 2014b; Mank, 2017; Ruzicka et al., 2019). The application of population genomic tools has proven useful in identifying and characterizing putative sexual conflict in natural populations (reviewed in Mank, 2017) and the identification of challenges and their associated solutions in this application has received much attention in recent years (Bissegger et al., 2020; Ruzicka et al., 2020). These novel approaches have also sparked recent debate over the interpretation of previously established signs of potential sex-specific selection, such as intersexual allele frequency differences (Cheng & Kirkpatrick, 2016, Cheng and Kirkpatrick, 2020; Kasimatis et al., 2019; Mank et al., 2020) and sex-biased gene expression (Cheng & Kirkpatrick, 2016; Wright et al., 2018). Sexual antagonism is thought to be common in *D. melanogaster* (Cheng & Kirkpatrick, 2016; Innocenti & Morrow, 2010); however, the identification of individual loci under sexually antagonistic selection has remained a major challenge (reviewed in Mank, 2017). One recent study used a combination of experimental data, population genomics and bioinformatics to identify thousands of sexually antagonistic single nucleotide polymorphism (SNP) variants in *D. melanogaster* and found that variation at sexually antagonistic loci is maintained among global *D. melanogaster* populations (Ruzicka et al., 2019). For a discussion of several genes thought to be under sexually antagonistic selection in *Drosophila*, see sections 2.3 and 3 below.

Another mechanism that can help maintain polymorphism within a species is polygenic adaptation, in which a population adapts via small or large allele frequency shifts across many loci. Polygenic adaptation has received much attention in recent years (Barghi et al., 2020; Hayward & Sella, 2022; Höllinger et al., 2019; Jain & Stephan, 2017) and can proceed quite rapidly under certain circumstances via rapid shifts in allele frequency (Jain & Stephan, 2017). The hallmarks of polygenic adaptation are non-parallelism between populations (i.e. populations with initially similar underlying genetic architecture will follow different adaptive trajectories under similar selection regimes) and heterogeneity among selected loci (i.e. adaptive allele frequencies will vary among populations) (Barghi et al., 2020), which results in the maintenance of variation among populations within a species. Polygenic adaptation is thought to underlie temperature adaptation in studies using experimentally evolved populations of *Drosophila simulans* (Barghi et al., 2019; Barghi & Schlötterer, 2020). Such selection has remained difficult to identify in natural *Drosophila* populations, but has been detected in a Pennsylvania population (Bergland et al., 2014; but see Buffalo & Coop, 2020) as well as *D. melanogaster* populations evolving under semi-natural conditions in the context of rapid seasonal selection (Rudman et al., 2019; Rudman et al., 2022; see also sections 1.2 and 2.2 below).

Heterogeneity of selection over time and/or space can also maintain polymorphism within a species. When spatially varying

selection occurs, genetic variation can be maintained between populations as local adaptation occurs across the species' range. In *Drosophila* populations, latitudinal clines in allozyme, inversion and allele frequency have been well-documented and are thought to be maintained by spatially varying selection (Anderson et al., 2005; Durmaz et al., 2018; Durmaz et al., 2019; Kapun et al., 2016; Lange et al., 2022; Oakeshott et al., 1982; Verrelli & Eanes, 2001; Yu & Bergland, 2022). Indeed, inversions are thought to be particularly important in the maintenance of genetic variation as they prevent linked adaptive variants from recombining away from one another, sometimes leading to the evolution of so-called 'supergenes' (reviewed in Llaurens et al., 2017). One of the best-known examples of balancing selection in *D. melanogaster* is driven by spatially varying selection on the alcohol dehydrogenase (ADH) enzyme, which catalyses the first step in the breakdown of environmental ethanol. Two allelic ADH variants, called Fast and Slow due to their differing migration speeds during gel electrophoresis (Johnson & Denniston, 1964), segregate in natural populations along a latitudinal cline, with the Fast variant more frequent at higher, more temperate latitudes (Cogni et al., 2017; Oakeshott et al., 1982; Umina et al., 2005), although this cline is thought to have rapidly shifted in recent years (Umina et al., 2005). This cline is hypothesized to be maintained by balancing selection (Hudson et al., 1987; van Delden et al., 1978) via a trade-off between catalytic activity versus stability at higher temperatures due to an amino acid polymorphism between these variants (Day et al., 1974; McKay, 1981; Sampsell & Sims, 1982; van Delden et al., 1978); however, a recent study suggests that this variation is not maintained by temperature but due to another unknown environmental factor (Siddiq & Thornton, 2019).

1.2 | Mechanisms that maintain variation and can lead to repeated allele frequency fluctuations

Another form of heterogenous selection is temporally varying selection, which occurs when the strength and/or type of selection varies over time. This temporal selection can occur in regular cycles due to changing seasons or other environmental variables with regular, repeating patterns or it can shift more irregularly over time, both of which can under certain circumstances lead to the long-term maintenance of variation (Bell, 2010; Gillespie, 1978; Pfenninger & Foucault, 2022; Wittmann et al., 2017). In the latter case, rapid adaptation via repeated, aperiodic shifts in allele frequency can occur in response to non-cyclic environmental changes (Bell, 2010; Pfenninger & Foucault, 2022); while, in the former case, rapid, repeated, cyclic shifts in allele frequency can occur in response to periodically changing environmental conditions, such as seasons, if the selected alleles are sufficiently dominant (Wittmann et al., 2017; reviewed in Johnson et al., 2023). It should be noted, however, that theoretical studies have suggested that temporally fluctuating selection can also decrease polymorphism in unlinked, non-selected regions (Park & Kim, 2019; Taylor, 2013; Wittmann et al., 2023). Indeed, a study using replicate experimental populations of *D. melanogaster* under various selection regimes found that while temporal selection led to an increase in genetic variation at selected loci, unlinked, neutral loci showed the largest decrease in polymorphism of all tested selection regimes (Huang et al., 2014). Until recently, it has been difficult to identify more subtle allele frequency fluctuations in natural *D. melanogaster* populations that may result from temporally varying selection, as it requires large population genomic time series datasets; however, advances in sequencing technology have made such large-scale sequencing projects more technically and economically feasible in recent years (see section 2.1 below).

Another mechanism that can help maintain variation and produce repeated allele frequency shifts is negative frequency-dependent selection, in which the selection an allele experiences is dependent upon its relative frequency in the population, with fitness increasing when the allele is rare, resulting in repeated shifts in allele frequency. One of the best-known cases of negative frequency-dependent selection in *D. melanogaster* is driven by selection on larval competition during low nutrient conditions in the *foraging* gene, which is involved in larval food searching behaviour and has two allele variants, named *sitter* and *rover*, segregating in natural populations. The *rover* larvae move more when foraging and are more likely to explore new food patches than *sitter* larvae and when raised under low nutrient conditions the fitness of each allele is highest when it is rare (Fitzpatrick et al., 2007).

The mechanisms that affect allele frequency dynamics and rapid adaptation are not mutually exclusive and can act simultaneously on the same locus, which can complicate the identification and characterization of these forces. Below, we discuss how large-scale population genomic studies in *D. melanogaster* can yield insight into rapid evolutionary change as well as how single-gene studies can complement genome-wide methods and help further our understanding of the functional and mechanistic bases of rapid adaptation. As an example, we also consider a case of putative temporally varying and sexually antagonistic selection on a regulatory polymorphism of a single gene in order to better understand the selective forces and constraints acting on this locus.

2 | ALLELE FREQUENCY VARIATION AND DYNAMICS IN NATURAL *DROSOPHILA* POPULATIONS

2.1 | Population genomics

The recent advent of community resources providing genome-wide allele frequency estimates from hundreds of *D. melanogaster* populations across multiple seasons and years has allowed for in-depth tracking of allele frequencies over short evolutionary timescales (Kapun et al., 2020; Kapun et al., 2021; Machado et al., 2021). In recent years, improvements in genome sequencing technology and affordability have led to an increasing number of studies using and producing whole genome datasets from natural *D. melanogaster* populations; however, such datasets are usually produced using

different sequencing technologies or analysis pipelines, making direct comparisons between populations difficult. The recently released *Drosophila* Evolution Over Space and Time (DEST) dataset, which contains 271 pooled sequencing (pool-seq) samples from 100 locations, 55 of which were sampled over multiple time points per year for at least 1 year, representing more than 13,000 flies collected across more than 20 countries and four continents, is the largest such dataset to date (Kapun et al., 2021). Intended as a community resource, this SNP dataset was a joint effort between the European DrosEU (Kapun et al., 2020) and the North American DrosRTEC (Machado et al., 2021) consortia and is easily accessed via a web-based genome browser and web portal (https://dest.bio). Such resources allow us to directly track evolution over time, including the response to natural environmental fluctuations, which can provide insights into the tempo and dynamics of evolution and adaptation. Indeed, a growing number of studies in various species have leveraged this type of population time series data to characterize genetic variation and evolution in natural populations over time (Mathieson et al., 2015; Hofmanová et al., 2016; Castañeda-Rico et al., 2020; Machado et al., 2021; Lange et al., 2022; Pfenninger & Foucault, 2022; reviewed in Johnson et al., 2023).

For example, one study using DrosRTEC data, found evidence of parallel seasonal adaptation via parallel allele frequency shifts among North American and European *D. melanogaster* populations (Machado et al., 2021). This study was also able to link environmental variation with changes in allele frequency, specifically weather conditions in the weeks prior to sampling predicted the direction of allele frequency shifts (Machado et al., 2021). Similarly, another study using DrosEU data was able to identify variants associated with putative local climate adaptation in European populations (Kapun et al., 2020). Interestingly, this study also found that European populations vary along longitudinal clines (Kapun et al., 2020) rather than the latitudinal clines that are well-documented in North America and Australia. Another study using North American populations found seasonal variation in immune response and that several previously identified (Bergland et al., 2014), seasonally fluctuating, immunity-associated alleles underlie some of the detected differences between seasons (Behrman et al., 2018). Recent studies using time series data spanning longer periods of time (i.e. over decades) have also yielded insights into how allele frequency clines evolve over time, with one study using a North American population finding that putatively adaptive allele frequency clines have strengthened in the past 35 years (Lange et al., 2022), while another study found that changes in latitudinal clines tend to be more gene- and continent-specific (Cogni et al., 2017).

As datasets such as DEST grow, it has been an ongoing effort to develop scalable pipelines to map and analyse such large datasets as well as to make them easily accessible (Hwang et al., 2019; Kapun et al., 2021). This is a particular issue for the growing number of long-read sequencing datasets, which often contain structural variants not present in the *D. melanogaster* reference genome, making direct comparisons between various genome assemblies difficult. The recently released, scalable, open-access browser DrosOmics (http://www.gonzalezlab.eu/drosomics) provides a solution to this problem by allowing the visualization of multiple genome assemblies at once (Coronado-Zamora et al., 2023). The current version contains 52 high-quality *D. melanogaster* genomes (Coronado-Zamora et al., 2023) and users can contribute their own datasets as well as use and view their own custom tracks.

2.2 | Temporal evolution under semi-natural conditions

Although the availability of high-quality spatiotemporal allele frequency data from natural *D. melanogaster* populations is improving, it remains difficult to obtain simultaneous, direct phenotypic and allele frequency estimates from the same natural population over multiple timepoints for an extended period of time. One recent study used 10 replicate *D. melanogaster* field populations founded from the same 80 isofemale strains, but allowed to evolve in independent outdoor cages in the same orchard, to track the evolution of genome-wide allele frequencies and fitness-associated phenotypes over the course of one growing season, spanning from summer to late fall and covering 10 generations (Rudman et al., 2022). The authors detected parallel, rapid and repeated phenotypic adaptation as well as widespread parallel genomic adaptation, with large, rapid shifts in phenotype and allele frequency occurring repeatedly and at multiple loci over time, which is consistent with strong and rapidly fluctuating selection (Rudman et al., 2022). Thus, phenotypic and genetic adaptation can be both rapid (occurring over just a few generations) and highly temporally dynamic, suggesting that adaptive tracking, that is, continuous and rapid adaptation to a rapidly changing environment, may be an important mechanism through which *D. melanogaster* populations cope with environmental changes. Indeed, previous studies in natural and experimental populations have suggested that phenotypic and the underlying genetic adaptation can proceed quite rapidly over short evolutionary timescales (Behrman et al., 2018; Rudman et al., 2019). Such rapidly fluctuating selection has previously been found to help maintain polymorphism within *D. melanogaster* populations over time via seasonal allele frequency fluctuations of selected and linked genetic variants (Bergland et al., 2014; Wittmann et al., 2017; Machado et al., 2021; but see Buffalo & Coop, 2020).

2.3 | Single-gene studies

Genome-wide studies have become an integral part of the characterization of selection in natural populations of *D. melanogaster*; however, single-gene studies remain an important and complementary part of the field. These individual examples of selection in action play a vital role as benchmarks in population genomic studies aiming to identify signatures of selection in the genome. Indeed, many studies utilizing genome-wide scans of selection have recovered signatures of well-documented selection at genes such as *Cyp6g1*, *Ace*

and *CHKov1* (e.g. see Duneau et al., 2018; Garud et al., 2021; Harris & Garud, 2023; Kapun et al., 2020). Single-gene studies are also important in their own right, as they can help us to better understand the myriad of mechanisms through which selection can act as well as the molecular functions and environmental conditions that drive it. Natural selection and the other forces that shape genetic variation are complex (Sella et al., 2009), and single-gene studies give us a window into this complexity. For some examples, see the discussion of ADH and *foraging* above.

One of the best described cases of selection in *D. melanogaster* occurs at the cytochrome P450 gene, *Cyp6g1*, where a resistance allele (DDT-R) containing an *Accord* transposable element insertion is associated with increased expression and resistance to the pesticide DDT (Daborn et al., 2002). The DDT-R allele spread rapidly in non-African populations and is thought to have undergone a selective sweep in response to the heavy use of DDT during the 1950s and 1960s (Catania et al., 2004). However, in the absence of DDT, the DDT-R allele is associated with increased fecundity and decreased developmental times in females, but genetic background-dependent, decreased reproductive success in males (Smith et al., 2011). Thus, the wild-type and DDT-R alleles are thought to have been maintained long-term in *D. melanogaster* populations due to sexually antagonistic balancing selection at this locus (Smith et al., 2011).

Another, more recent study documented the action of both sexually antagonistic and spatially varying selection on *a fatty-acyl CoA reductase* gene, *DsFAR2-B*, that affects cuticular hydrocarbon (CHC) traits in *Drosophila serrata* (Rusuwa et al., 2022). In *Drosophila*, CHCs serve as mating signals as well as protectants against abiotic stress and exhibit latitudinal clines that are thought to be maintained by spatially varying selection (Frentiu & Chenoweth, 2010; Rouault et al., 2000). In *D. serrata* populations, CHC phenotypes can be categorized into a *northern* and a *common* phenotype. Males with the *common* phenotype have increased mating success in comparison to *northern* males, while *northern* females show increased abiotic stress resistance (Rusuwa et al., 2022). These differences were narrowed down to polymorphism in the *DsFAR2-B* gene, which shows a signature of balancing selection in northern populations and similar phenotypes when its orthologue is knocked down in *D. melanogaster* (Rusuwa et al., 2022). Thus, a combination of sexually antagonistic and spatially varying selection appears to underlie the maintenance of variation at this locus and in this trait in natural *D. serrata* populations.

3 | THE *FEZZIK* GENE: A CASE STUDY

3.1 | Selection at the *fezzik* locus

The *fezzik* (*fiz*) gene is located on the X chromosome and its expression has been shown to affect a variety of traits, including larval growth rate, body size determination, cold and insecticide tolerance (Glaser-Schmitt & Parsch, 2018). In a study using replicate experimental populations of *D. melanogaster*, *fiz* was strongly down-regulated in populations adapted to larval malnutrition (Kawecki et al., 2021); while another study detected its orthologue as highly up-regulated in actively migrating *Episyrphus balteatus* in comparison to the non-migratory summer morph (Doyle et al., 2022). The *fiz* protein shows evidence for adaptive protein evolution since *D. melanogaster*'s divergence from *D. simulans* (Langley et al., 2012; Saminadin-Peter et al., 2012), with *fiz* ranked among the top candidates for adaptive evolution on the *D. melanogaster* lineage (Langley et al., 2012).

Fiz expression is typically two to five times higher in derived, cosmopolitan populations than in ancestral, sub-Saharan African populations (Glaser-Schmitt & Parsch, 2018; Glaser-Schmitt & Parsch, 2023; Hutter et al., 2008; Meiklejohn et al., 2003) and this expression difference is driven by variation in an upstream *cis*-regulatory element known as the *fiz* enhancer (Glaser-Schmitt & Parsch, 2018). The *fiz* enhancer has previously been shown to be a target of positive selection in derived populations for this expression increase (Glaser-Schmitt et al., 2013; Saminadin-Peter et al., 2012), which is driven by three SNPs. Two of these SNPs affect expression only in larvae, are fixed in cosmopolitan populations, and are thought to have been the targets of a selective sweep (Glaser-Schmitt & Parsch, 2018).

The third SNP, which is located 67 base pairs upstream of the *fiz* gene, is polymorphic in global populations: the derived variant (G) is associated with increased *fiz* expression and is common in cosmopolitan populations (average frequency of 41% in the examined populations; Glaser-Schmitt et al., 2021), while the ancestral variant (C) is in high frequency in sub-Saharan Africa (100% in the examined populations; Glaser-Schmitt & Parsch, 2018). Hereafter, we refer to this polymorphism as SNP67 and its two variants as SNP67G and SNP67C. The SNP67G variant has been present at intermediate frequency in derived, cosmopolitan populations for at least several decades, although it has likely been segregating in non-African populations since before *D. melanogaster*'s colonization of Europe (Glaser-Schmitt et al., 2021) approximately 1800 years ago (Sprengelmeyer et al., 2020). A previous study examining allele frequencies at SNP67 in a derived population from Munich, Germany sampled biannually for 5 years found overall differences in allele frequency between males and females as well as consistent changes in female but not male allele frequencies across seasons (Glaser-Schmitt et al., 2021). Modelling based on these data suggested that a combination of sexually antagonistic and temporally varying selection may help maintain polymorphism at this site, but other scenarios for its long-term maintenance could not be ruled out (Glaser-Schmitt et al., 2021). It has been proposed that this selection may be due to the sex-specific effects *fiz* expression has on adult starvation resistance (Glaser-Schmitt et al., 2021).

3.2 | Variation in SNP67 allele frequencies between sexes and over time

Figure 1a shows the allele frequencies at SNP67 for wild-caught *D. melanogaster* males and females collected from a population in

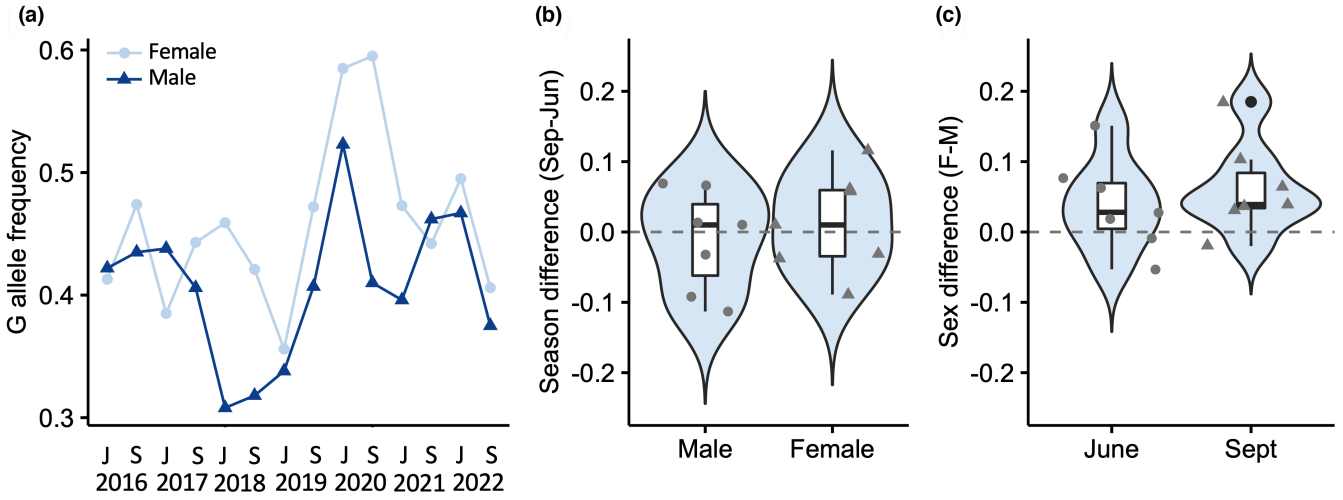

FIGURE 1 Variation at *fiz* position 67. (a) G allele frequency in males (dark, triangles) and females (light, circles) across all collections. (b) Difference in G allele frequency between September (Sep) and June (Jun) in males (circles) and females (triangles). No significant effect of season on the G allele frequency was detected for either a bootstrapping or a CMH test ($p > .5$ for both). (c) Difference in G allele frequency between males (M) and females (f) for June (circles) and September (Sept, triangles). Differences in allele frequency between sexes were detected as significant with both a bootstrapping test ($p = .0196$) and a CMH test ($p = .0472$).

Munich, Germany over a period of 7 years, with flies being collected in June and September of each year from 2016 to 2022, which includes the collections of Glaser-Schmitt et al. (2021) plus two additional collections made in 2021 and 2022 (Box 1). The frequency of the SNP67G variant was higher in females than in males for 11 of the 14 collections (Figure 1a,c). For all collections combined, this difference between the sexes was significant (Figure 1b). In contrast, there was no significant effect of season on the SNP67G allele frequency (Figure 1b). Similar to the previous study (Glaser-Schmitt et al., 2021) there was also no significant deviation from HWE in any of the collections (Figure S1).

For all of the collections shown in Figure 1, SNP67 allele frequencies were estimated from a greater number of female alleles than male alleles (Figure 2a). There are two reasons for this difference in sample size between the sexes. First, because *fiz* is X-linked, each female has two alleles, while each male has only one. Second, a greater number of females than males were sampled in 13 of the 14 collections. Previous work has shown that this imbalance is not caused by an unequal sex ratio at the time of eclosion (Glaser-Schmitt et al., 2021), but may instead be a result of increased female attraction to food sources for oviposition or increased male mortality in nature. Given this difference in sample size between sexes, it is unexpected that significantly higher variance in allele frequency across collections was detected in females than in males (Figure 2b).

3.3 | SNP67 allele frequencies at a second sampling location

To test for potential local population structure, which would be indicative of partially isolated populations of small size, we genotyped flies from a second Munich location approximately 12.4 km from the main sampling site in July 2022, which is during the peak season for *Drosophila* abundance in the area (Box 1). In contrast to the majority of collections at the primary sampling site (Table S1), we collected similar numbers of males and females (Table S2) at the second sampling location, resulting in a larger proportion of male alleles (Figure 3a). This difference between sampling locations may be due to sampling variation, different sampling time, or bait. In this population, SNP67G also segregated at intermediate frequency (65.6%), which was significantly higher than the SNP67G frequency for the 2022 collections (44.1%) as well as across all collections (44.6%) at the primary sampling site ($p < 10^{-7}$ for both, Chi-Square test). Similar to overall trends at the primary collection site (Figure 1c), the frequency of the SNP67G allele was higher in females (68.6%) than in males (58.7%) (Figure 3b) which was marginally non-significant. However, it should be noted that because only a single collection was made at this location, statistical power is lacking to detect differences between sexes in comparison to the primary collection site.

A significant deviation from HWE was detected at the second site, with there being a significant deficiency of heterozygotes (Figure 3c, Table S2), which could be indicative of underdominant selection (also known as homozygote advantage), inbreeding or population subdivision (also known as the Wahlund effect). If this deficiency of heterozygotes were driven by homozygote advantage or inbreeding, we would expect the effect would be more widespread in Munich leading to deficiencies in heterozygotes in some of the other collections, which makes these scenarios less likely. Together with the frequency difference we observed between sampling locations, this deficiency may reflect population substructure within Munich (i.e. a spatial Wahlund effect), with there being relatively small, local populations similar to what has been suggested for a North American population (Lange et al., 2022). It is also possible that this difference is due to slight changes in genetic background and dominance between these

BOX 1 ***D. melanogaster*** **samples and SNP67 genotyping**

From 2016 to 2022, wild *D. melanogaster* were sampled from a population in Munich, Germany (latitude: 48.18, longitude: 11.61) twice per year in late June and early September, which represents the approximate beginning and end of the breeding season in Munich. Sampling was performed at the same time each year and season, with approximately 2.5 months between the June and September collections and approximately 9.5 months between the September collection and June collection of the following year. Data from 2016 to 2020 have previously been described (Glaser-Schmitt et al., 2021). We further sampled from a second Munich site (latitude: 48.12, longitude: 11.47) 12.4 km from the first sampling site in late July 2022, which represents the approximate peak of the breeding season and lies halfway between the June and September collections. Genotyping of SNP67 was carried out using DNA extraction and PCR followed by a restriction enzyme-based assay (Glaser-Schmitt & Parsch, 2018). For the June and September collections, we tested for differences in allele frequency between seasons or sexes using both a Cochran–Mantel–Haenszel (CMH) test and a bootstrapping test, which allow for the detection of consistent directional patterns across collections but may give slightly different *p*-values due to the different nature of these tests (see Glaser-Schmitt et al., 2021; Supplemental Methods). For female genotypes, we tested for deviations from expectations under HWE using a Chi-Square test. At the second sampling site, we tested for differences in allele frequency between the sexes using a Fisher's exact test. For more details, see the Supplemental Methods.

sampling locations modulating the selection coefficient and therefore shifting the allele frequencies that we detected. However, given the low level of differentiation that has been detected among both geographically proximate as well as more distant European populations (Kapun et al., 2020), these scenarios seem less likely. Another possibility is that this difference is due to sampling time. Previous modelling work has suggested that polymorphism at SNP67 behaves nonmonotonically, with large, rapid frequency jumps occurring relatively often between the two annual sampling points. Thus, the observed difference in SNP67G frequency between the two Munich sampling sites suggests that there may be larger, even more rapid allele frequency shifts within this population that we have not been able to detect with our sampling scheme. Indeed, the detected deficiency of heterozygotes specifically at the second sampling location may be a temporal Wahlund effect driven by a rapid, large change in selection resulting in a sampling of individuals from two divergent selection regimes during the same collection.

3.4 | Evolutionary scenarios for the maintenance of the SNP67 polymorphism

To explore evolutionary scenarios that could result in patterns of allele frequency variation similar to those observed at SNP67 over 7 years in the Munich population (Figure 1a), we performed individual-based, forward simulations that accounted for both genetic drift and the sample sizes used to estimate allele frequencies for each sex and collection (Box 2). We considered a neutral model, which included only genetic drift and sampling variance, as well as models that included either sexually antagonistic selection, temporally fluctuating selection, or both (Table 1). Assuming an initial allele frequency of 0.40 and a population size of 100,000, which is a conservatively low estimate for the X chromosome of *D. melanogaster* in Europe (Hutter et al., 2007; Kapopoulou et al., 2020; Laurent et al., 2011), genetic drift was able to maintain a polymorphism at intermediate frequency (minor allele frequency ≥ 20%) for well over 500 generations (50 years), which exceeds the age (ca. 30 years) of the oldest collection for which SNP67 allele frequency data are available (Glaser-Schmitt & Parsch, 2018). However, genetic drift cannot explain two other aspects of our data: (i) the higher frequency of SNP67G in females than in males (Figure 1c), and (ii) the higher variance in SNP67G frequency in females than in males (Figure 2b). This is illustrated in Figure 4, which shows the simulated distributions of the female:male ratio of SNP67G frequency and its variance in relation to the observed values (Figure 2a). Although genetic drift could produce a sex difference in allele frequency in rare cases (approximately 5% of the simulated distribution lies at or above the observed value), it could not cause a sex difference in variance as extreme as the observed value (Figure 4). Indeed, the simulated female:male variance values fall well below the observed value, which is expected given that the number of alleles sampled at each collection was much lower for males than for females (Figure 2a).

Models of sexually antagonistic selection, particularly when SNP67G has a dominant or co-dominant (additive) effect on female fitness, were able to produce sex differences in allele frequency similar to the observed value; however, like the drift model, they did not lead to a sex difference in variance as extreme as the observed value (Figure 4). In the case of the fully recessive model, polymorphism could only be maintained when selection was relatively weak ($s \leq 0.015$). This is because the detrimental effect of SNP67G is always expressed in hemizygous males but its beneficial effect is expressed only in homozygous females. When selection is stronger or more generations are considered, the recessive male-deleterious/female-beneficial allele is lost from the population.

Temporally fluctuating selection can slightly increase the median female:male ratio of SNP67 frequency and broaden its distribution (Figure 4). This results in greater overlap with the observed value, with 8–15% of the simulated values being as (or more) extreme than the observed value, depending on dominance. However, temporally fluctuating selection leads to only a modest increase in the female:male ratio of SNP67 variance, with only slight overlaps with the observed value in the co-dominant (2%) and recessive

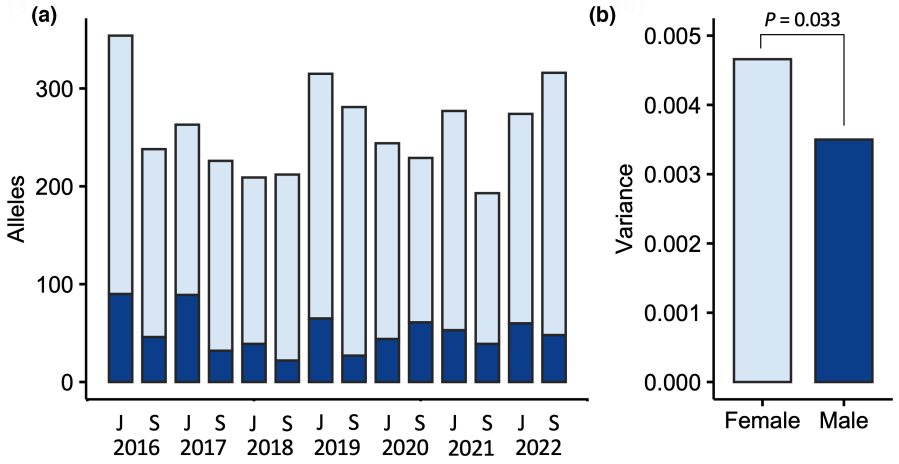

FIGURE 2 Sex differences in allele number and variance. (a) Total number of alleles genotyped in females (light) and males (dark) in each collection (J, June; S, September) and year. (b) Variance in G allele frequency in females (light) and males (dark) across all collections. Significance was assessed with a bootstrapping test to account for differences between the number of female and male alleles sampled at each collection.

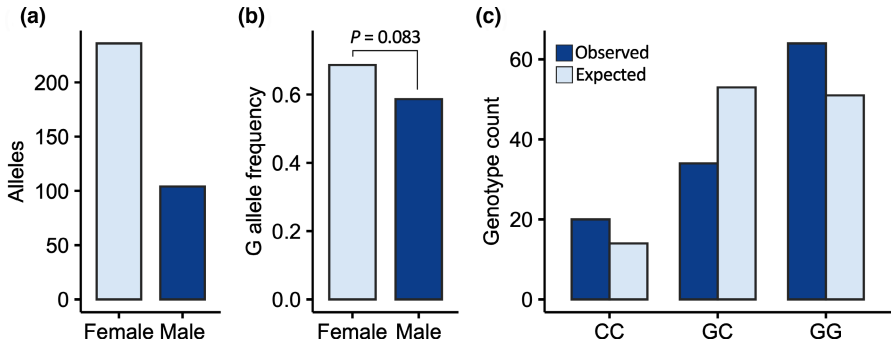

FIGURE 3 Data from a second sampling site in July 2022. (a) Total number of alleles genotyped in females (light) and males (dark). (b) Frequency of the G allele in females (light) and males (dark). Significance was assessed with a Fisher's exact test. (c) Observed (dark) and expected (light) female genotypes. A significant deficiency of heterozygotes was detected ($p = .0355$, Chi-Square test).

TABLE 1 Models and fitness values.

	Phase 1					Phase 2				
Model	G_M	C_M	GG_F	GC_F	CC_F	G_M	C_M	GG_F	GC_F	CC_F
Drift	1	1	1	1	1	1	1	1	1	1
SA_dom	$1-s$	1	1	1	$1-s$	$1-s$	1	1	1	$1-s$
SA_co	$1-s$	1	1	$(2-s)/2$	$1-s$	$1-s$	1	1	$(2-s)/2$	$1-s$
SA_rec	$1-s$	1	1	$1-s$	$1-s$	$1-s$	1	1	$1-s$	$1-s$
TF_dom	$1-s$	1	$1-s$	$1-s$	1	1	$1-s$	1	1	$1-s$
TF_co	$1-s$	1	$1-s$	$(2-s)/2$	1	1	$1-s$	1	$(2-s)/2$	$1-s$
TF_rec	$1-s$	1	$1-s$	1	1	1	$1-s$	1	$1-s$	$1-s$
SA+TF_dom	$1-s$	1	$1-t$	$1-t$	1	$1-s$	1	1	1	$1-t$
SA+TF_co	$1-s$	1	$1-t$	$(2-t)/2$	1	$1-s$	1	1	$(2-t)/2$	$1-t$
SA+TF_rec	$1-s$	1	$1-t$	1	1	$1-s$	1	1	$1-t$	$1-t$

Note: Shown are fitness values and selection coefficients (s and t) for male (M) and female (F) genotypes under the genetic drift model and models including sexually antagonistic (SA) and/or temporally fluctuating (TF) selection. For the selective models, the effect of the G allele in females is assumed to be dominant (dom), co-dominant (co) or recessive (rec). For models including TF selection, fitness values vary over time, with two selective phases (Phase 1 and Phase 2) per year.

(6%) models. For the dominant model, the polymorphism is stable only when selection is weak ($s \leq 0.1$) relative to the other models. Stronger selection leads to a rapid reduction in the frequency of the deleterious allele during one of the phases and its eventual loss from the population.

The models most likely to produce allele frequency patterns similar to the observed data were those that incorporated both sexually antagonistic and temporally fluctuating selection, particularly when the effect of SNP67G on female fitness was co-dominant or recessive (Figure 4). These models could increase the female:male

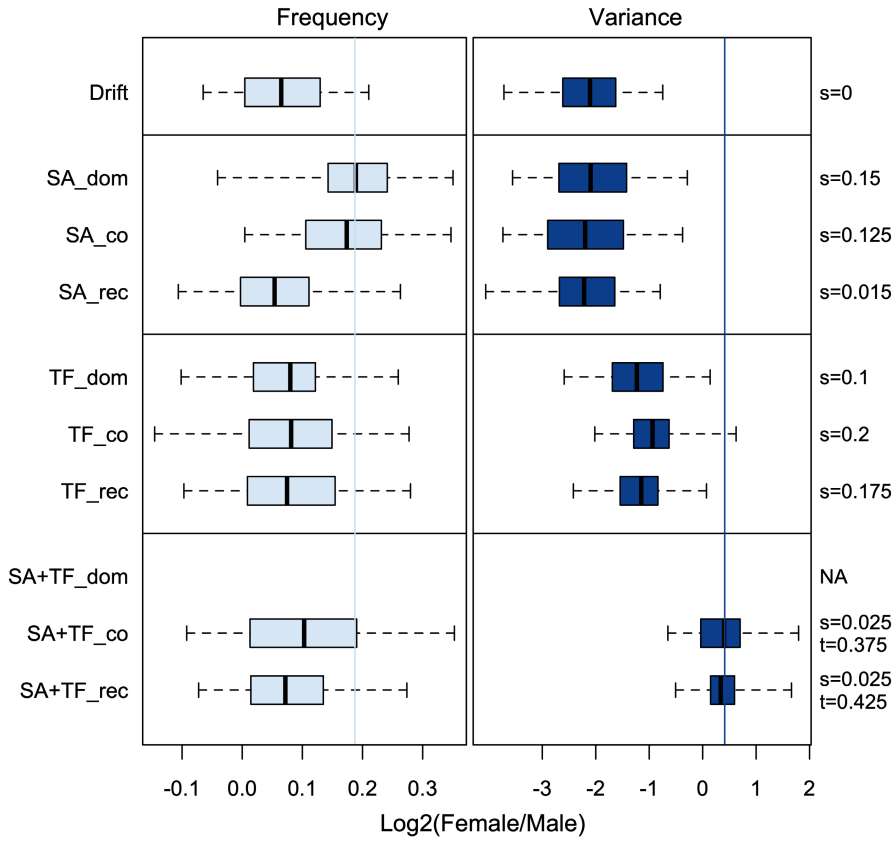

FIGURE 4 Results of simulations using the models shown in Table 1. The boxplots represent the distributions of simulated values of the female:male ratio of SNP67G frequency (light blue) or the variance in SNP67G frequency (dark blue) across 14 biannual collections with the sample sizes depicted in Figure 2a. Boxes indicate the interquartile distance and dashed lines span the 2.5% and 97.5% quantiles. The observed values for the Munich population are indicated by vertical lines of corresponding colour. The selection parameters (*s* and *t*) resulting in the greatest overlap with the observed values were used for the plots and are shown at the right. The simulations assumed a population size of 100,000 and 10 generations per year. The SA + TF_dom model did not lead to a stable polymorphism and was not plotted. NA, not applicable.

ratio of SNP67G frequency similar to the models that included only temporally fluctuating selection, with 10–23% of their distributions overlapping with the observed value. Importantly, these were the only models that could consistently generate female:male ratios of SNP67G frequency variance similar to the observed values, with approximately 50% of the simulated values being as (or more) extreme than the observed value. These models performed best when the effect of the sexually antagonistic allele was weak in males ($s = 0.025$), but the effect of temporally fluctuating selection was strong in females (*s* in the range 0.375–0.425). In the case of the dominant model, polymorphism could not be maintained, even for values of *s* and *t* as low as 0.01. This is because there is always selection against males bearing the male-deleterious allele and, in one of the phases, selection against females bearing the same allele, whether they are homozygous or heterozygous. This leads to a rapid loss of the male-deleterious allele.

3.5 | Temporally varying and sexually antagonistic selection most likely act on SNP67

Collectively, the patterns we observed at SNP67 (rapid allele frequency shifts, significant differences in allele frequency between sexes and higher variance in females despite much larger sample sizes) are highly unlikely to be caused by genetic drift alone, or by the individual action of sexually antagonistic or temporally fluctuating selection. Instead, the observations are much better explained by a model that includes the combined action of both forms of selection, with the effect of the sexually antagonistic allele being relatively weak in males, but the effect of temporally fluctuating selection being strong in females (Figure 4). Such strong selection is required in order to see differences in allele frequency or its variance between the sexes over the short time scales considered here. In the case of sexual antagonism, the difference in allele frequency between sexes must be established every generation, as the X chromosome passes between the sexes at each generation. In the case of temporally fluctuating selection, differences can accumulate during each selective phase. However, for temperate populations of *D. melanogaster*, each phase is likely to comprise only five to eight generations. For these reasons, our simulation results are robust to differences in population size or generation time (Figures S2 and S3).

The main driver of the differences in allele frequency between the sexes at SNP67 appears to be female-specific temporally fluctuating selection. In line with this observation, previous work found that *fiz* expression affects adult starvation resistance in a sex-specific manner, and that in the genetic background of a natural population, variation at SNP67 has a stronger effect in females than in males (Glaser-Schmitt et al., 2021), suggesting that adult starvation resistance may be the phenotype under selection. However, it is possible that a combination of it and/or other traits that *fiz* expression has been proposed or demonstrated to affect (see Glaser-Schmitt & Parsch, 2018; Kawecki et al., 2021; Doyle et al., 2022 for examples) are the target(s) of selection. Similarly, it is important to note that there are many factors relevant to selection in nature that are not accounted for in the models we considered. For example, in nature variable selection is unlikely

BOX 2 Simulating allele frequency dynamics

To evaluate potential neutral or selective scenarios that could generate allele frequencies consistent with the *fiz* SNP67 data, we performed simulations following the general framework of Glaser-Schmitt et al. (2021), but with two major modifications. First, we assumed a finite population of size 100,000, in contrast to the previous deterministic model, which assumed an infinite population. This allowed us to account for the effects of genetic drift, which can alter allele frequencies over time. Second, we accounted for the sampling variance inherent in estimating allele frequencies from a small subset of the population that has been genotyped, whereas the previous model assumed perfect knowledge of the allele frequencies. To achieve this, we performed random binomial sampling of alleles based on their population frequencies to generate samples of male and female alleles of the same sample size as our observed data (Figure 2a). The subsampled data were then used to estimate allele frequencies for each sex and collection.

We considered an X-linked locus with two alleles (G and C) and random mating. The initial frequency of the G allele was set to 40% to match the SNP67G frequency in the first Munich collection and the average frequency across multiple European populations (Glaser-Schmitt et al., 2021; Kapun et al., 2020; Kapun et al., 2021). We assumed a total of 10 generations per year, divided equally into two phases of five generations each, with samples being collected for genotyping at the end of each phase. Within each phase and for each sex, a fitness value was assigned to each genotype, with the most fit genotype having the value of 1 and the least fit genotype having a value of $1 - s$. For models with two selection parameters, the fitness parameter t was included and used analogously to s. Viability selection was simulated at each generation by randomly eliminating individuals from the population, with the probability of elimination being equal to s (or t). Alleles of the next generation were then randomly drawn from the surviving parental genotypes, with males receiving one allele from a female parent and females receiving one allele each from a male and a female parent.

We focused on four major models, with each model having three sub-models depending on the dominance of the G allele when appropriate (Table 1). The first model considered only random genetic drift, with all genotypes being assigned a fitness value of 1 in both phases and sexes. The second model allowed for sexually antagonistic selection, with the G allele conferring higher fitness in females and the C allele conferring higher fitness in males in both phases. The third model allowed for temporally fluctuating selection, with the C allele conferring higher fitness in phase 1 and the G allele conferring higher fitness in phase 2 for both sexes. The fourth model incorporated both sexually antagonistic and temporally fluctuating selection, allowing the fitness associated with the two alleles to differ between sexes and between phases in females.

For each of the selective models, we considered a range of values for the selection parameter s (and t) ranging from 0 to 0.5. For each parameter value we performed 100 simulations and determined the resulting distributions of two summary statistics: (i) the mean female:male ratio of G allele frequency over all collection, and (ii) the female:male ratio of the variance in G allele frequency over all collections. The parameters leading to distributions with the greatest overlap with the observed values are shown in Figure 4. To assess how uncertainty in the generation time or local population size of free-living *D. melanogaster*, which may vary among collection sites (Lange et al., 2022; Pool, 2015), affects our results, we repeated the simulations using a total of 16 generations per year (8 per phase) or a population size of 5000 (Figures S2 and S3). All simulations were carried out in R (R Core Team, 2022) using custom scripts (see Supplemental R scripts S1–S3).

to occur in discrete phases of equal length that remain constant every year and align perfectly with sample collection points. Furthermore, the strength of selection may vary within a phase, or between corresponding phases in different years. It is also possible that the effect of an allele on fitness and/or its dominance will be modified by the genetic background. For these reasons, we do not expect our models to perfectly capture the situation in nature. Indeed, our results suggest that the detected fluctuations in allele frequency are not purely seasonal, but sometimes may be annual (Figure 1a). Furthermore, it has been reported that the degree of phenotypic dominance at SNP67 can vary depending on the genetic background (Glaser-Schmitt et al., 2021). Thus, it is possible that in natural populations dominance at SNP67 varies temporally and/or spatially, which may also contribute to the maintenance of polymorphism at this locus.

4 | CONCLUSION AND OUTLOOK

As demonstrated by the *fiz* example in section 3 above, temporally fluctuating and sexually antagonistic selection can lead to rapid changes in allele frequency, but can also constrain these changes, leading to a balanced polymorphism that is maintained over long periods of time. Indeed, single-gene studies, such as those discussed here, demonstrate the complexity of the mechanisms that help maintain polymorphism in natural populations and that these forces are not mutually exclusive and can act simultaneously on the same locus. These single-gene studies serve as complements to population genomic studies, which can give us insight into how polymorphism in natural populations is maintained across many loci. As the number of studies documenting spatiotemporal genetic variation in wild *D. melanogaster* populations continues to

grow, these population genomic and single-gene studies should help us to refine our understanding of the forces driving and limiting adaptation in nature.

AUTHORS' CONTRIBUTIONS

JP conceptualized the study. JP and AGS collected the samples. AGS and TJSR processed the samples and collected the data. JP and AGS analysed the data. JP performed the simulations. AGS and JP wrote the manuscript. All authors edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

DATA ACCESSIBILITY AND BENEFIT-SHARING STATEMENT

All data are included in the main text or in Supplemental Tables S1 and S2.

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