Key words: lethal, mutation-selection balance, heterozygote advantage, associative overdominance, linked selection, supergene

Abstract

For nearly a century, evolutionary biologists have observed chromosomes which cause lethality when made homozygous persisting at surprisingly high frequencies (>25%) in natural populations of many species. The evolutionary force(s) responsible for the maintenance of such detrimental mutations have been heavily debated – are some lethal mutations under balancing selection? We suggest that mutation-selection balance alone cannot explain lethal variation in nature and the possibility that other forces play a role. We review the potential that linked selection in particular may drive maintenance of lethal alleles through associative overdominance, linkage to beneficial mutations, or by reducing effective population size. Over the past five decades, investigation into this mystery has tapered. During this time, key scientific advances have been made that give us the ability to collect more accurate data and analyze it in new ways, making the underlying genetic bases and evolutionary forces of lethal alleles timely for study once more.

Introduction

Mutation introduces genetic diversity, and genetic drift decreases diversity by eventually fixing or eliminating mutations in natural populations. Because genetic drift is stronger in smaller populations, the Neutral Theory of Molecular Evolution predicts neutral genetic diversity to be positively correlated with effective population size (M. Kimura, 1968). However, the prevalence and type of natural selection also influence genetic diversity. Positive and purifying selection reduce genetic diversity, while balancing selection increases genetic diversity. Lastly, low recombination rate (increased linkage) can increase the effect of selection on genetic diversity (whether reducing or maintaining diversity) in areas around the selected sites.

While a subset of genetic variants can be beneficial, mutations with deleterious effects curiously often contribute to standing genetic variation in natural populations (Simmons & Crow, 1977). Individual deleterious mutations, even if recessive in their negative effects, should only persist at low frequencies due to the balance between recurrent new mutations and purifying selection (mutation-selection balance), but what forces could be at play in creating the higher frequencies documented for some detrimental alleles? Naturally segregating recessive lethal alleles (hereby referred to as 'lethals') are of particular interest because, even at low frequencies, such an extreme detrimental fitness effect can contribute greatly to heritable variation in fitness.

Numerous studies in multiple species of *Drosophila* estimated that over half of the fitness variation in natural populations, and thus effects of inbreeding depression, are produced by lethal mutations (Deborah Charlesworth & Charlesworth, 1987; Simmons & Crow, 1977). The curiously high overall frequencies (>25%) of lethal chromosomes within various species (Simmons & Crow, 1977) challenge evolutionary biologists to distinguish evolutionary forces that allow their persistence in the face of extraordinarily strong purifying selection. Although research on this question dates to the 1930's, we still know remarkably little about the genetic basis of lethals and what evolutionary forces maintain their abundance. Surprisingly high overall lethal frequencies have been observed across numerous species, including humans, zebrafish and

bluefin killifish (Ballinger & Noor, 2018), but lethals have been most intensely studied in *Drosophila* due to their ease of laboratory culturing and genetic tools that allow sampling of entire wild chromosomes to test for homozygous lethal effects. In this review, we will focus on evolutionary forces that could maintain lethal alleles in any natural population, and we will primarily discuss results from studies of *Drosophila*, which comprises most of the data available.

Although many recessive lethal chromosomes have been isolated, few of the underlying genes have been identified (Derks et al., 2019). Researchers have assumed that recessive lethal effects in wild chromosomes are largely due to single locus, loss-of-function mutations rather than combined polygenic effects. Our review will assume the same throughout. While this hypothesis has not been tested directly in most systems, some observations suggest the assumption is true (Lewontin, 1974) and other studies found that many lethal alleles result from loss-of-function effects (e.g., Derks et al., 2018, 2019). Furthermore, unpublished results of an ongoing study in our laboratory also suggest that naturally occurring lethal alleles in *Drosophila melanogaster* are often loss-of-function and due to single locus mutations (as opposed to some sort of multigenic effects, "synthetic lethals" (Spiess et al., 1963)).

Multiple evolutionary forces might allow lethal mutations to reach or persist at non-zero frequencies. Previous debate focused on force(s) that act directly on lethal mutations: e.g., whether individual lethal frequencies are determined by a push and pull between recurrent lethal mutations and purifying selection (mutation-selection balance). Alternatively, some lethal alleles may be maintained at higher than expected frequencies because of selective forces like classical overdominance (Simmons & Crow, 1977). The contributions of mutation-selection balance versus classical overdominance on lethal allele frequencies were heavily researched between the 1930's and 1970's. However, other forces that may *indirectly* allow lethal alleles to reach high frequencies through linked selection remain unchartered territory. For example, lethal allele frequencies may be influenced through linkage with loci under different forms of selection (e.g. associative overdominance) or through the impact of recombination rate on N_e variation throughout the genome.

In this review, we consolidate previous research exploring three underlying questions and propose how to use modern ideas and techniques to determine the answers to each:

1. Does *mutation-selection balance alone* explain lethal variation in natural populations?
2. Does *classical overdominance* (or other forms of direct, single-locus, balancing selection) play a role in maintaining some lethal variation?
3. How might *linked selection* and *genetic drift* play a role in allowing persistence of lethal variation?

1. Does *mutation-selection balance alone* explain lethal variation in natural populations?

The continuous battle between recurrent lethal mutations increasing and purifying selection decreasing lethal allele frequency (mutation-selection balance) often results in an equilibrium frequency of individual lethal alleles. All other potential evolutionary forces would act on top of the baseline created by mutation-selection balance and ever-present genetic drift. The expected equilibrium frequency of a recessive allele (in the absence of drift, which will be

explored later) is dependent on the lethal mutation rate (μ) and the lethal selection coefficient (s = 1) (Haldane, 1937).

$$q_l = \sqrt{\frac{\mu}{s}} = \sqrt{\mu}$$

Average *Drosophila* mutation rates per nucleotide (combining point mutations and indels) are estimated by mutation accumulation (MA) experiments to be on the order of 10^{-9} (Haag-Liautard et al., 2007; Huang et al., 2016; Keightley et al., 2009; Schrider et al., 2013). Even if the lethal mutation rate by itself was as high as this estimate, the expected equilibrium frequency of any lethal mutation due to mutation selection balance alone should be very low, on the order of 10^{-5}. However, MA experiments may underestimate mutation rate because they do not allow for the accumulation of extremely deleterious mutations (Huang et al., 2016). Importantly, 10^{-9} is an *average* across all nucleotides, and some regions likely having higher lethal mutation rates than others. We know of no studies that have determined the landscape of mutation rates across the genome, but recent evolutionary simulations suggests that intra-chromosomal mutation rate variation plays a measurable role in shaping genomic diversity (Barroso & Dutheil, 2021). Another consideration is that the "phenotypic" mutation rate may be higher than the per nucleotide rate because different mutations in the same gene may be capable of recessive lethal phenotypes. Older studies estimated 0.0060 lethals per chromosome per generation (Mukai et al., 1972) and estimated a *per locus* (i.e., band on a salivary chromosome) lethal mutation rate on the order of 10^{-6} per generation. Even combining mutations and ~~across this larger swath of the genome and~~ inferring them all to affect a single locus would still result in an equilibrium lethal phenotypic frequency per locus far less than 1%. If no other forces besides mutation and deleterious selection were acting on lethal alleles, most lethals sampled from natural populations (assuming a sample size not exceeding millions) would be expected to appear only once (singletons). If certain lethal alleles did appear at higher frequencies due to drift, these high frequencies would likely not persist over many generations.

Numerous studies in the mid-1900's found high overall proportions of lethal chromosome frequencies in the second and third chromosomes of *D. melanogaster* (typically between 15% - 30%, but ranging from 8% to as high as 40%) (Simmons & Crow, 1977). High overall frequencies of lethal chromosomes ranging between 20% to 40% have been reported in other *Drosophila* species including *D. pseudoobscura*, *D. willistoni* and *D. subobscura* (T. Dobzhansky et al., 1963; Malogolowkin-Cohen et al., 1964; F. Mestres et al., 1990).

Although there were high overall rates of lethality in many populations, many studies found low allelism rates and low frequencies of nearly all *individual* lethal alleles, consistent with equilibrium frequencies predicted by a mutation-selection balance model (Table 1).

Table 1. Overall and Individual Lethal Allele Frequencies in Natural Populations in *Drosophila melanogaster* Chromosome II*

References	Population	Year	N	Lethal Chromosome Frequency	Highest Individual Lethal Allele Frequency	Average Allelis Frequency
(Hoensgsberg et al., 1969)	Hungary	June, 1964	961	0.08	NA	NA

(T. K. Watanabe, 1967; T. K. Watanabe, 1969)	Katsunuma, Japan	October 1963 – October 1968	4267	0.16	2.16%	3.50% (range 1.95%-6
(Kosuda, 1971)	Yamanashi, Japan	Autumn, 1968	289	0.16	NA	NA
(Band, 1964; Band & Ives, 1963)	South Amherst, MA, USA	September 1960-1962	469	0.22	1.32%	No average repo (range 0.4%-1%
(Temin et al., 1969)	Madison, Wisconsin, USA	Fall, 1962, 1963, 1965	1855	0.23	NA	NA
(Hoensgsberg et al., 1969)	Colombia	November 1963 - October 1964	1115	0.26	NA	NA
(Dawood, 1961)	Egypt	Summer, 1957	301	0.26	NA	NA
(Ives & Band, 1986)	South Amherst, MA, USA	October - November, 1970-1977	2295	0.29	NA	
(Ives & Band, 1986)	South Amherst, MA, USA	June - July, 1970-1977	1788	0.29	NA	
(Takao K. Watanabe et al., 1976)	Katsunuma, Japan	October 1970-1973	1377	0.32	NA	3.43% (range 1.46%-5
(Ives, 1945)	MA, USA	1940's	151	0.32	1.99%	
(Mukai & Yamaguchi, 1974)	Raleigh, NC, USA	August, 1970	691	0.40	NA	0.04%
(Ives, 1945)	FL, USA	April, 1940	227	0.44	0.88%	

*The more limited data on lethal allele frequencies in D. melanogaster chromosome 3 showed similar frequencies to the chromosome 2 data.

The typically low lethal allele frequencies (less than 1%) are consistent with expectations from a mutation-selection balance model, but some lethal alleles were more common than others and *persisted* for many years (Table 1). For example, in a natural Japanese populations of *Drosophila melanogaster*, multiple lethal alleles were found to persist at measurable frequencies across several years (T. K. Watanabe & Oshima, 1970). In fact, one lethal allele persisted at a relatively stable 1% frequency over an 8-year span. The average allelism rate for lethal second chromosomes was much higher at ~3.48% for lethals sampled in the same year and ~1.9% on average if sampled one year apart (T. K. Watanabe & Oshima, 1970), and they estimated that ~40% of lethal genes persist in this population for at least one year.

These results are not unique to *Drosophila melanogaster*. High frequencies of *individual* lethal alleles have been reported in *D. subobscura* (~3.6%) (F. Mestres et al., 1990). Beyond *Drosophila*, individual lethal frequencies have been estimated between 2% to 15% in orchard grass and >5% in pigs (Apirion & Zohary, 1961; Derks et al., 2018). Even if only present at

~1% frequencies, unless lethal mutation rates were on the order of 10^{-4} (and the lethal alleles are completely recessive), such persistent frequencies cannot be explained easily by mutation-selection balance alone.

2. Does *classical overdominance* (or other forms of direct, single locus balancing selection) play a role in maintaining lethal variation?

In contrast to a mutation-selection model, if a lethal mutation persisted via some form of balancing selection, the equilibrium frequency would be likely much higher than that of mutation-selection balance. The most well-studied type of balancing selection, heterozygote advantage, results in a lethal equilibrium frequency negatively correlated with the fitness difference between a lethal heterozygote and homozygous wild type (t) (Th. Dobzhansky, 1970). The following equation is specific to lethal alleles where the fitness difference between the heterozygous individual and homozygous lethal is 1 (s). Even a heterozygote advantage of 1% ($t = 0.01$) would result in ~0.01 lethal allele frequency (q_l).

$$q_l = \frac{t}{t+s} = \frac{t}{t+1}$$

In addition to measuring lethal allele frequencies, researchers calculated lab-assayed fitness and found that individuals heterozygous for lethal-bearing chromosomes showed, on *average*, reduced lab-assayed viability of ~0.02-0.03, but also found significant variance across lethal lines (Hiraizumi & Crow, 1960; Mukai et al., 1972). One study that showed viability estimates for each lethal heterozygote line reported that 23 out of 53 *D. melanogaster* lethal lines had *higher* heterozygote viability than the average control viability (Hiraizumi & Crow, 1960), though it remains unclear how much this reflected effects of the lethal alleles themselves vs. other alleles across the chromosome. In another study, lethal heterozygotes on average had reduced fitness in a Japanese population of *D.melanogaster*, but chromosomes that carried persistent lethal alleles (in a population for at least one year) tended to be completely recessive or have heterotic effects (T. K. Watanabe & Oshima, 1970). Further studies found either no evidence for reduced fitness of heterozygotes or found evidence that external factors, such as high temperature, narrow temperature range, and genetic background can result in lethal heterosis (Sturtevant, 1937). And outside of Drosophila, recent research discovered a high-frequency (>4%) lethal gene disruption in a commercial pig population that persisted for at least 7 years (Derks et al., 2018, 2019). While homozygous lethal, individuals carrying one lethal haplotype gain fitness advantage due to increased fertility. Crested newts show a balanced lethal system due to extreme heterozygote advantage. All adults are carry complementary lethal haplotype in a region of no recombination, resulting in 50% of all offspring dying in development (Grossen et al., 2012). Overall, the oft-quoted statement that lethal heterozygotes have lower fitness on average loses much of the nuance of the reported empirical results.

Box 1: Conditions that could favor lethal heterozygote advantage

Genetic background	Temperature

Local Background	Narrow temperature range
Species: *D. melanogaster* **Study:** Longitudinal study of homozygous vs. heterozygous viabilities of laboratory flies with radiation induced homozygous lethality. **Results:** Heterozygote lethal and semi-lethal viabilities increased over time in some laboratory populations of *Drosophila melanogaster* but only when wildtype background was local (Wallace, 1962). **Species:** *D. pseudoobscura* **Study:** Compare viabilities of heterozygotes carrying a wild lethal chromosome with heterozygotes carrying a wild non-lethal second chromosome (on both local and foreign backgrounds). **Results:** Lethal chromosomes heterozygous with local backgrounds showed an average advantage over the control, but on a laboratory or foreign background, heterozygotes were at a disadvantage (Anderson, 1969; T. Dobzhansky & Spassky, 1968).	**Species:** *D. melanogaster* **Study:** Compare viabilities of semi-lethal and le heterozygous and non-lethal heterozygotes in fl varying ranges of daily temperatures (with a cor average temperature across populations). **Results:** Lethal and semi-lethal allele frequenci negatively associated with daily temperature ra natural populations. Semi-lethal and lethal heter had higher viabilities than non-lethal heterozygo range conditions, but non-lethal heterozygotes h viabilities in wide-range environment (Band, 19
Presence of inversion	High temperature
Species: *D. melanogaster* **Study:** Compare viabilities of heterozygotes carrying a wild lethal chromosome with heterozygotes carrying a wild non-lethal second chromosome (with both inversion-carrying and inversion-free wild second chromosomes). **Results:** In non-inversion flies, average viability of lethal heterozygotes is significantly less than non-lethal heterozygotes. In inversion-carrying flies, average viability of lethal heterozygotes was no different than average viability of control (Mukai & Yamaguchi, 1974).	**Species:** *D. melanogaster* **Study:** Compare viabilities of lethal heterozygo lethal heterozygotes in flies kept at different tem (17º, 25º, and 29º C). **Results:** Lethal heterozygote viability was direc temperature: at 17º C and 25º C heterozygotes fo chromosomes had, on average, reduced viability relative to the control. At 29ºC, however, the av viability was on average higher for lethal hetero ~ 1.5% (Tobari, 1966).

Although the evidence was mixed, by the 1970's, most researchers concluded that mutation-selection balance was the primary force behind lethal chromosome maintenance in natural populations and that contributions from balancing selection are negligible (D. Charlesworth & Charlesworth, 1987; Simmons & Crow, 1977). For the following reasons, we propose that the results summarized above (Table 1 and Box 1) do not reject the potential for other forces besides mutation-selection balance to allow lethal variation to persist in natural populations:

1) Some lethal allele frequencies were well above expected values from mutation-selection alone and the forces behind these high frequencies remain unexplained (Table 1).
2) Most studies reported average heterozygote viabilities; mean values do not account for the variation in heterozygote fitness and obscure specific lethal heterozygote viabilities, some of which have been shown to be heterotic (Box 1).
3) Lab environments are not reflective of nature and heterozygote advantage may be specific to different environments. Multiple studies found evidence of heterozygote advantage only under certain environments (Box 1).
4) Balancing selection broadly refers to selection that maintains genetic variation in a population, but only one form of balancing selection, heterozygote advantage, was previously studied. Other forms of balancing selection (negative frequency dependent selection) have not been thoroughly investigated.
5) Indirect effects of linkage were not considered (see next section).

3. Does *linked selection* or *genetic drift* play a role in allowing persistence of lethal variation?

While previous research has primarily focused on mutation-selection balance and heterozygote advantage, other forces that may *indirectly* allow lethal alleles to reach high frequencies through linked selection remain unchartered territory. Lethal allele frequencies may be influenced through linkage with loci under positive or negative selection, and linkage effects on varying N_e across the genome (and thus strength of genetic drift) could have a measurable influence on lethal allele frequencies.

A. Balanced Deleterious Haplotypes (i.e. Associative Overdominance)

While classical overdominance was the focus of previous studies, another form of balancing selection, associative overdominance (AOD), has been comparatively underexplored in relation to lethal allele maintenance in natural populations. Associative overdominance results in higher nucleotide diversity (pi) of neutral loci linked to loci experiencing selection (Becher et al., 2020; Zhao & Charlesworth, 2016), somewhat resembling the pattern predicted by heterozygote advantage. While there is a well-accepted positive correlation between recombination rate and nucleotide diversity due to background selection (BGS) and positive selection, AOD may prevail when recombination is low and increase pi compared to neutral expectations in low recombination regions (Comeron, 2014; Gilbert et al., 2020; Stephan, 2010). Theoretical models established conditions that favor AOD over BGS: low recombination rate (strongest effect show between 0-0.001 cM/Mb), recessive (or partially recessive) deleterious mutations ($0 \leq h \leq 0.25$), intermediate selection coefficients ($0.1 < Ns < 100$), high mutation rate, and a high number of deleterious sites (Gilbert et al., 2020). Such conditions would not only result in higher than expected neutral diversity, but also higher than expected frequencies of deleterious alleles because they become 'balanced' in complementary haplotypes.

While the selection coefficients of lethal alleles are arguably beyond 'intermediate', suggesting lethal alleles may not be the initial driving mutations of AOD, little research has been done into whether they can accumulate or be maintained by incorporation into one or both

balanced deleterious haplotypes. The role of AOD in maintaining lethal alleles has not been studied explicitly, but multiple studies investigating the distribution of lethal alleles have found evidence consistent with the possibility that AOD may influence some lethal allele frequencies (e.g., Spiess et al., 1963; T. K. Watanabe & Oshima, 1966; Ytterborn, 1968). If lethal alleles persist because of their linkage to complementary deleterious haplotypes, one expects these lethal alleles to be found in regions of low recombination. A few studies in the 1960's utilized recombination mapping with recessive marker stocks to examine the distribution of lethal alleles along a chromosome and found clustering in an area of extremely low recombination - the centromere (Spiess et al., 1963; T. K. Watanabe & Oshima, 1966; Ytterborn, 1968). One study contrasted this pattern to the random distribution of newly arisen spontaneous lethals, suggesting that the differences between the distributions could be due to a form of natural selection (T. K. Watanabe & Oshima, 1966).

Inversions provide an exceptionally favorable environment in which lethal alleles may be maintained via AOD because many arrangements persist at high frequencies and because recombination products are not recovered from inversion heterozygotes. The lack of recombination in heterozygotes prevents gene flow between arrangements, which allows for the accumulation of fixed differences between the two haplotypes. If recessive deleterious mutations accumulate in a rare inversion arrangement that is otherwise beneficial or neutral, the arrangement may increase in frequency due to the initial advantage and, once it reaches a frequency high enough to expose its deleterious homozygous effect, stop its spread at a stable equilibrium due to AOD (Kirkpatrick, 2010). If somehow a recessive lethal mutation becomes fixed within either arrangement, then the accumulation of further recessive lethal or other deleterious alleles becomes untethered.

Studies have observed multiple lines of evidence that suggest inversions could drive some high frequency lethal alleles. Some researchers have found complete and partial LD between lethal alleles and inversions within *Drosophila subobscura*, *Drosophila pseudoobscura*, and *Drosophila melanogaster* (Albornoz & Domínguez, 1994; Coyne et al., 1991; F. Mestres et al., 2001; Francesc Mestres et al., 2009). In one intriguing example, Coyne et al. (1991) discovered a high frequency lethal allele in *D. melanogaster* (present in 6 out of 20 wild lines from Mauritius) that was in complete LD with a second chromosome inversion. A different lethal allele was found in LD with the same inversion on another island (present in 2 out of 8 wild lines from Mauritius). Coyne determined that the cause of lethality was not due to the inversion breakpoint locations nor due to segregation distortion, and he speculated that associative overdominance may be the cause. A similar example was found in *D. subobscura*, where a single lethal was associated with the O_5 inversion in every sample. A different lethal was in partial LD with the O_{3+4+7} arrangement (i.e. the lethal was only found on the inverted arrangement but the inversion sometimes carried other lethals or no lethals) (F. Mestres et al., 2001; Francesc Mestres et al., 2009).

Lastly, some researchers have found lethal alleles clustering near inversion breakpoints (Francesc Mestres et al., 2009; T. K. Watanabe & Oshima, 1966; Yang et al., 2002). Regions encompassing inversion breakpoints in heterozygotes tend to have extremely low recombination rates in inversion heterozygotes ((Hasson & Eanes, 1996; Navarro et al., 1997), making them prime candidates for lethal accumulation via AOD. Particularly interesting results were those that compared distributions of naturally occurring lethals with that of spontaneous, newly arisen lethals. Naturally occurring lethals were concentrated around the centromere and somewhat concentrated around inversion breakpoints as opposed to the random distribution of spontaneous

lethals (T. K. Watanabe & Oshima, 1966). This contrast hints at AOD may play an essential role over time in maintaining or allowing for the accumulation of lethal alleles.

B. Linkage to Beneficial Loci and Haplotypes

While recessive lethals may accumulate in a polymorphic system created by complementary mildly deleterious haplotypes, another mechanism for lethal accumulation in regions with suppressed recombination is through linkage to adaptive haplotypes (Barton, 1995; Chun & Fay, 2011; Hartfield & Otto, 2011). A partially dominant beneficial mutation may arise on a haplotype near an existing recessive detrimental (or even lethal) allele. Given the abundance of such lethals in segregating chromosomes (see Table 1), such a scenario is quite feasible. While any sort of linkage to adaptive variants has the potential to produce a higher-than-expected lethal allele frequency, the best examples are studied via supergenes (aka 'coadapted gene complexes'). Supergenes are linked sets of multiple adaptive loci that segregate at intermediate frequencies in many species (Schwander et al., 2014). In such systems, the initial increase in frequency of a particular linked haplotype would be driven by positive selection and but the polymorphism is maintained by the accumulation of recessive deleterious alleles on the different haplotypes (AOD).

Studies across multiple species have found evidence for mutation accumulation in supergenes, which are commonly associated with inversions. A recent study demonstrated that deleterious mutations have accumulated and presumably maintain a polymorphic wing pattern supergene in the butterfly species, *Heliconius numata* (Jay et al., 2019). Another found that a homozygous lethal inversion in the wading bird, *Philomachus pugnax*, carries a polymorphic supergene controlling male mating morphs (Küpper et al., 2015). While it appears that the lethality of this inversion is due to the breakpoint location, one fixed lethal mutation in a linked supergene allows for unlimited accumulation of more recessive deleterious mutations and lethal alleles in the arrangement (Schwander et al., 2014).

While not positive selection, linkage to meiotic drive elements, such as segregation distorter (SD) loci, can be associated with lethal alleles and may also allow some lethals to persist at higher frequencies. A classic example of segregation distorter meiotic drive lies in *D. melanogaster* where male SD heterozygotes produce over 95% SD offspring (Larracuente & Presgraves, 2012). Two loci critical to segregation distortion are tightly linked – one of which, the distorter gene, produces a protein that disrupts any sperm cells without the SD haplotype. This allows for the tightly linked SD haplotype to be passed on at a rate above Mendelian expectation. Interestingly, SD loci are homozygous lethal and strongly associated with different inversions. While the reduced recombination of SD chromosomes from numerous inversions provides short-term evolutionary benefits (Larracuente & Presgraves, 2012), it also results in the accumulation of recessive lethal alleles. The homozygous lethality of many SD chromosomes limits the frequency at which they appear in natural populations, but the segregation distortion allows lethals to appear at higher frequencies than mutation-selection would predict. Also found in mice, segregation distorter t-haplotypes contain recombination suppressing inversions and commonly carry homozygous lethal mutations (Schimenti, 2000).

Studies focusing on supergenes and segregation distortion have found specific examples of how these two forces may aid in the preservation of deleterious or lethal mutations, but few have

focused their research on natural lethal frequencies and the extent to which linkage plays a role in the maintenance of such detrimental mutations.

C. Genetic Drift and effective population size

Genetic drift, if strong enough, has the potential to increase lethal allele frequencies higher than one might expect. In fact, a simulation of a commercial pig population with an effective size of 150 estimated that a lethal mutation could potentially rise to a 10% frequency solely due to drift (Derks et al., 2019). While the effective size of many populations is much greater than 150, the effects of drift on lethal frequencies must not be overlooked and its potential strength should be assessed in different populations.

Not only can effective size vary between populations, but it also can vary across chromosomes within populations. Recombination rate can affect lethal frequencies in complex ways. It may work directly through linked selection, as discussed, or indirectly via the effects of linked selection on the strength of drift. Strong drift could allow some lethal alleles to reach (but not persist) at higher-than-expected frequencies. The strength of drift depends on multiple factors and varies across regions of the genome. Typically there is a *positive correlation between recombination rate and nucleotide diversity (thus N_e)* (Campos et al., 2014). Theoretical and empirical data show that both nucleotide diversity and N_e are reduced in regions of low recombination and elevated in regions of high recombination. This pattern is due to effects of background selection and selective sweeps (Comeron, 2014; Stephan, 2010). Lower recombination, thus smaller N_e, could allow drift to overcome selection and perhaps result in sporadically higher frequencies of lethal alleles. Lethal allele frequencies may be influenced through the impact of recombination rate on N_e variation throughout the genome. While N_e would need to be very small to have a measurable impact on lethal allele frequency, the combination of strong drift and other evolutionary forces (e.g. some forms of selection) could provide telling interactions.

4. Lethal alleles in humans

While the vast majority of research on lethal allele frequencies in nature has been done in *Drosophila*, high lethal (or nearly lethal) allele frequencies have also been explored in humans extensively. In fact, humans have been estimated to carry between 0.58 – 1.6 lethal mutations per individual via studies using very different approaches (Bittles & Neel, 1994; Gao, Waggoner, et al., 2015; Narasimhan et al., 2016). Some studies utilized inbreeding to estimate lethal proportions: an early study found the correlation between consanguinity and offspring mortality (Bittles & Neel, 1994), while a later study utilized loss-of-function (LOF) genotype data of offspring from related parents and compared the number of homozygous knockout genotypes to an expected amount, generating an estimated 1.6 recessive lethal LOF mutations per individual (Narasimhan et al., 2016). Another recent study estimated 0.58 lethal mutations per individual in a founder population through pedigrees, disease incidence data and simulations (Gao, Waggoner, et al., 2015). Importantly, most of these studies examined phenotypic data, which would not take into account any recessive lethals that resulted in death during embryonic development.

Primarily studied in the context of human disease, there are multiple classic examples of lethal disease alleles that have been found to occur at greater than 1% in certain populations

(Overall & Waxman, 2020). In the European Union, the cystic fibrosis mutation, ΔF508, averages a frequency of 2.2%, far above the expected equilibrium due to mutation-selection balance (Farrell, 2008). Ashkenazi Jewish populations carry the Tay Sachs disease allele at an estimated frequency of 1.1%-1.5% (Myrianthopoulos & Aronson, 1966). While these provide classic and long-standing examples of lethal alleles in human populations, it is difficult to distinguish which evolutionary forces are behind the maintenance of such disease alleles.

A 2017 study examined 417 recessive lethal disease mutations and found that the majority were present at frequencies much higher than expected from a purely mutation-selection model, but suspected this pattern may have been due to ascertainment bias rather than repeated cases of overdominance (Amorim et al., 2017). More recent simulations inspired by these findings have provided evidence that *some* lethal disease alleles (such as Cystic Fibrosis and Tay Sachs) are maintained by intermittent periods of overdominance (Waxman & Overall, 2020).

Fewer studies in humans have looked at the relationship between lethal alleles and recombination rate, but some have demonstrated that deleterious alleles are maintained in populations partially through hitchhiking in low recombination regions (Chun & Fay, 2011). We do not know of studies that have looked at recombination rate association with lethal alleles in humans to a broad degree.

It should be noted that human lethal allele frequency data are not easily comparable to those in *Drosophila* and other species because of variance in medicine and medical access and estimations must be inferred from population genetic analyses or disease frequency rather than direct population sampling.

5. Utilizing Modern Tools to distinguish between evolutionary forces

The evolutionary forces maintaining lethals in nature remain unresolved. Given this gap in our understanding and modern population genetic tools now at our disposal, this area is ripe for study. Previous research in *Drosophila* measured overall lethal chromosome frequencies, individual lethal mutation frequencies through allelism crosses, and broad locations via recombination mapping, but with modern genetic tools, fine mapping numerous naturally occurring lethal alleles and getting sequence level characterization is within reach. Not only would fine mapping and sequencing lethal mutations give us essential insight into the genetic basis of fitness variation in nature but would also provide the groundwork for using modern population genetic tests to infer the evolutionary forces that maintain them.

A. Distinguishing between mutation selection balance and balancing selection

If fine-mapping and sequence specific characterization of natural lethal alleles were generated, two broad approaches could tease apart the evolutionary forces maintaining such extreme fitness variation in nature: 1) sequence data from *regions bearing specific lethal mutations at high frequencies* and 2) broader patterns in abundant *lethal allele distributions along the genome*.

The most straightforward direction would be to investigate regions of the genome with the most abundant lethal mutations. Theory predicts that regions tightly linked to sites undergoing long-term balancing selection will show higher polymorphism compared to regions linked to neutral sites or those undergoing positive or purifying selection (Gao, Przeworski, et al., 2015). Other signatures include increased LD around the target site, longer genealogies and more level

allele frequency distribution than expected under neutral conditions. While such evidence may be easily detected for ancient balancing selection, these signatures are harder to detect for more recent balanced polymorphisms (Fijarczyk & Babik, 2015). If balancing selection is recent, the signature may resemble that of a soft sweep (a slight reduction in variation around the lethal site) (Fijarczyk & Babik, 2015; Hermisson & Pennings, 2005; Lee et al., 2016). While this similarity poses complications to detecting balancing selection more generally, it highlights a potential advantage of studying lethal alleles. Intermediate fitness estimations are always relative, but the detrimental effects of lethality are often absolute. Thus, a signature of recent balancing selection around a lethal target should not be easily confused for evidence of positive selection.

While explicit tests and predictions have not been developed, population genetic theory hints that the *distribution* of lethal alleles relative to nucleotide diversity and other relevant parameters may also provide key insight into distinguishing between mutation selection advantage and balancing selection. Recombination rate can affect lethal frequencies in complex ways. It may work directly through linked selection or indirectly via the effects of linked selection on the strength of drift. With limited historical research on the relationship between linked selection and lethal allele frequencies, it is crucial to take advantage of modern approaches to explore this connection. Population genetic simulations can be used to model expected distributions of lethal alleles relative to recombination rate and related factors such as inversions and N_e. For example, the differing strengths of drift due to linkage effects on N_e could affect lethal alleles under heterozygote advantage differently than those under purely mutation and purifying selection. There may be diagnostic patterns of balancing selection versus mutation selection balance that can be revealed through differences in lethal distributions between these models.

Lastly, while overall mutation rate estimates have been developed, lethal mutation rates and mutation rate variation across the genome is relatively unknown. We cannot rule out that unusually high mutation rates in different regions could result in higher frequencies of some lethal alleles, potentially resembling predictions from balancing selection. More information on mutation rate variation within *Drosophila* genomes would be useful for comparison in analyzing the distribution of high frequency lethal alleles. Many researchers have estimated average mutation rates per individual genome or chromosome using mutation accumulation lines (Haag-Liautard et al., 2007; Keightley et al., 2014; Schrider et al., 2013), but only recently has anyone attempted to infer the landscape of mutation rate across a chromosome (Barroso & Dutheil, 2021).

B. Distinguishing between classical overdominance and linkage based overdominance

Upon finding evidence of balancing selection, differentiating between classical overdominance and linkage-based overdominance is an even more challenging task. To the best of our knowledge, no empirical test can totally differentiate signatures of classical overdominance from those of associative overdominance (or other forms of balancing selection, e.g. negative frequency dependent selection).

The recombination rate present in the region of a particular high frequency lethal allele has the potential to provide insight into what form of balancing selection could be acting. Lethal alleles in high recombination regions that persist at measurable frequencies for a very long time are more likely *not* due to linkage-based overdominance, but lethal alleles that persist in low

recombination regions or inversions cannot be so easily assigned to a specific form of balancing selection.

By definition, all forms of balancing selection will result in higher than expected nucleotide diversity (*pi*) than neutral expectations. While *pi* alone cannot distinguish between linkage-dependent overdominance and classical overdominance, research is beginning to explore how to use patterns of pi along with other measurements of diversity to identify evidence of associative overdominance. Gilbert et al. measured *pi* and average derived allele frequency within individuals ($\overline{DAF_i}$) in simulations of deleterious mutations with differing selection coefficients. They found that recessive deleterious alleles with stronger selection coefficients results higher than expected by but lower $\overline{DAF_i}$ (due to a larger number of rare complementary haplotypes across loci) and suggested that this pattern has the potential to be useful in distinguishing between AOD and classical overdominance (Gilbert et al., 2020). While this pattern was not identified in human genome data, such patterns have not yet been explored in other species.

Patterns in the *distribution of lethal alleles* relative to recombination rate and inversions could provide evidence for or against linkage as a driving force for the maintenance of many lethal alleles. While previous studies on this provided tantalizing results, these studies were limited in number, precision, and accuracy by their use of recombination mapping. Current collections of Drosophila deficiency stocks, gene-knock out lines and modern sequencing technology will allow us to map naturally occurring lethal alleles to a much finer scale and more accurately investigate their distribution. Modern tools also make it easier to locate multiple distinct lethal alleles in a single chromosome, opening the possibility of exploring LD between lethal alleles and haplotype structuring. While the role of associative overdominance is difficult to test, it is crucial to consider and explore the potential effects it may have on deleterious variation in nature.

Conclusion

Surprisingly high lethal frequencies in many species have puzzled evolutionary biologists for nearly a century, sparking debate over what evolutionary forces may be responsible for such extreme deleterious variation persisting within and across species. Given low average frequencies of any particular mutation and, on average, reduced lethal heterozygote viabilities, most evolutionary biologists dismissed the role of balancing selection in maintaining extremely harmful variation in nature. We propose that there are cases of lethal mutations persisting at much higher frequencies than cannot be explained by mutation-selection balance.

Over the past few decades, investigation into the underlying genetics of naturally occurring recessive lethals and evolutionary forces behind them have tapered. During this time, key scientific advances have been made that give us the ability to collect more accurate data and to analyze it in new ways, leaving the underlying genetic basis and evolutionary forces of lethal alleles ripe for study. Previous work made little headway into the genetic underpinnings of naturally occurring lethal mutations, and the mapping data were limited in precision by the use of recombination mapping. The ability to pinpoint lethal mutation locations and sequences would not only serve as novel characterizations of naturally occurring lethal alleles but allow us to utilize modern analyses to determine evolutionary forces maintaining lethal alleles in nature (both at such high overall frequencies and higher than expected individual lethal allele

frequencies). Given the right data, there are numerous directions of research that could elucidate evolutionary forces behind deleterious variation in nature. For example, if high frequency lethal mutations are identified and sequences, patterns in sequence variation in regions surrounding high frequency lethal alleles could expose the presence or absence of balancing selection. If enough lethal mutations are pinpointed, their distribution relative to recombination rate or inversion locations may provide insight into the effects of linked selection. With novel tools to generate and analyze more extensive and precise data, evolutionary geneticists can now revisit the question of balancing selection with modern analyses and explore new explanations including the role of linked selection.

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