Identifying and characterising sexually antagonistic polymorphisms in *Drosophila melanogaster*

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Master's of Science by Research



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Abstract

Sexual antagonism arises when alleles at the same locus have opposing fitness effects in males and females. Identifying such loci is key to understanding the evolutionary maintenance of genetic variation and the genomic basis of sexual conflict. Here, I aim to identify sexually antagonistic (SA) loci in *Drosophila melanogaster* by comparing allele frequencies between successful sperm and egg pools using mass breeding Pool-Sequencing design. I identified 232 candidate SA single nucleotide polymorphisms (SNPs). These SA SNPs were enriched for chromosome arm 2L whereas chromosome arm 3R was significantly depleted of candidate SA SNPs. Neither the combined autosomes or X chromosomes showed significant enrichment or depletion. Candidate SA SNPs showed significant enrichment in regulatory regions including upstream, downstream and synonymous and significant depletion in intergenic regions. Genes with a candidate SA SNP within their coordinate interval (i.e. candidate SA genes) were significantly enriched for previously published SA candidate genes and exhibited significantly higher intersexual genetic correlations (r_{MF}) for gene expression compared to the average background genes in the *D. melanogaster* genome. These results provide new insight into the genomic architecture of sexual antagonism and highlight several key patterns, consistent with both previous theoretical predictions and empirical studies.

Section 1- Literature Review.

1 Introduction

One of the main questions in evolutionary biology is how genetic variation is maintained within populations. Mutation-selection balance is an inevitable evolutionary process that maintains low-frequency deleterious alleles through a

dynamic equilibrium between their introduction via mutation and removal by purifying selection (Zhang and Hill, 2005). Whilst this mechanism is expected to contribute to genetic variation, it does not fully explain the magnitude of genetic variance observed in natural populations, indicating that additional processes are involved.

One mechanism is balancing selection, which includes processes such as heterozygote advantage or negative frequency-dependent selection. Balancing selection preserves allelic diversity, maintaining alternative alleles at intermediate frequencies over time (Charlesworth, 2006). Sexually antagonistic (SA) selection occurring when alleles at a given locus have opposite fitness effects in each sex (Bonduriansky and Chenoweth, 2009) represents another process that can result in balancing selection. SA selection has been linked to a range of evolutionary consequences, including the maintenance of genetic variation (Kidwell *et al.*, 1977), the generation of a fitness load across the sexes (Berger *et al.*, 2016) and shaping genetic variation within and between species (Glaser-Schmitt *et al.*, 2021).

Numerous population genetic models (Kidwell *et al.*, 1977; Rice, 1984) demonstrate how alleles under opposing selective pressures in males and females can persist over time, especially when the fitness trade-offs between sexes are balanced. Empirical studies further suggest that SA selection is likely to be widespread, particularly for polygenic traits influenced by alleles of varying effect sizes (GlaserSchmitt *et al.*, 2021). Despite this, the extent to which SA selection contributes to genetic variation remains poorly understood.

1.1 Sexual conflict and its evolutionary consequences

Sexual conflict arises when males and females experience different optimal phenotypes (Arnqvist and Rowe, 2005). These differences are rooted in anisogamy, the fundamental differences in gamete size and reproductive investment between the sexes (Parker *et al.*, 1972). Male gametes (sperm) are typically small and numerous, whereas female gametes (eggs) are larger and are a limiting resource (Chapman *et al.,* 2003). As a result, male reproductive success is primarily determined by access to

mates, while female reproductive success is more strongly limited by gamete production and reproductive investment.

Empirical studies of sex-specific reproductive strategies in *D. melanogaster* have demonstrated that male reproductive success is more variable than female reproductive success because males gain increased reproductive success from successive matings, whereas female reproductive success does not increase substantially with additional matings (Harano *et al.*, 2010). Sexual conflict can arise through two distinct mechanisms. Interlocus sexual conflict arises when different traits with distinct genetic bases in males and females lead to antagonistic outcomes in reproductive interactions, often leading to SA coevolutionary dynamics-such as evolutionary arms races (Rowe and Day, 2006). In contrast, intralocus sexual conflict (IaSC) occurs when selection acts in opposing directions on the same genetic locus in males and females preventing either sex from reaching its phenotypic optima (Bonduriansky and Chenoweth, 2009).

One potential resolution to IaSC is the evolution of sexual dimorphism, where males and females diverge in the expression of a trait despite a common genetic basis to the trait in both sexes. Much of this divergence is achieved through differences in their gene regulation, particularly sex-biased gene expression (SBGE), where genes are expressed at different levels in males and females (Grath and Parsch, 2016). Studies have shown that SBGE is widespread across taxa. One study in humans suggests that up to 60% of autosomal genes exhibit sex-biased expression patterns (Chen *et al.*, 2016) and similar patterns have been reported in mice (Karp *et al.*, 2017) birds (Naurin *et al.*, 2011) and fish (Huang *et al.*, 2018). These widespread patterns suggest that SBGE plays a key role in sex-specific adaptation and the evolution of dimorphic traits.

However, this resolution is often incomplete. For genes with pleiotropic or essential functions the evolution of sex-specific regulation may be constrained, limiting the ability of each sex to independently reach its optimum, which can manifest as standing SA genetic variation.

Despite its importance, many aspects of SA genetic architecture remain poorly understood. There is no consensus on the number of SA polymorphisms present in the genome of any organism, nor on their genomic distribution, functional classifications (e.g. protein-coding vs. regulatory), or the types of phenotypic traits shaped by this form of selection (Ruzicka *et al.*, 2019; Flintham, 2025). These knowledge gaps limit our ability to fully understand the genomic and evolutionary consequences of sexual antagonism.

Quantifying intralocus sexual conflict (IaSC) at the phenotypic and genetic level remains a major challenge in evolutionary biology. Quantitative genetic approaches have been widely used to infer SA genetic variation by estimating sex-specific selection gradients and genetic correlations between male and female fitness. For example, studies in *Callosobruchus maculatus* have provided strong evidence for the presence of SA genetic variation within populations (Berger *et al.*, 2014; Grieshop and Arnqvist, 2018). Approaches such as mapping fitness landscapes for varying phenotypes, including traits like body size or mating latency, can also reveal contrasting patterns of selection between the sexes (Hosken *et al.,* 2019). However, while these methods provide evidence that SA genetic variation exists, they rarely identify the specific loci underlying this conflict. Distinguishing true SA loci from statistical noise and sex-limited expression, therefore, remains challenging and typically requires large sample sizes, high-resolution fitness data, or unique experimental manipulations.

Recent efforts have attempted to link genetic variation directly to sex-specific fitness outcomes. For example, Innocenti and Morrow (2010) combined transcriptomic data with fitness assays in *D. melanogaster* to identify candidate genes under SA selection. Ruzicka *et al.* (2019) used genome-wide association to identify candidate SA variants in *D. melanogaster.* Similar approaches in other systems have identified loci with sex-specific effects, such as a maturation-associated locus in salmon (Barson *et al.*, 2015). Nevertheless, distinguishing true SA loci from statistical noise, pleiotropy or sex-limited effects remains difficult.

1.2 Balancing selection

SA selection can potentially generate balancing selection that maintains alternative alleles, especially under the conditions that selection is strong and/or evenly matched between sexes (Prout, 2000) there is sex-specific dominance reversal (Kidwell *et al.*, 1977; Grieshop *et al.,* 2024), SA epistasis linkage (Arnqvist *et al., 2014)* between SA loci (Patten *et al*., 2010), linkage to sex chromosomes (Rice, 1984) or linkage to meiotic drivers (Haig *et al.,* 2014). Balancing selection preserves multiple alleles within a population over long time periods, preventing any single allele from becoming fixed, therefore preserving genetic diversity. One of the most well-characterised forms of balancing selection is overdominance, where heterozygotes at a locus have higher fitness than either homozygote (Prout, 2000). A classic example is the β-haemoglobin locus in humans, where individuals heterozygous for the sickle-cell allele (HbA/HbS) are resistant to malaria without developing sickle-cell disease (Hedrick, 2011). In regions where malaria is prevalent, this heterozygote advantage maintains both alleles within the population (Salih *et al.*, 2010; Hedrick, 2011).

SA selection is predicted to generate similar patterns, as alleles with opposing fitness effects in males and females can be maintained at intermediate frequencies. Empirical evidence supports this prediction, for example Ruzicka *et al.* (2019) identified over 230 candidate SA regions in *D. melanogaster* that were enriched for potential signatures that are consistent with balancing selection, including elevated heterozygosity and trans-species polymorphism in sister species like *D. simulans*. While not conclusive, these findings support theoretical models predicting that sexual conflict can maintain polymorphisms through SA balancing selection (Connallon and Clark, 2014). Population genomic approaches commonly attempt to detect balancing selection by identifying loci exhibiting signatures, such as elevated heterozygosity or intermediate allele frequencies (Charlesworth, 2006; Fijarczyk and Babik, 2015). Such genomic patterns can provide evidence for balancing selection at individual SNPs or loci. However, multiple evolutionary processes, including overdominance and frequency-dependent selection, can produce similar genomic signals (Bitarello *et al.*, 2023). Therefore, while individual SNPs may provide evidence consistent with

balancing selection, distinguishing the specific mechanism responsible for maintaining a polymorphism can be challenging using genomic data alone.

Together, these limitations highlight a key challenge: although balancing selection is one possible mechanism through which SA variation may be maintained, SA selection can influence allele frequencies and evolutionary dynamics even when variation is not stably maintained over time. Genomic evidence can therefore provide information about patterns of genetic variation associated with selection, but it may not by itself establish the fitness consequences or evolutionary mechanisms underlying SA polymorphisms. Integrating population genetic signals with direct measures of sex-specific fitness is therefore important for identifying candidate SA loci and understanding their contribution to sex-specific selection and genetic variation.

1.3 *Drosophila* as a model organism

To investigate these processes, a reliable model system is required. *D. melanogaster* is particularly well suited for studying the genetic basis of SA variation due to its well characterised genome, short generation time and suitability for experimental evolution. Laboratory populations provide controlled environments in which sex specific selection and fitness variation can be studied with high precision.

Two populations LH_m and Dahomey have been widely used to investigate IaSC and SA genetic variation. The LH_m is a laboratory-adapted population of *D. melanogaster* established in 1991 from 400 inseminated females collected from an orchard near Modesto, California (Rice *et al.*, 2006)**.** Since 1996, the LH_m population has been maintained under a strictly controlled rearing regime with a large effective population size (~1,800 breeding adults). This long-term maintenance under stable laboratory conditions provides a consistent environment in which fitness and sex-specific fitness variation can be measured meaningfully across experiments, allowing LH_m to be extensively used in studies of intralocus sexual conflict and sex-specific fitness variation (Innocenti and Morrow, 2010; Ruzicka *et al.*, 2019).

The Dahomey population is another laboratory-adapted population of *D.*

melanogaster, originally established in the 1970s from wild-caught flies collected in Dahomey (now Benin) in West Africa. In contrast to LH_m the population is maintained with overlapping generations (Shenoi *et al.*, 2022). Like LH_m the Dahomey population has been maintained under controlled laboratory conditions for many generations and has been widely used to study sex-specific selection and the evolution of reproductive traits (Grieshop *et al.*, 2025).

1.4 Location and characteristics of SA loci

A longstanding question in the study of SA variation concerns where in the genome such polymorphisms are most likely to occur. Early theory predicted that the X chromosome should disproportionately harbour SA polymorphisms, particularly under certain dominance conditions. Rice (1984) proposed that male-beneficial recessive alleles are more likely to spread on the X chromosome because they are exposed to selection in hemizygous males, while remaining partially masked in females.

Initial empirical studies provided support for this prediction. For example, work in *D. melanogaster* suggested that a substantial proportion of SA fitness variation mapped to the X chromosome (Gibson, Chippindale and Rice, 2002), and the genes with antagonistic expression patterns were enriched on the X chromosome (Innocenti and Morrow, 2010). These findings led to the view that the X chromosome may act as a reservoir for sexual conflict.

However, subsequent theoretical and empirical work has painted a more complex picture. Models show that autosomes, which are equally inherited by both sexes, may provide a more stable foundation for antagonistic alleles, particularly when dominance differs between the sexes (Fry, 2010). Consistent with this, genome-wide studies have also identified SA variation distributed across autosomes, particularly on chromosomes 2 and 3 in *D. melanogaster* rather than concentrated solely on the X chromosome (Ruzicka *et al.*, 2019).

Beyond their genomic location, the biological characteristics of genes harbouring SA polymorphisms have also received some attention. One hypothesis is that SA variation is more likely to persist in genes where sex-specific expression is constrained. In particular, genes with essential or pleiotropic functions may be less able to evolve sex-specific regulation limiting the resolution of conflict (Zajitschek and Connallon, 2018). Similarly, protein-coding regions may be more constrained than regulatory regions, reducing the potential for sex-specific optimisation (Connallon and Clark, 2014)

Another obvious constraint to sexually dimorphic gene expression is the intersexual genetic correlation in gene expression (r_{MF}), which reflects the degree to which gene expression is genetically correlated between the sexes. High r_{MF} can indicate that gene expression is strongly shared between the sexes, which can constrain their ability to evolve independent sex-specific patterns of gene expression. Therefore high r_{MF} may limit the potential for each sex to optimise gene expression independently in response to sex-specific selection. Loci with high r_{MF} may be more likely to experience IaSC because selection favouring an expression pattern that increases fitness in one sex may constrain the ability of the other sex to achieve its own optimal expression level (Griffin *et al.*, 2013; Grieshop *et al.*, 2025).

Despite growing theoretical and empirical interest, identifying SA loci in genomic data remains challenging. One key difficulty is that the genomic signatures of SA selection are often subtle and easily confounded, as alleles typically segregate according to Mendelian inheritance. To address this, a range of population genomic approaches can be used to identify candidate SA loci. One widely used method is pooled sequencing (pool-seq). Pool-seq allows allele frequency estimation by sequencing a DNA pool from many individuals. This approach has been successfully applied in both experimental and natural systems to detect sex-specific allele frequency differences (Bissegger *et al.*, 2020; Lirakis *et al.*, 2022). In parallel, statistics such as Tajima's D can be used to identify deviations from neutrality, with positive values indicating an excess of intermediate-frequency alleles that can be consistent with balancing selection (Tajima, 1989). However, positive Tajima's D values are not specific to balancing selection and can also arise from demographic processes such

as population contraction. Therefore, Tajima's D can provide evidence consistent with balancing selection but should be interpreted alongside other population genetic evidence.

However, these approaches have limitations. Many genomic signatures associated with SA selection, such as intermediate allele frequencies or elevated diversity, can also arise from demographic events, bottlenecks often lead to positive Tajima's D values, especially if followed by periods of low diversity (Gattepaille *et al.*, 2013) and background selection typically reduces diversity, producing negative Tajima's D values (Comeron, 2017). As a result, no single statistic provides definitive evidence for SA selection, and distinguishing true SA loci from false positives remains difficult.

1.5 Link to diseases and health problems

SA selection may play an important role in shaping the genetic architecture of diseases. Alleles that are deleterious in one sex can persist in populations if they provide a fitness advantage in the other, offering an explanation as to why certain disease-associated variants remain at higher frequencies than expected given their negative effects (Morrow and Connallon, 2013). Under this framework, some alleles that increase disease risk in one sex may be maintained if they enhance reproductive success in the other.

A well-studied example of this evolutionary paradox is polycystic ovarian syndrome (PCOS), one of the most common endocrine disorders affecting women. Despite its negative effects on female fertility and metabolic health, PCOS-associated alleles may persist because they provide fitness benefits in males, such as improved metabolic efficiency or reproductive traits (Casarini *et al.,* 2016). Patterns of geographic variation in these alleles further suggest that SA selection may operate in a context dependent manner (Casarini *et al*., 2016)**.**

Evidence for SA effects has also been proposed in a range of other complex diseases (Harper *et al*, 2021). For instance alleles associated with schizophrenia may have sex specific effects, increasing risk in males while being neutral or protective in females

(Tiihonen *et al.*, 2019). Similarly, genes involved in immune function or autoimmune diseases often exhibit sex-dependent effects, likely reflecting differences in hormonal and immunological environments between males and females (Kamitaki *et al.*, 2020; Bose and Jefferies, 2022). More recently SA mechanisms have also been suggested to contribute to the persistence of alleles associated with late-onset disorders such as Alzheimer's disease (Morrow and Harper, 2026).

Together, these findings highlight the potential for SA selection to maintain disease associated genetic variation in human populations. Recognising this perspective may help explain sex differences in disease prevalence, progression and treatment response, highlighting the importance of considering sex-specific evolutionary pressures in studies of human health.

1.6 Aims and objectives

The challenges associated with identifying SA loci highlight the need for approaches that combine robust genomic tools with careful experimental design to minimise confounding effects and accurately identify candidate SA loci. Here, I aim to contribute to this goal by using pool-seq samples and permutation-based statistical frameworks to detect SA polymorphisms in *D. melanogaster*. By refining detection methods and evaluating the genomic distribution of candidate loci, this work helps address key gaps in our understanding of how sexual antagonism shapes variation.

Specifically, I aim to advance our understanding of the number, location and characteristics of SA genetic polymorphisms in the *D. melanogaster* through the development of a novel experimental genomic method. Below, I describe the method and analysis pipeline for identifying allele frequency change within a single generation that is caused by sex-specific selection, and how I isolate the fraction of that genetic variation that is explicitly SA. I then characterise these candidate SA SNPs in the context of four other studies that have attempted to reveal SA genetic variation. I discuss the implications of my findings for the study of SA genetic

variation, and I conclude with some recommendations for future applications of this method and its potential value to the broader field of evolutionary biology.

Section 2. Identifying sexually antagonistic polymorphisms.

2.1 Methods

2.1.1 Experimental design

DNA samples used in this study were previously generated as part of Jacob Neal's Postgraduate dissertation, *A novel method for identifying candidate regions of sexual conflict in the Drosophila melanogaster genome,* in the Grieshop lab at the University of East Anglia (Neal, 2024).

Briefly, two genetically distinct laboratory-adapted populations of *D. melanogaster (*Dahomey and LH_m, described above) were used to generate hybrid offspring in a competitive mass cage mating arena. Approximately 1,500 virgin females from one population were placed with approximately 1,500 males from the alternative population in cages, allowed to court, compete, mate and remate for three days. Fertilised eggs (>5,000 per sample) were collected within six hours of oviposition over days 3-5 and frozen for downstream DNA extraction. This experiment was always conducted in pairs of cages, with the population by-parent-sex assignment reversed between the main cage and the reciprocal cage.

In addition, "pure" population samples were generated by collecting eggs from large mating cages with all individuals from one or the other population under the same conditions. These samples were used to construct population-specific reference genomes.

In total, 2 blocks of a main and reciprocal pair of cages (four cages) were conducted. 14 hybrid samples (7 using Dahomey fathers and LH_m mothers, and 7 using Dahomey mothers and LH_m fathers), each consisting of > 5,000 fertilised eggs) and 1

pure sample each of the 2 populations (16 samples in total) were sent for sequencing.

2.1.2 DNA extraction, sequencing and competitive read mapping

DNA extraction was performed by Jacob Neal (Neal, 2024). Briefly, DNA was extracted from 16 Pool-seq samples (14 hybrid samples and 2 “pure” samples) using the Qiagen MagAttract HMW DNA kit. Eluted DNA was sent to Novogene for paired end PCR-free library prep and whole genome paired-end sequencing on an Illumina NovaSeqX plus to ~150x coverage on average across the genome. Pure Dahomey and LH_m sample FASTQ files were aligned to the latest *D. melanogaster* reference genome (BDGP6.46, Ensembl) using BWA (Li and Durbin, 2009)**,** and the consensus command in BCFtools (Danecek *et al.*, 2021) was used to generate population specific reference genomes (PSRGs). These PSRGs were subsequently concatenated together to produce a combined reference genome for downstream mapping.

Competitive read mapping of hybrid samples was conducted by aligning paired-end sequencing reads to the concatenated PSRGs using BWA, and alignment outputs were converted to BAM format and processed using SAMtools. Reads were therefore forced to align to whichever PSRG they mapped to better, which due to the design (above), identified the parent-sex-of-origin of each read. Reads mapping equally well to both PSRGs would map randomly to one or the other but would receive an alignment score of 0. Following competitive read mapping, reads with an alignment score of 0 were discarded, and reads were partitioned into 28 “sperm” and “egg” sub-samples according to whether they mapped better to the paternal or maternal population, respectively. Sperm and egg sub-sample BAMs were realigned to the standard *D. melanogaster* reference genome (BDGP6.46). This resulted in 28 (14 pairs) of sperm and egg BAM alignment files (of ~75x coverage averaged across the genome) representing the successful male gamete pool (hereafter “sperm pool”) and the successful female gamete pool (hereafter “egg pool”) that gave rise to the fertilised zygotes resulting from a single generation of competitive reproductive success among thousands of recombinant genotypes. SNPs were called across the

full set of sperm and egg pool samples using VCFtools, where allele frequencies in the successful sperm and egg pools were inferred from the depth of coverage for downstream contrasts using PoPoolation2 (see below).

Across the complete set of 14 hybrid samples, the sperm-derived sub-samples contained equal contributions from Dahomey and LH_m fathers, while the egg-derived sub-samples contained equal contributions from Dahomey and LH_m mothers. The comparison was therefore not between the sperm and egg pools within a single hybrid cross, where differences in population of origin could produce an apparent allele-frequency difference. Instead, the CMH analysis compared the balanced set of 14 sperm-derived sub-samples with the balanced set of 14 egg-derived sub-samples across the replicate crosses. In the absence of sex-specific selection, allele frequencies would not be expected to show a consistent difference between two balanced gamete groups. In contrast, SNPs subject to sex-specific selection, including SA selection, could show consistent differences between successful sperm and egg sub-samples across the replicate crosses. The reciprocal design is important because any allele-frequency difference caused solely by population of origin is reversed between the two cross directions and therefore does not produce a consistent sperm-versus-egg association across the complete dataset.

2.1.3 Allele frequency estimation and CMH testing

The aim of the allele-frequency analysis was to identify genomic variants showing consistent differences between successful sperm and successful egg pools following competitive reproduction. A key consideration was that the Dahomey and LH_m populations differ in their allele frequencies before the experiment. Consequently, a difference between an individual sperm and egg sub-sample cannot automatically be interpreted as evidence of sex-specific selection because the two sub-samples may have different population-of-origin frequencies. The reciprocal-cross design addressed this by ensuring that the population of origin was balanced between the sperm and egg pools across the complete dataset.

Specifically, seven replicate crosses used Dahomey males and LH_m females, whereas seven reciprocal crosses used LH_m males and Dahomey females. Following competitive read mapping, each hybrid sample produced one sperm derived and one egg-derived sub-sample. Across all 14 sperm sub-samples, half originated from Dahomey fathers and half from LH_m fathers. Similarly, across all 14 egg sub-samples, half originated from Dahomey mothers and half from LH_m mothers.
The statistical comparison therefore considered the balanced set of Dahomey and LH_m derived sperm sub-samples against the corresponding balanced set of Dahomey and LH_m derived egg sub-samples.

This distinction is important when considering the expected allele-frequency patterns. In the absence of selection, allele frequencies among successful sperm and eggs should not show a consistent difference once the population of origin is balanced across the two gamete classes. Any difference between the source populations is present in both the sperm and egg dataset and is therefore not expected to generate a consistent sperm versus egg association across reciprocal crosses. For example, a SNP in which allele A is more common in Dahomey but rare in LH_m would show in a Dahomey-male x LH_m -female cross, allele A would initially be more common among sperm than eggs because the sperm originate from Dahomey males whereas eggs originate from LH_m females. However, in the reciprocal LH_m -male x Dahomey-female cross the pattern would be reversed, where allele A would initially be more common among eggs than sperm. Therefore, the population-of-origin differences alone generate opposite sperm-egg contrasts in the two reciprocal cross directions. When both reciprocal crosses are included in equal numbers, these effects should cancel rather than produce a consistent genome-wide association with gamete sex.

The hypothesis tested here measures selection during competitive reproduction. If an allele has a fitness advantage in males but a disadvantage in females, as expected under sexually antagonistic selection the allele should become disproportionately represented among successful sperm relative to successful eggs. As the reciprocal cross design balances population-of-origin this sex-associated difference should be consistent across the two reciprocal cross directions rather than simply reflecting which population the mother or father comes from. In contrast, if the same allele is

favoured in both sexes (sexually concordant selection), selection should favour the same allele in males and females, rather than producing opposing allele-frequency differences between their gamete pools. Sex-limited selection in which selection acts through one sex, could also produce a sex-associated allele frequency difference and therefore represents an alternative explanation for a significant sperm-egg contrast. Consequently, the CMH analysis identifies loci showing consistent sex associated allele frequency differentiation following reproductive competition; these loci are treated as candidate SA loci when interpreted in the context of the experimental design and the expected biological mechanism.

Only SNPs present in both populations were eligible for allele frequency contrasts between sperm and egg pools. This ensured that the variants analysed were segregating in both source populations and could therefore be compared across the balanced reciprocal design. Allele frequency contrasts were quantified using Popoolation2 (Kofler *et al*., 2011). First, BAM files of the sperm and egg pools were realigned to the common *D. melanogaster* reference genome (BDGP6.46, Ensembl) and converted into pileup format using SAMtools (Li *et al.*, 2009). These mpileup files were synchronised using PoPoolation2's mpileup2sync.jar with a minimum base quality threshold of 20. The Cochran-Mantel-Haenszel (CMH) test was then performed using Popoolation2's cmh-test.pl script across the 14 matched sperm-egg pairs. The CMH test is appropriate for this design because it tests for an association between the two categorical variables, in this case gamete type (sperm or egg) and allele frequency whilst stratifying the analysis by replicate. This allows the test to assess whether an allele is consistently differentiated between successful sperm and eggs across replicate crosses rather than relying on a single pooled comparison. The reciprocal cross structure is retained within these paired replicates so that population-of-origin effects are accounted for by the balanced experimental design.

Parameters included a minimum allele count of 5, minimum coverage of 40x and maximum coverage of 200x. Sample groups were defined using the --population flag, to pair egg and sperm pools for each replicate.

Pairwise F_{st} estimates between egg and sperm pools were calculated using

Popoolation2's merged_fst_pwc.pl script. Significant SNPs were annotated using Ensembl's Variant Effect Predictor (VEP) (McLaren *et al.*, 2016) referencing the BDGP6.46 genome annotation. The input VCF files contained chromosomal coordinates and allele information generated from PoPoolation2's merged_fst_pwc.pl script. VEP was used to assign functional consequences to each variant (e.g., synonymous, missense, intronic, intergenic). SNPs were then mapped to genes using the biomaRt package (Durinck *et al.*, 2009) to facilitate downstream gene-level analyses.

2.1.4 Permutation-based significance thresholding

To identify SNPs with statistically significant allele frequency differences, an empirical null distribution was generated using a permutation-based approach following established methods (e.g Bastide *et al.*, 2013). Ten permuted datasets were generated by randomly selecting 7 of the 14 pairs of samples and swapping their "sperm/egg" labels in the –population flag of PoPoolation2's CMH test (see above). This generated a null distribution of $-\log_{10}(P)$ values across the genome. Any SNPs in the real data with $-\log_{10}(P)$ values greater than the 95th percentile of this empirical null distribution would be significant by conventional standards in biology, with differences beyond that cut-off being attributable to chance 5% of the time. 18 SNPs exceeded this threshold, which (following the further filtering criteria below) yielded one SNP on each of two peaks near the centromere of Chromosome 3. This did not provide enough material for downstream analyses that aim to detect statistical associations and broad-scale evolutionary patterns in a set of candidate SA SNPs and genes. Therefore, the remaining analysis below uses the 90th percentile of the empirical null as the cut-off, increasing both false-positive and true positive rates such that 10% of SNPs exceeding this threshold are expected by chance. SNPs from the dataset exceeding these thresholds were retained as candidate loci for further downstream analysis. This permutation-based approach helps control for false positives and is well-suited to pool-seq data, where standard multiple testing corrections may not be reliable due to underlying overdispersion and sampling variance (Camargo *et al.*, 2008; Wang *et al.*, 2012; Viljanen and Boshuizen, 2022).

To further refine the candidate set and reduce false positives, an additional clustering filter was applied to filter for “true peaks”. Only SNPs that (i) had at least one neighbouring SNP within a 500-base pair (bp) window, and (ii) had a neighbour with a P-value within one order of magnitude (i.e a $-\log_{10}(P)$ of $\pm$ 1) were retained. This approach was used to identify groups of nearby SNPs showing similar levels of statistical support, which may provide greater confidence in those isolated significant SNPs. Although linkage disequilibrium (LD) can contribute to local clustering of signals, its extent can vary between populations and demographic histories, therefore a fixed LD decay distance should not be assumed for the laboratory adapted Dahomey and LH_m populations. Combining the permutation-based significance threshold with this clustering criteria helped provide an additional filter against isolated significant SNPs and helped refine the set of candidate SA SNPs. For the 90th percentile cut-off, initially 2,327 SNPs were identified as significant, and after filtering for true peaks this left 323 candidate SA SNPs.

Results were visually inspected using a Manhattan plot of $-\log_{10}(P)$ values. A QQ plot was generated using the qqman R package (D. Turner, 2018) to assess genomic inflation, quantified using the lambda (λ) statistic.

2.1.5 Enrichment tests and gene-level analyses

To provide a consistent baseline for all downstream analysis, a background SNP set was defined as all SNPs retained in the CMH analysis following quality control and filtering. This background therefore represents the full set of SNPs subjected to the same analytical pipeline as the candidate SA SNPs.

Chromosomal enrichment of these 323 candidate SA SNPs was assessed using a two-sided Z-test, comparing their distribution across chromosome arms to that of the genome-wide background SNP set. This analysis was repeated after grouping all autosomal sites into a single category to test for differences in enrichment between autosomes and the X chromosome.

Candidate SA genes were identified by mapping candidate SA SNPs to gene IDs via the biomaRt package in R (Durinck *et al.*, 2009). Overlap between candidate SA genes identified in this study and previously published candidate SA gene sets was evaluated using Fisher's exact tests. For these analyses, the background gene set consisted of 13,178 annotated protein-coding genes in *D. melanogaster*.

Gene ontology (GO) enrichment analysis was conducted using the clusterProfiler R package (Xu *et al.*, 2024) across three categories: biological process (BP), molecular function (MF) and cellular component (CC). GO terms with adjusted P-values <0.05 were considered statistically significant.

2.1.6 Gene-level annotations and external datasets

To characterise the functional and evolutionary properties of candidate SA genes, multiple published gene-level datasets were integrated into downstream analyses.

SBGE classifications were from Mishra *et al. (*2024) based on whole-body expression data. Genes were assigned to five categories according to $\log_2$FC (male/female) expression contrasts: highly female biased ($\log_2$FC ≤ -2), moderately female biased (-2 < $\log_2$FC ≤ -0.5), unbiased (-0.5 < $\log_2$FC ≤ 0.5), moderately male biased (0.5 < $\log_2$FC ≤ 2) and highly male biased ($\log_2$FC >2).

Tissue specificity (τ) was obtained from Fraisse *et al.* (2019), which quantifies gene expression (τ) from broadly expressed across all included tissues (τ = 0) to narrowly expressed in only one tissue (τ = 1). Tissue specificity was measured across three adult tissues in *D. melanogaster*: carcasses, digestive systems and heads.

The r_{MF} estimates were obtained from Singh and Agrawal (2023), derived from the *D. melanogaster* genetic reference panel (DGRP). High r_{MF} values close to 1 indicate that genes are constrained from responding independently in each sex, a

prerequisite for intralocus sexual conflict. Low r_{MF} values near 0 indicate genes are able to exhibit sexually dimorphic expression either indicating an absence of sexual conflict or resolved (Huang *et al.*, 2015).

The relative level of nonsynonymous diversity ($\tilde{\pi}_{rN}$) for each gene was obtained from Singh and Agrawal (2023) and are from African lines. It is a derived metric rather than a standard measure of nucleotide diversity and is calculated as pi_N / (pi_N + pi_S), where the nonsynonymous diversity within a gene was measured as the mean nucleotide diversity at 0-fold degenerate sites within that gene, and synonymous diversity within a gene as the mean nucleotide diversity at 4-fold degenerate sites within each gene. High $\tilde{\pi}_{rN}$ values reflect weaker purifying selection, allowing more polymorphisms, whereas lower $\tilde{\pi}_{rN}$ values indicate stronger purifying selection, suggesting that changes in the protein-coding sequences are likely to be deleterious and removed through selection (Singh and Agrawal, 2023).

Direction of selection (DoS) values were obtained from Fraisse et al (2019) and are from African lines. An excess of nonsynonymous substitutions relative to nonsynonymous polymorphisms (DoS > 0) within a population indicates positive selection, whereas an excess of nonsynonymous polymorphisms relative to nonsynonymous substitutions within a population (DoS < 0) indicates purifying selection (Stoletzki and Eyre-Walker, 2011).

Finally, Tajima's D values were obtained from Singh and Agrawal (2023) and are from African lines. These values were estimated using 4-fold degenerate sites, accounting for the sample size and number of 4-fold sites in each gene (Walsh and Lynch, 2018).

2.1.7 Data analysis

Bioinformatic processing and analysis were performed using command-line sbatch scripts submitted to the Slurm job scheduler (Yoo *et al*, 2003) on the University of

East Anglia's high-performance computing cluster (ADA) and following the CMH test, all downstream statistical analyses were conducted in *Rstudio* (version 2025.05.1). A range of packages were used including dplyr (Wickham *et al.*, 2026) for data manipulation, ggplot2 (Wickham, 2016) was used for visualisation and biomaRt (Durinck *et al.*, 2009b) was used for gene mapping and assigning Flybase IDs to their gene names. Once exploratory analyses were performed, statistical analyses were conducted to assess whether candidate SA genes differed from background genes. Permutation tests were used to compare the frequency of overlap in SA candidate genes versus background genes, assessing whether the presence of these SA candidate genes were observed more often than expected by chance. Each permutation test was run using 10,000 iterations between candidate and background groups to generate a distribution of differences. The observed difference in gene groups was then compared against the distribution to calculate a p-value. Permutation tests were applied across all genes and within each SBGE category separately to further inspect any overall and category-specific differences.

A Fisher's exact test was used to test the statistical significance of whether SA candidate genes were enriched for the SA genes identified in other studies. These were compared to a background set of genes compiled using the biomaRt package (Durinck *et al.*, 2009b) that provided Flybase IDs for 13,178 protein-coding genes of the *Drosophila* genome.

Gene ontology enrichment analysis was performed using the clusterProfiler package (Yu *et al.*, 2012) in RStudio using 311 candidate genes relative to the genomic background.

2.2 Results

2.2.1 Identification of candidate SA loci

A genome-wide CMH test identified SNPs exhibiting consistent allele frequency differentiation between successful sperm and egg pools across the five major

chromosome arms of *D. melanogaster (*2L, 2R, 3L, 3R and X; Figure 1). The analysis compared the balanced set of sperm-derived sub-samples containing equal representation from the Dahomey and LH_m fathers, with the corresponding balanced set of egg-derived sub-samples containing equal representation from Dahomey and LH_m mothers. The CMH analysis used the 14 matched sperm-egg pairs to test whether allele frequencies consistently differed between successful sperm and eggs across replicate crosses, while accounting for variation between replicates.

Using a stringent threshold based on the 95th percentile of the permutation-derived null distribution of CMH -$\log_{10}$(P) values, 18 SNPs were initially identified as significant. After applying clustering filters to retain only “true peaks”, this was reduced to 2 SNPs located on chromosome arm 3L, both outside annotated coding regions. This limited number of SNPs was insufficient for downstream analyses.

To increase power, a more permissive 90th percentile was applied. This identified 2,327 SNPs, which were reduced to 323 independent SNPs after filtering, mapping to 311 genes. These were retained as candidate SA SNPs for all subsequent analyses.

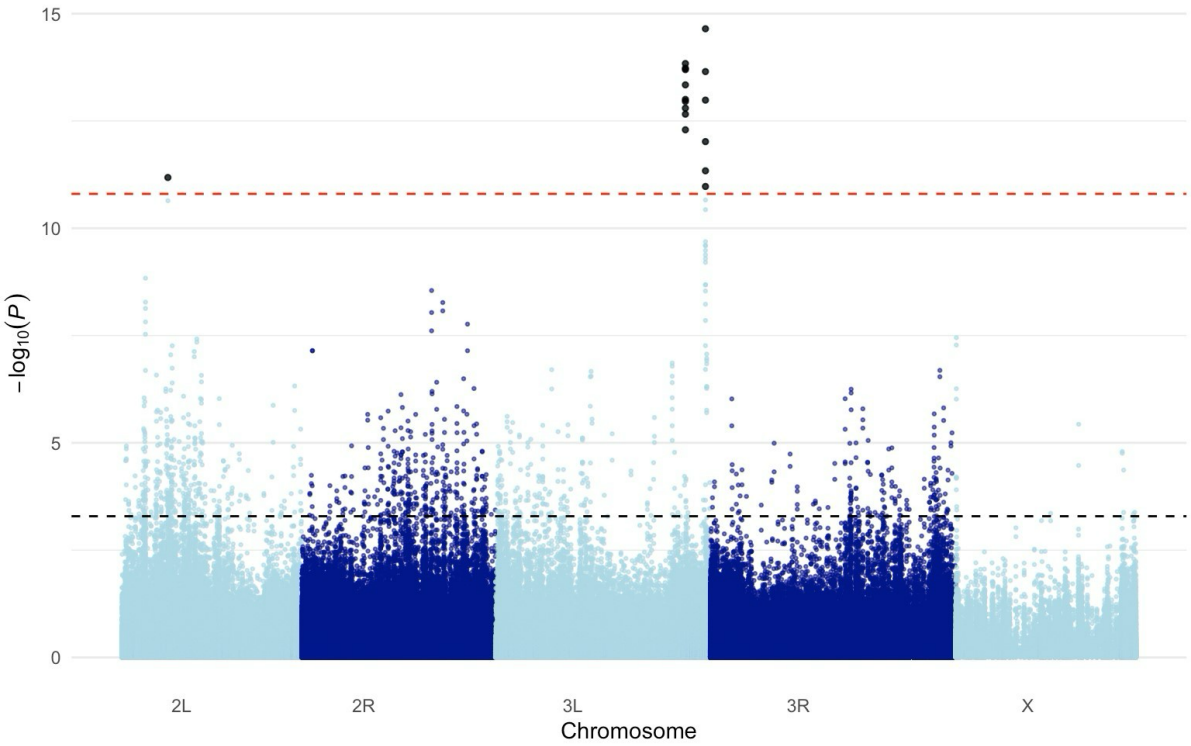

Figure 1: Association of each SNP with sex-specific selection along the five major *D. melanogaster* chromosome arms. Each point represents the -log10(P) value from a pool-seq CMH test between the successful sperm versus eggs that gave rise to tens of thousands of fertilisation events after a single generation of selection among thousands of genetically diverse parental genomes. Horizontal dashed red and black lines represent the 95th and 90th percentiles of the permuted empirical null, respectively.

2.2.2 Validation of significance threshold

Two approaches were used to validate the arbitrary second choice 90th percentile cut-off. First, a quantile-quantile (QQ) plot (Figure S1) showed that deviation from the null expectation was restricted to the upper tail, consistent with a subset of true outlier loci rather than genome-wide inflation. Second, r_{MF} was examined across a range of percentile thresholds (99th-79th) (Figure S2). Although the strongest r_{MF} signal occurred at stricter thresholds (94th percentile), this would have yielded too few

SNPs for robust downstream analysis as well. The 90th percentile therefore represented a compromise between statistical power and biological signal (Wickham *et al.*, 2026).

2.2.3 Genomic distribution of candidate SNPs

Candidate SA SNPs were non-randomly distributed across chromosome arms (Figure 2A). Chromosome arm 2L exhibited significant enrichment relative to the genomic background ($Z = 5.24$, $P < 0.001$), whereas chromosome arm 3R showed significant depletion of candidate SA SNPs ($Z = -4.59$, $P < 0.001$). No other chromosome arm deviated significantly from expectation.

When autosomes were pooled and compared against the X chromosome, no significant difference in enrichment or depletion was detected (Figure 2B), indicating that enrichment patterns are arm-specific rather than driven by X-linkage.

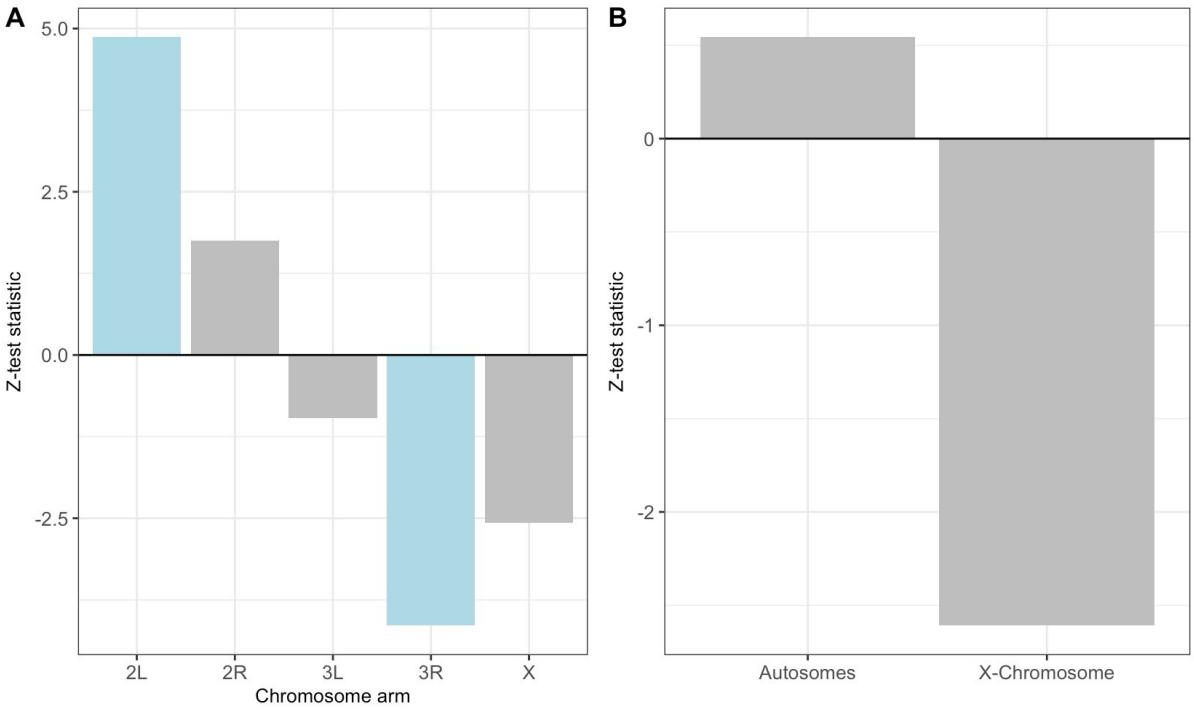

Figure 2: Enrichment of candidate SA SNPs across chromosome arms. Results of Ztest that compare (A) the number of candidate SA SNPs on each chromosome arm relative to all SNPs identified across the genome, and (B) the number of candidate SNPs on autosome and X chromosome, relative to the total SNPs in each of those categories. Blue = statistically significant Z-test(P<0.05), Grey = non significant Z-test (P ^{3} 0.05).

2.2.4 Functional annotation and overlap with previous studies

Functional annotation revealed that candidate SNPs were significantly enriched in regulatory regions and transcript-proximal regions, including upstream, downstream and synonymous regions (Figure 3). These regions are typically associated with gene regulation and mRNA processing rather than changes to amino acid sequences. In contrast, intergenic and intronic regions were significantly depleted. This suggests that SA variation is more strongly associated with regulatory changes than protein-coding variation.

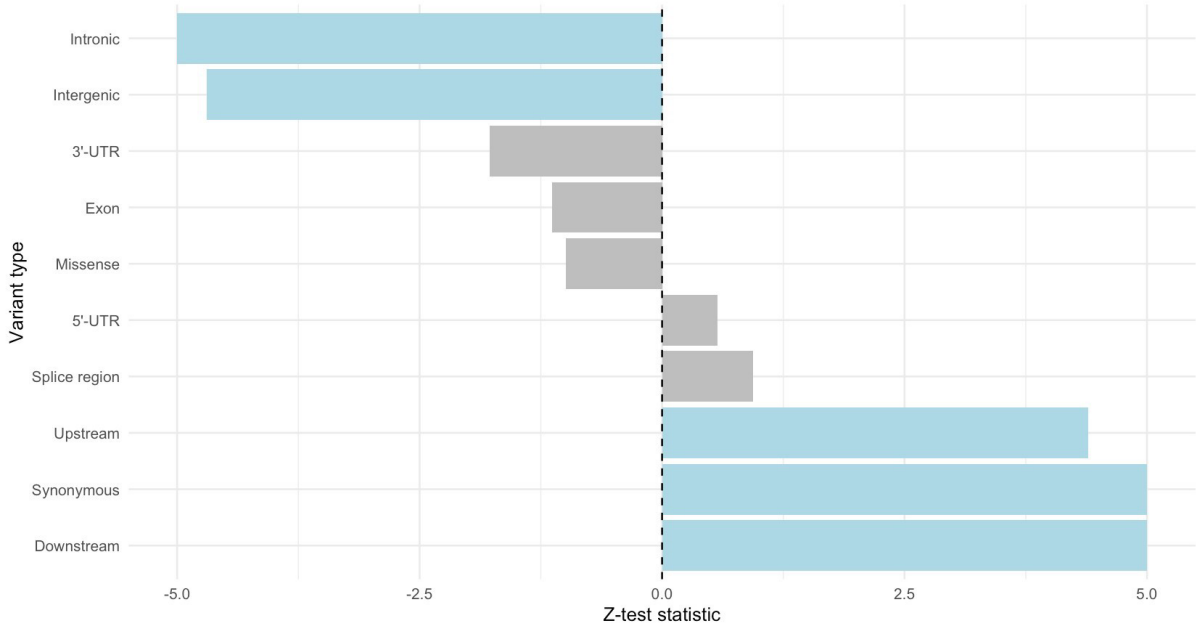

Figure 3: Enrichment of variant types among candidate SA SNPs. Results of Z-test comparing the number of candidate antagonistic SNPs falling into each functional category against the representation of each category among all SNPs. Blue= statistically significant Z-test ($P < 0.05$), Grey = non- significant Z-test ($P \geq 0.05$).

Comparison with previously identified candidate SA gene sets revealed significant overlap with Mishra *et al.* (2024) (P=0.005) and Grieshop *et al.* (2025)(P=0.003), but not with earlier studies (Table 1). Detailed results for individual comparisons are provided in Supplementary figures S3-S6. These results indicate partial congruence with recent genomic studies of sexual antagonism.

Table 1. Fisher's exact test results for overlap between candidate SA genes of this study and those of other published studies.

Study	Frequency of their candidates among my candidate genes	Frequency of their candidates among background genes	P-value
Innocenti & Morrow (2010)	7% 7/311	5% 640/13,178	0.08
Ruzicka *et al.* (2019)	3% 10/311	3% 350/13,178	0.48
Mishra *et al.* (2024)	3% 10/311	1% 158/13,178	0.005
***Grieshop et al.* (2025)**	7% 22/311	3% 458/13,178	0.003

2.2.5 Gene ontology enrichment

Gene ontology analysis identified a single significantly enriched term "collagen containing extracellular matrix" (GO:0062023), represented by eight genes (Table S1). No additional GO terms remained significant after multiple testing correction, suggesting either limited functional clustering among candidate genes, and/or that candidate SA genes are very uniformly distributed across many biological functions and molecular processes.

2.2.6 Evolutionary Signatures of SA genes

Candidate SA genes exhibited significantly higher r_{MF} than background genes (P <

0.001; Figure 4). This overall pattern appears to be driven by consistently higher (though non-significant) r_{MF} values across most SBGE categories and a stand-alone significantly higher r_{MF} in the highly-male biased category.

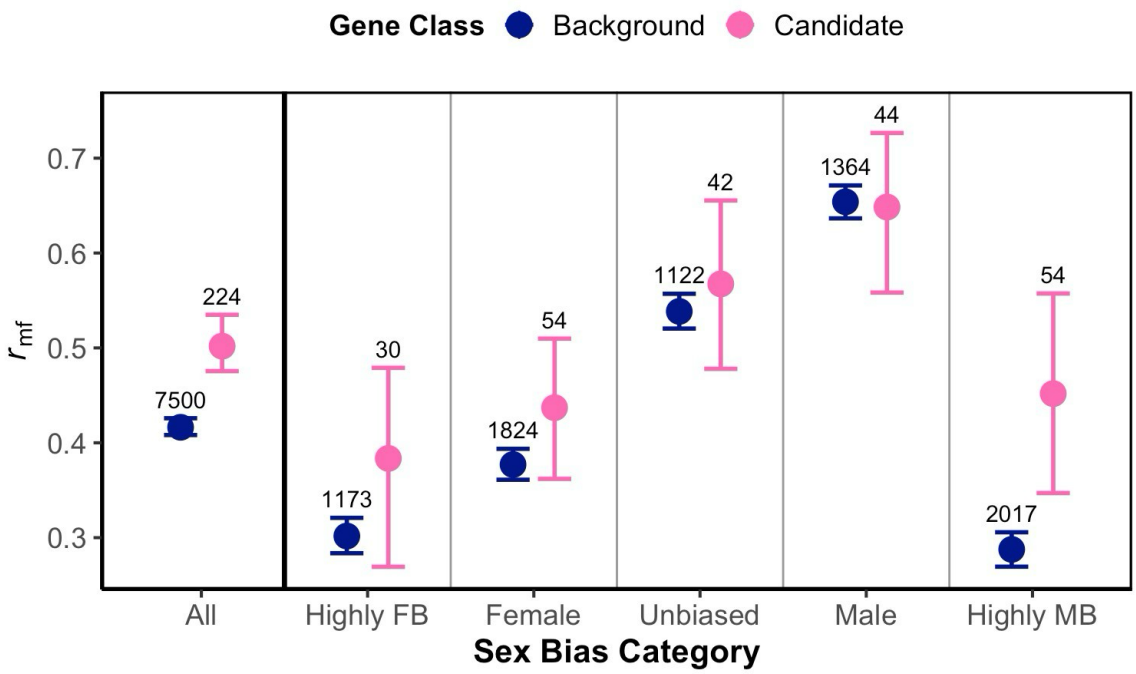

Figure 4. Comparison of r_{MF} between candidate SA genes and background genes in *Drosophila.* The "All" panel shows results irrespective of SBGE, whilst the remaining panels are binned into SBGE categories: Highly Female-Biased (Highly FB), Moderately Female-Biased (Female), Unbiased, Moderately Male-biased (Male) and Highly Male-Biased. Points represent median values with pink representing candidate SA genes and blue representing all genes in *D . melanogaster.* Error bars are bootstrapped 95% confidence intervals, and the numbers above represent the sample sizes (number of genes).

The direction of selection (DoS) did not differ significantly between candidate and background genes overall or within any SBGE categories (Figure 5). Therefore, candidate genes did not show evidence of positive or negative selection relative to the genomic background.

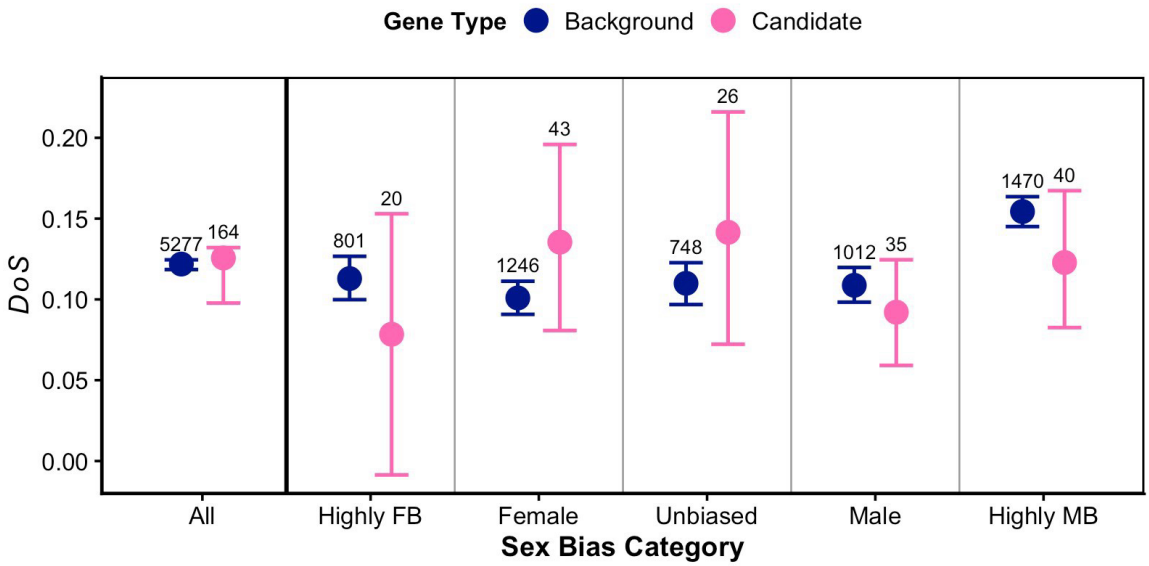

Figure 5. Comparison of DoS between candidate SA genes and background genes in *Drosophila.* The "All" panel shows results irrespective of SBGE whilst the remaining panels are binned into SBGE categories: Highly Female-Biased (Highly FB), Moderately Female-Biased (Female), Unbiased, Moderately Male-biased (Male) and Highly Male-Biased. Points represent median values with pink representing candidate SA genes and blue representing all genes in *D. melanogaster.* Error bars are bootstrapped 95% confidence intervals, and the numbers above represent the sample sizes (number of genes).

Tajima's D values did not differ significantly between candidate SA and background genes in any category (Figure 6). Although most SBGE categories showed slightly elevated Tajima's D among candidate genes, these differences were not statistically significant. Further, the slightly elevated value is still below 1, suggesting an excess of low-frequency polymorphisms.

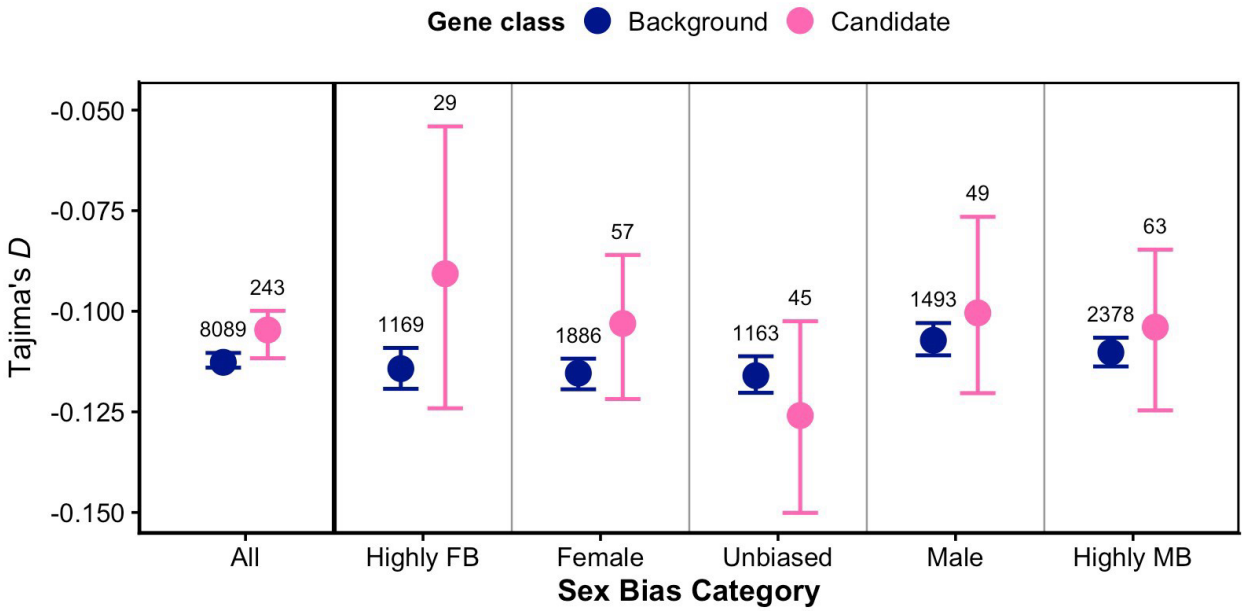

Figure 6. Comparison of Tajima's D between candidate SA genes and background genes in *Drosophila.* The "All" panel shows results irrespective of SBGE whilst the remaining panels show SBGE: Highly Female-Biased (Highly FB), Moderately Female-Biased (Female), Unbiased, Moderately Male-biased (Male) and Highly Male-Biased. Points represent median values with pink representing candidate genes and blue representing all genes in *D.melanogaster.* Error bars are bootstrap 95% confidence intervals and the numbers above represent the sample sizes.

Median tissue specificity did not differ significantly between candidate and background genes across all genes (observed median difference = -0.03) or within any SBGE categories (Figure 7). This indicates that on average, candidate genes did not exhibit broader or more restricted tissue expression patterns relative to the background genome.

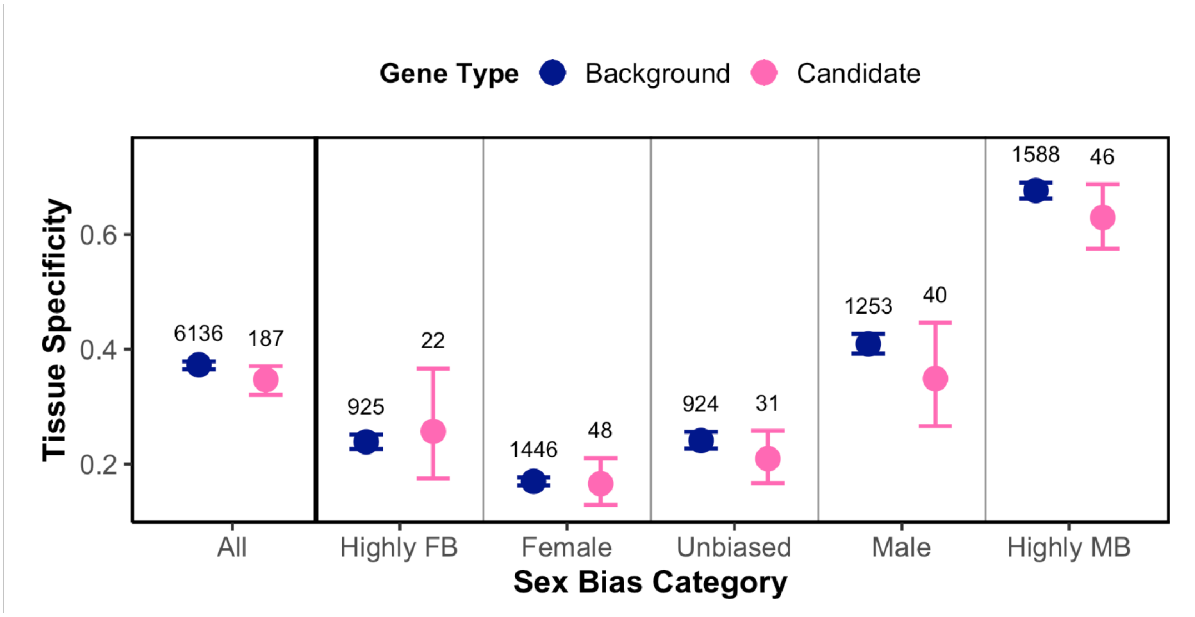

Figure 7. Comparison of Tissue specificity between candidate SA genes and background genes in *Drosophila.* The "All" panel shows results irrespective of SBGE whilst the remaining panels are binned into SBGE categories: Highly Female-Biased (Highly FB), Moderately Female-Biased (Female), Unbiased, Moderately Malebiased (Male) and Highly Male-Biased. Points represent median values with pink representing candidate genes and blue representing all genes in *D. melanogaster.* Error bars are bootstrapped 95% confidence intervals and the numbers above represent the sample sizes.

Candidate genes exhibited significantly reduced $\tilde{\pi}$rN compared to background genes ($P < 0.001$; Figure 8). Within SBGE categories moderately female-biased (P-value =0.005) and moderately male-biased (P-value = 0.01) categories showed significantly reduced $\tilde{\pi}$rN. Reduced nonsynonymous diversity typically indicate purifying selection of amino acid sequence variation.

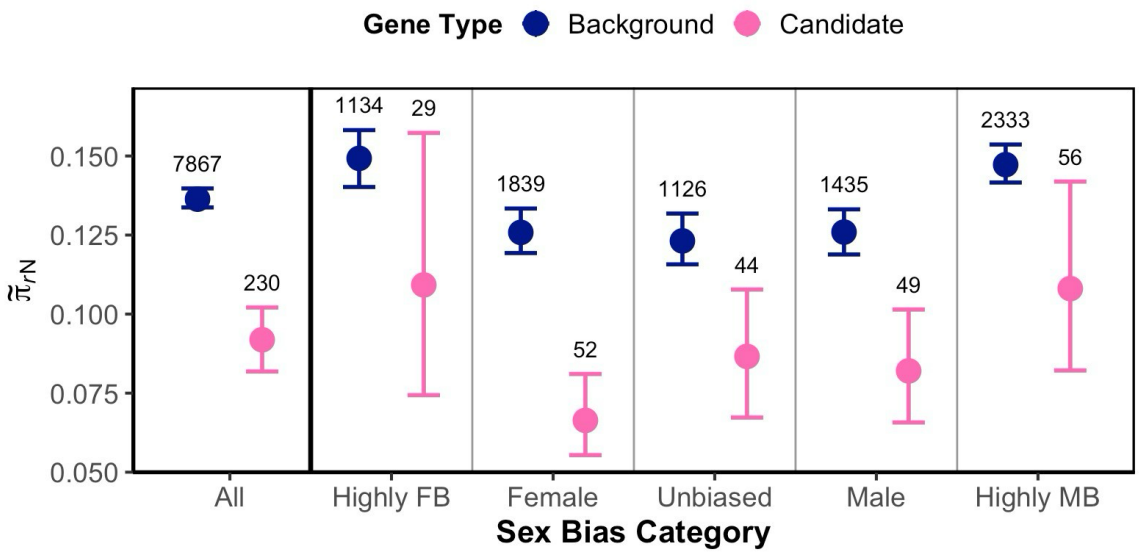

Figure 8. Comparison of $\tilde{\pi}_{rN}$ between candidate SA genes and background genes in *Drosophila.* The "All" panel shows results irrespective of SBGE whilst the remaining panels are binned into SBGE categories: Highly Female-Biased (Highly FB), Moderately Female-Biased (Female), Unbiased, Moderately Male-biased (Male) and Highly Male-Biased. Points represent median values with pink representing candidate genes and blue representing all genes in *D. melanogaster.* Error bars are bootstrapped 95% confidence intervals and the numbers above represent the sample sizes.

2.3 Discussion

Despite significant theoretical interest, the genomic distribution and evolutionary dynamics of sexually antagonistic (SA) polymorphisms still remain poorly understood. Here, SA loci in *D. melanogaster* were explored by comparing allele frequencies between male and female successful gamete pools. This provides a direct measure of the strength and direction of reproductive selection acting on alternative alleles in each sex.

This approach offers several advantages over established methods. First, this method offers a greater degree of causal inference compared to methods that aim to associate sex-specific fitness estimates with gene expression (Innocenti and Morrow, 2010) or SNPs (Ruzicka *et al.*, 2019). The experimental design of this present

method does not rely on linear modelling assumptions and leaves only the causal explanation of allele frequency divergence between sperm and egg subsamples to parent-sex-of-origin. Further, my approach is genome-wide and does not require prior selection of specific candidate genes or genomic regions. This provides a complementary approach to experimental evolution studies, including male-limited or sex-specific selection experiments, which can also investigate genome-responses to selection but typically require multiple generations of selection and subsequent genomic analysis (Grieshop *et al.*, 2025).

There has been interest in comparing allele frequencies between adult males and females in an effort to identify candidate SA genetic variation (Lucotte *et al.*, 2016)**.** Such approaches can identify allele-frequency differences associated with sex specific-survival or viability, but these differences arise before the stage of reproductive competition. Therefore, adult male-female comparisons cannot directly distinguish allele-frequency differences arising from sex-specific survival from those associated with subsequent reproductive success. In contrast, compared with all alternative methods of which I am aware of, the empirical effort (e.g. fitness statements, generational culturing protocols, etc.) would scale linearly with statistical power and resolution. However, the present approach generates many recombinant parental genotypes and fertilisation events (increasing genomic resolution) in exchange for relatively few virgin female collections and small adjustments to the cage size and number of oviposition plates. A similar concept applies to improving resolution for low-frequency variants, provided that the number of parental genotypes far exceeds the sequencing depth, then increasing sequencing depth improves the accuracy of allele frequency estimation. This can be adjusted post hoc with the same eluted DNA samples.

However, this approach is not without limitations. Limitations to my approach include that it is restricted to convenient laboratory organisms, such as *D. melanogaster* or perhaps other insects or fish. It also requires some background knowledge about the two alternative populations used or would require some background investigation to ensure sufficient divergence between alternative populations to minimise the percent of ambiguous DNA reads discarded whilst not having them be so divergent that

parent-sex-of-origin is confounded with local adaptation in ways that compromise the experiment. Lastly, my approach could require correction among sex-linked loci, since for example the X-linked allele frequencies are estimated with twice the accuracy (sequencing depth) in the successful egg pool relative to the successful sperm pool. This could be more complicated in organisms with more complicated sex chromosome evolution, such as the pseudo-autosomal regions of guppies (Charlesworth *et al.*, 2020)

Despite these limitations, the results provide new insight into the genomic architecture of sexual antagonism and highlight several key patterns. Some patterns are consistent with both previous theoretical predictions and empirical studies. Other findings of mine provide opposing evidence to some of that same theory and empirical work. These details are discussed below considering the previously mentioned methodological advantages and disadvantages across methods.

2.3.1 Methodological Challenges

Detecting SA polymorphisms using pooled gametic sequencing presents multiple challenges. Threshold selection involves balancing false positive risk against statistical power, while permutation-based thresholds reduce reliance on arbitrary cut-offs; the selection of percentile thresholds inevitably involves a trade-off between stringency and power. Conservative thresholds (e.g. 95th percentile) minimise false positives but substantially reduce the number of candidates, limiting downstream enrichment and evolutionary analyses. Conversely, more liberal thresholds increase power but introduce a higher expected false positive discovery rate. Although optimisation using intersexual genetic correlation provided an empirical basis for threshold choice, the true proportion of false positives among candidates remains uncertain. Distinguishing genuine signals from regular allele frequency variation remains challenging.

Pool-seq provides efficient allele frequency estimation across many individuals but lacks individual-level genotypic information. Therefore, sampling noise and unequal coverage may inflate apparent allele frequency differences, particularly for low

frequency alleles. Although filtering and minimum coverage thresholds were applied, technical variance may persist.

The CMH test assumes independence among SNPs and consistent effect direction across populations. However, linked SNPs in regions of reduced recombination may generate clusters of significant markers rather than independent selection targets. Although sliding window analyses indicated robust clustering patterns, establishing direct targets from linked variants remains challenging without haplotype-based analyses.

Classical signatures of balancing selection (e.g. elevated Tajima's D, increased relative nonsynonymous diversity or DoS) may lack sensitivity in polygenic systems where selection is weak or fluctuating. Some maintained SA polymorphisms may not produce strong equilibrium signals detectable using statistical methods. Therefore, the absence of strong balancing-selection signatures should not be interpreted as evidence against SA dynamics.

Functional enrichment analyses rely on existing gene annotations and GO classifications, which are biased towards well-characterised protein-coding genes. Regulatory variants, non-coding RNAs and distal enhancers may be underrepresented. Consequently, the apparent lack of strong pathway-level enrichment may only partly reflect annotation limitations rather than the biological absence of functional clustering.

This study focused on two laboratory-adapted populations (Dahomey and LHm). While replication across populations strengthens reliability, laboratory maintenance may alter allele frequencies through drift or adaptation to laboratory populations. Therefore, assumptions to natural populations should be made cautiously.

2.3.2 Identification and distribution of Candidate SNPs

Using a stringent 95th percentile of the null threshold, 18 SNPs were initially detected, with only two true peaks, both located on chromosome 3 outside annotated coding

regions. While conservative, this threshold likely underestimates the true number of SA loci. Applying a more permissive 90th percentile threshold enabled broader discovery, increasing the false positive rate and decreasing the false negative rate relative to the 95th percentile cut-off, with 2,327 candidate SNPs across 323 true peaks, enabling downstream functional and comparative analyses.

If r_{MF} is a good indicator of the potential for IaSC then the 94th percentile cut-off might have maximised our true-positive rate for SA SNPs (but with only 46 SNPs mapping to 42 genes). This cut-off would still have presented a substantial limit to the number of SNPs and genes available for analyses. It does, however, lend some indirect confidence that the 90th percentile threshold used is a reasonable balance between signal strength and downstream analytical power. As more liberal thresholds introduce additional noise, they are more likely to reduce statistical power in downstream analyses, making this approach conservative when identifying consistent patterns across studies.

Candidate SNPs were non-uniformly distributed across chromosomes with enrichment on chromosome arm 2L and depletion on 3R. These patterns indicate that candidate SA SNPs were not distributed uniformly across the genome, although the factors underlying this distribution cannot be determined from the analysis differences in local recombination rate, gene density genomic architecture or regulatory regions could potentially contribute to variation in the distribution. Notably, these results do not support a strong X-linked bias, challenging classical predictions that SA variation should accumulate specifically on the X chromosome. This pattern of enrichment supports the findings of Ruzicka *et al* (2019), who observed that X chromosome was neither enriched nor depleted for candidate SA SNPs.

Sliding window analyses (1kb window with 500bp steps and 500bp window with 250bp steps) produced highly consistent patterns in terms of the number and genomic distribution of candidate SNPs, indicating that the window size had minimal influence on the overall candidate SNP set (Figure S7). The main differences between the two approaches was the number of SNPs retained by the clustering filter. Based on these comparisons the original 1kb window with 500bp steps was

used for all subsequent analyses as it produced broadly consistent results with the alternative window size while retaining the original analysis parameters.

2.3.3 Functional insights and enrichment

Functional annotation of candidate SNPs revealed that a greater proportion were synonymous, with candidate SNPs also occurring in upstream and downstream annotated regions. The greater proportion of synonymous SNPs suggests that candidate SA variation was not predominantly located within protein-coding changes that alter amino acid sequences (Meiklejohn *et al.*, 2014). This pattern differs from Ruzicka et al. (2019), who reported a greater representation of nonsynonymous variants among their candidate SA loci. The differences between these findings may reflect differences in experimental design, candidate SNP identification or the populations and genomic data analysed. Therefore the functional annotation results provide evidence about the distribution of candidate SNPs across functional categories but further functional analysis would be required to determine whether regulatory variation contributes to the resolution of sexual conflict in these candidate loci.

In contrast, candidate SNPs were depleted in intronic and intergenic regions. However, intronic regions are not necessarily functionally neutral and in *Drosophila* the first intron can contain substantial regulatory information that contributes to gene expression. Similarly, intergenic regions may contain regulatory elements, including enhancers and other non-coding functional sequences (Ward and Kellis, 2012). Therefore, the depletion of candidate SNPs in these categories should not be interpreted as evidence that intronic or intergenic variation is generally less functionally relevant. Instead, it indicates that candidate SNPs were underrepresented in these annotated categories relative to the genomic background. The biological significance of this distribution would require more detailed analysis of the specific genomic locations and regulatory elements associated with the candidate SNPs.

These regions are typically more evolutionarily neutral and less likely to influence phenotypic traits unless they encompass long-range regulatory elements or noncoding RNAs (Ward and Kellis, 2012). This depletion therefore supports the hypothesis that functionally constrained regulatory elements are more likely to be the targets of SA selection, consistent with findings from studies in both *Drosophila* and other species (Ellegren, 2011; Mank, 2017).

Despite this regulatory enrichment, GO enrichment analysis only identified a single significantly enriched term, “Collagen-containing extracellular matrix”. This limited functional clustering suggests that candidate SA genes are broadly distributed across diverse functions, rather than concentrated within a few small biological pathways. Similar patterns have been reported in genomic studies of both *Drosophila* and humans, where candidate SA genes are spread broadly across the genome rather than confined to specific functional categories (Lucotte *et al.*, 2016; Ruzicka *et al.*, 2019). One possible explanation is that many antagonistic variants occur in regulatory or non-coding regions, which are not always well captured by standard GO enrichment approaches that primarily focus on protein-coding genes. (Haynes *et al.*, 2018). Alternatively, the lack of strong enrichment may reflect the complex nature of SA variation, where selection acts on many loci of small effect rather than few genes with large effect (Barson *et al.*, 2015; Rowe *et al, 2018)*.

2.3.4 Comparative overlap with independent studies

Significant overlap was observed between candidate genes identified here and those reported in independent studies, strengthening confidence in the biological relevance of the detected signals. Despite methodological differences, a subset of genes consistently emerges across datasets, suggesting that certain loci may represent robust targets of SA selection.

Several genes identified in this study were also reported in multiple independent analyses. For example, *pde11* was shared with both Ruzicka *et al., (2019)* and Grieshop *et al., (2025).* This gene encodes a dual-specificity phosphodiesterase

involved in cAMP and cGMP signalling, pathways that play central roles in cellular regulation (Day *et al.*, 2005). Similarly, *sulfateless* was identified in both Ruzicka *et al.,* (2019) and Mishra *et al.,* (2024) and is involved in the modification of heparan sulfate chains, which regulate morphogen signalling during tissue development (Lin *et al.*, 1999).

Notably, several overlapping genes are associated with developmental and regulatory processes. For example, *Hakai* which was also identified in Ruzicka *et al.,* (2019) and Mishra *et al.,* (2024), plays a role in sex determination through the m6A RNA modification pathway, ensuring correct female-specific splicing of *Sxl* transcripts (Bawankar *et al.*, 2021). Disruption to this pathway can lead to sexspecific phenotypic consequences, including the development of male abdominal pigmentation, and is often fatal, making it a potential target of SA selection. Likewise, *wmd,* also shared by Ruzicka *et al.,* (2019) and Mishra *et al.,* (2024), is involved in wing development, motor function and muscle integrity (Dworkin and Gibson, 2006). Finally, *claret,* identified here and in both Innocenti and Morrow (2010) and Ruzicka *et al., (2019)* is involved in eye pigmentation (Yamamoto *et al.*, 1989).

Overall, the recurrence of some genes across independent datasets indicates that there may be shared genetic targets associated with SA variation. However, the interpretation of this overlap should be treated cautiously, as similarities between datasets may be influenced by differences in gene annotation, gene length, genomic coverage of GO annotation bias. Therefore, the recurrence of genes does not by itself demonstrate that SA selection preferentially targets developmental or regulatory pathways. Nevertheless, the overlap provides evidence that some loci identified in the present study have also been implicated in previous studies of SA variation, supporting their potential relevance for further investigation.

2.3.5 Genomic signatures of SA selection

Across multiple analyses, candidate genes exhibited patterns consistent with shared genetic architecture between the sexes, alongside evidence of some sex-specific differentiation in gene expression. Candidate genes had significantly elevated

intersexual genetic correlation than background genes, although r_{MF} was not necessarily elevated in absolute terms across the candidate set. High r_{MF} is widely interpreted as evidence of unresolved IaSC, where alleles cannot independently optimise fitness in each sex due to genetic constraints (Griffin *et al.*, 2013; Collet *et al.*, 2016). However, the candidate genes showing the greatest differences in rmf were at least mildly sex-biased in their expression, suggesting some degree of sex specific resolution may occur despite the shared genetic architecture. These results therefore suggest that candidate SA loci may experience a combination of genetic constraint and partial resolution of sexual conflict, rather than representing loci at which SA selection remains entirely unresolved (Pennell *et al.*, 2016).

Candidate genes also exhibited significantly reduced relative nonsynonymous diversity, indicating stronger purifying selection acting on protein-coding sequences. This pattern was particularly noticeable in moderately sex-biased genes, suggesting that these genes are important and constrained at the amino acid level. Together, elevated r_{MF} and reduced nonsynonymous diversity support the idea that changes to protein sequence may be deleterious, therefore favouring the maintenance of shared alleles across sexes.

In contrast, no significant differences in DoS were detected between candidate and background genes. This indicates that candidate genes did not show a detectable difference in the relative contribution of positive selection to protein evolution compared with the genomic background. This result does not however indicate that candidate genes experience identical selective pressures or that they lack evidence of altered selective constraint. The higher relative nonsynonymous diversity observed among candidate genes is consistent with reduced purifying constraint, but this does not necessarily result in a detectable difference in DoS, which reflects the relative contribution of nonsynonymous substitutions compared with within population polymorphism. The absence of a significant DoS difference may therefore indicate that candidate genes have accumulated greater nonsynonymous variation without showing a corresponding increase in the relative rate of adaptive protein evolution. Additionally, SA selection may act on the standing variation without necessarily

producing a long-term divergence between populations, which could further limit the ability of DoS to detect its effects.

Patterns of Tajima's D provided limited support for balancing selection. Although candidate genes tended to show slightly elevated values, consistent with an excess of intermediate-frequency alleles, these differences were not statistically significant. This suggests that if balancing selection is acting on SA loci, it is likely to be weak or restricted to a subset of loci. Alternatively, the limited number of candidate genes may reduce the power to detect subtle deviations from neutrality. These findings align with broader evidence that long-term balancing selection is relatively rare at the genome-wide scale. This is consistent with evidence that long-term balancing selection is relatively rare at the genome-wide scale, even if the processes that generate it (e.g SA selection) are common (Fijarczyk and Babik, 2015).

No significant differences were observed in tissue specificity between the candidate and background genes, either overall or when stratified by sex-biased expression categories. This indicates that the candidate genes are not distinguished by broader or more restricted expression patterns, suggesting that tissue-specific constraints are unlikely to be the primary factor maintaining these polymorphisms. Instead, the persistence of SA variation appears to be more closely tied to shared genetic architecture.

Together, these results suggest that SA polymorphisms are most likely to persist in genes that are constrained and shared between the sexes. While clear genomic signatures of long-term balancing selection were not detected, other evidence, including elevated r_{MF}, reduced relative nonsynonymous diversity and genome-wide allele frequency differentiation, supports the presence of ongoing SA selection shaping genetic variation.

2.3.6 Conclusion

In conclusion, this study provides new insight into identifying SA polymorphisms in *D.*

melanogaster using a novel experimental approach. By directly comparing allele frequencies between successful sperm and egg pools, this method offers a framework for detecting SA selection over traditional approaches. Across multiple analyses, the results support the view that SA variation is widespread, polygenic and shaped by genomic constraint. Candidate genes were distributed non-uniformly across the genome but showed no significant enrichment on the X chromosome, challenging classical theoretical predictions.

Functional analyses indicate that SA selection is more likely to act through regulatory variation than through changes to protein-coding sequences. This is consistent with the observation that candidate genes exhibit elevated intersexual genetic correlation and reduced relative nonsynonymous diversity, suggesting that many loci are constrained and shared between the sexes, limiting the potential for conflict resolution through coding sequence divergence. The lack of strong signals in DoS and Tajima's D further suggests that SA selection does not consistently produce classic signatures of long-term balancing selection and instead may operate through weak, transient or context-dependent dynamics.

Importantly, overlap with independent studies highlights a subset of genes that repeatedly emerge as candidates for SA selection, reinforcing the biological relevance of these detected signals. However, the limited functional enrichment and absence of strong pathway clustering suggest that SA selection acts across diverse biological processes rather than being restricted to specific traits or pathways.

Overall, these findings support a view of sexual antagonism as complex, acting across many loci of small effect and often constrained by shared sexual genetic architecture. While clear genomic signatures of long-term balancing selection remain difficult to detect, multiple results indicate that SA selection contributes to shaping genome-wide patterns of variation. Future work integrating functional validation, higher-resolution genomic data and analyses across additional populations will be essential to further resolve the mechanisms maintaining SA polymorphisms and their evolutionary significance.

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Supplementary material

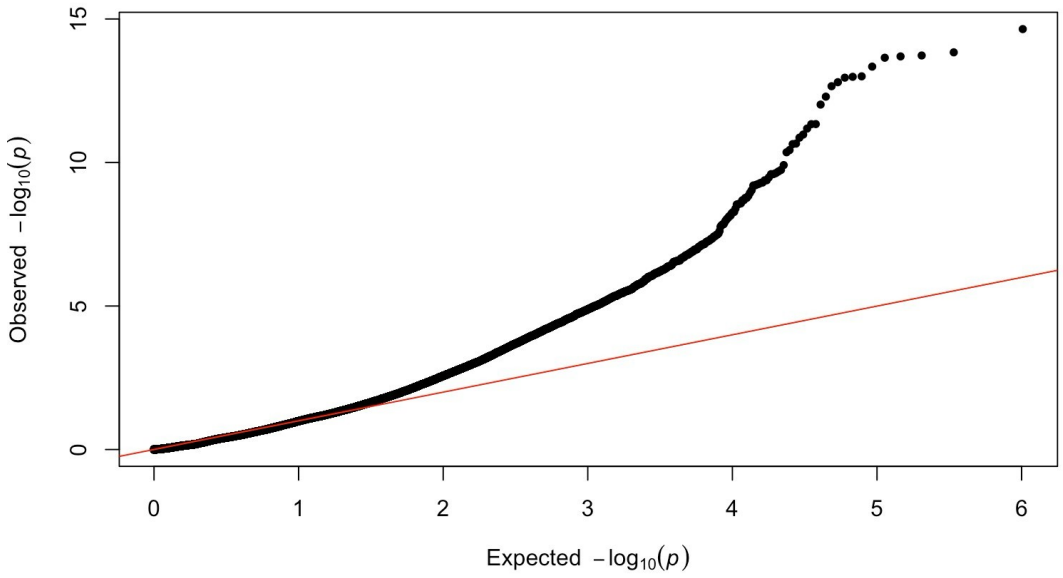

Figure S1: Quantile-Quantile (QQ) plot of observed vs expected -log10(p) values. Red line represents the expected distribution under the null hypothesis. Each black dot represents a SNP.

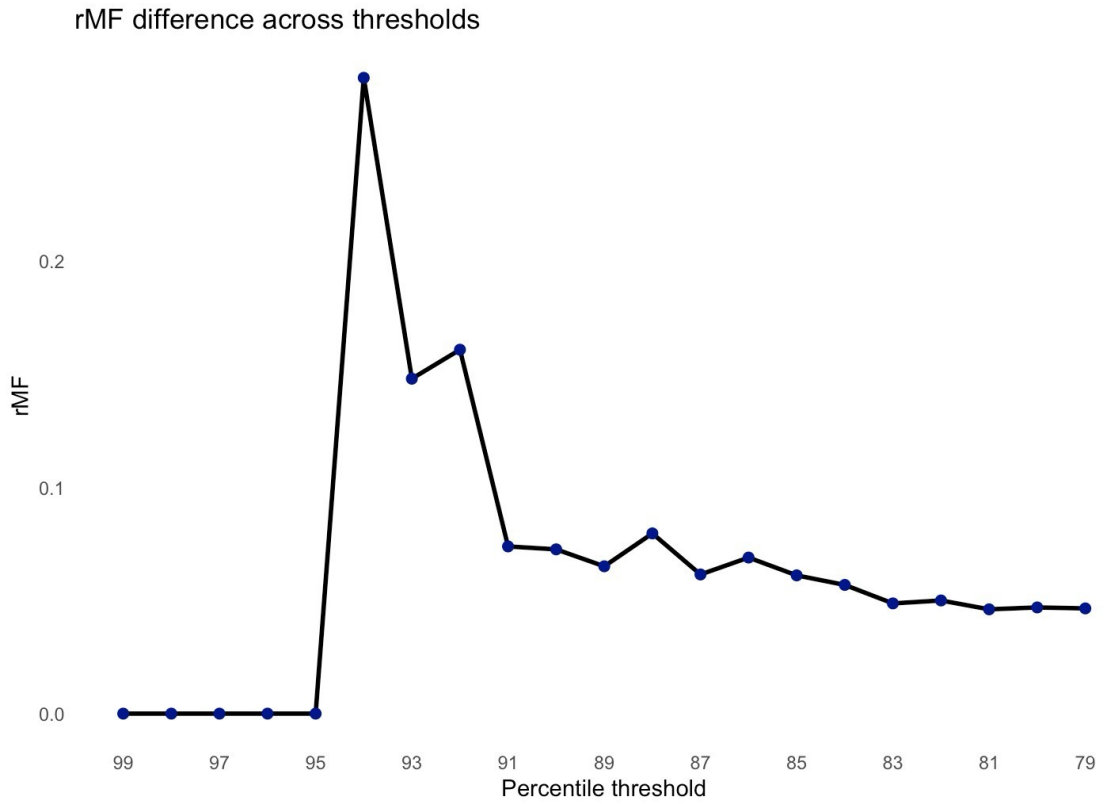

Figure S2: Intersexual genetic correlation (r_{MF}) across percentile thresholds for candidate SNP selection. The line graph shows the median r_{MF} calculated for candidate loci identified at each percentile threshold of CMH test -log10(P) values derived from permutation analysis. Each point represents the median r_{MF} for SNPs exceeding the indicated percentile.

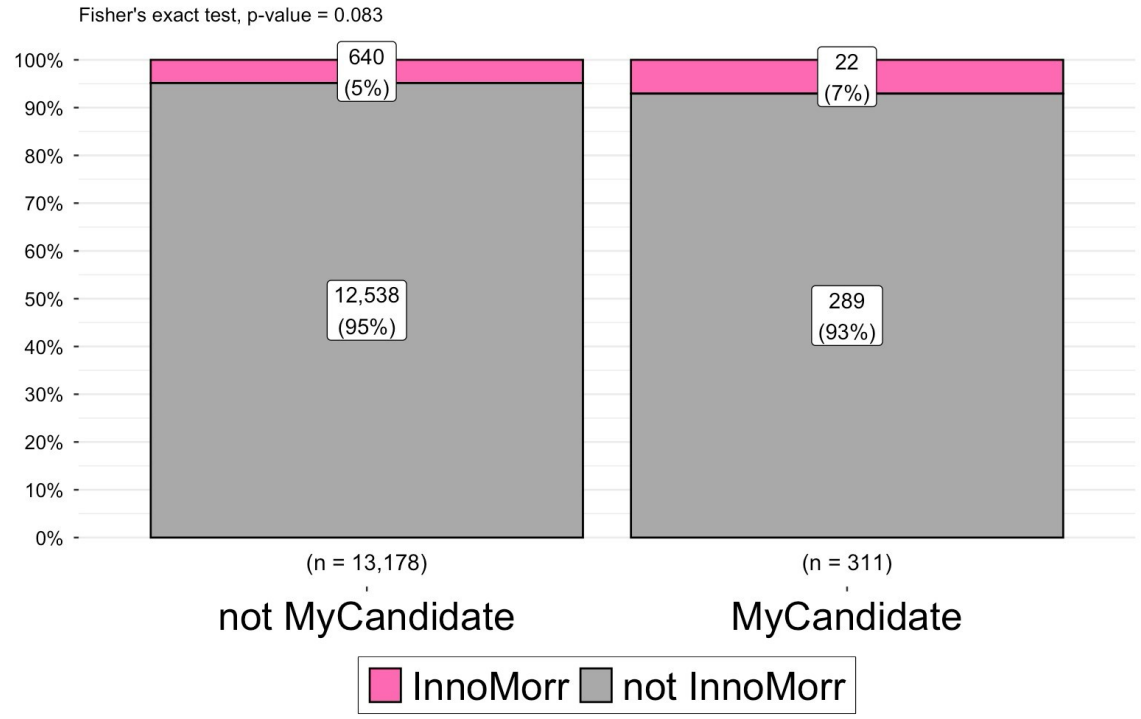

Figure S3: Proportion of candidate genes that overlap with candidate sexually antagonistic genes identified by Innocenti and Morrow (2010). A total of 22 out of 311 candidate genes (7%) overlapped with the Innocenti and Morrow candidate genes compared to 640 out of 13,178 background genes (5%)

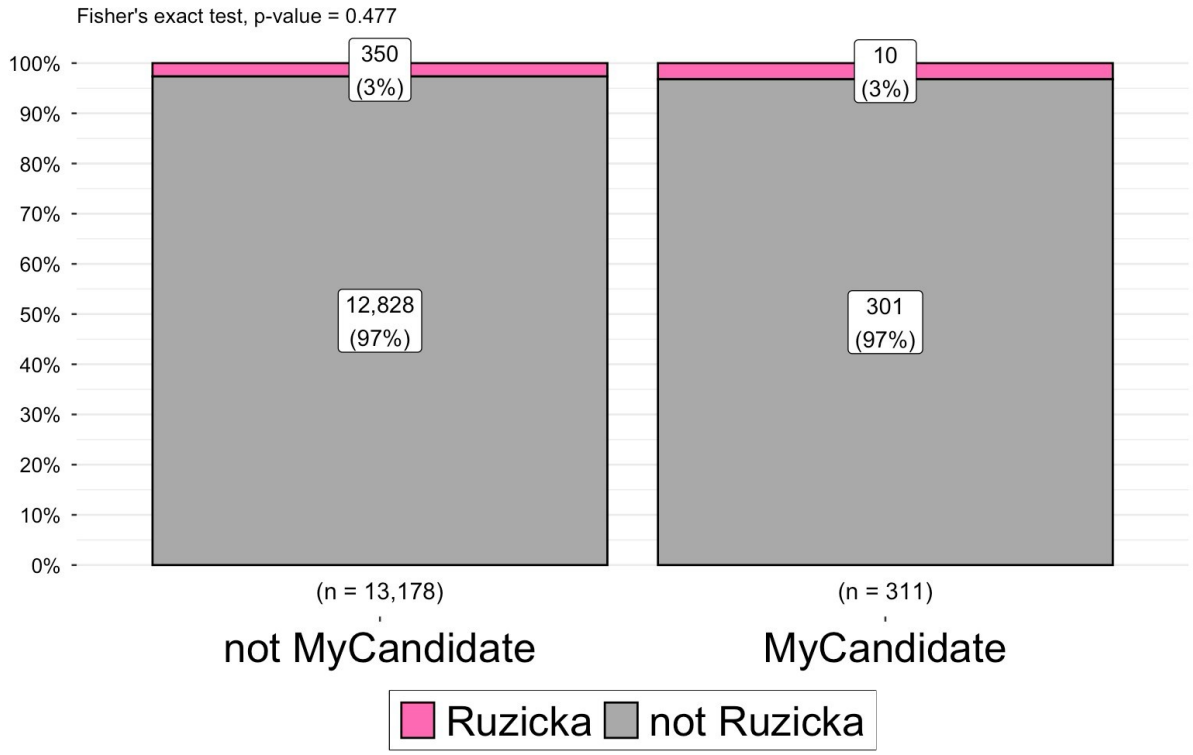

Figure S4: Proportion of candidate genes that overlap with candidate sexually antagonistic genes identified by Ruzicka *et al.* (2019). A total of 10 out of 311 candidate genes (3%) overlapped with Ruzicka *et al.* (2019) candidate genes, compared to 350 out of 13,178 background genes (3%).

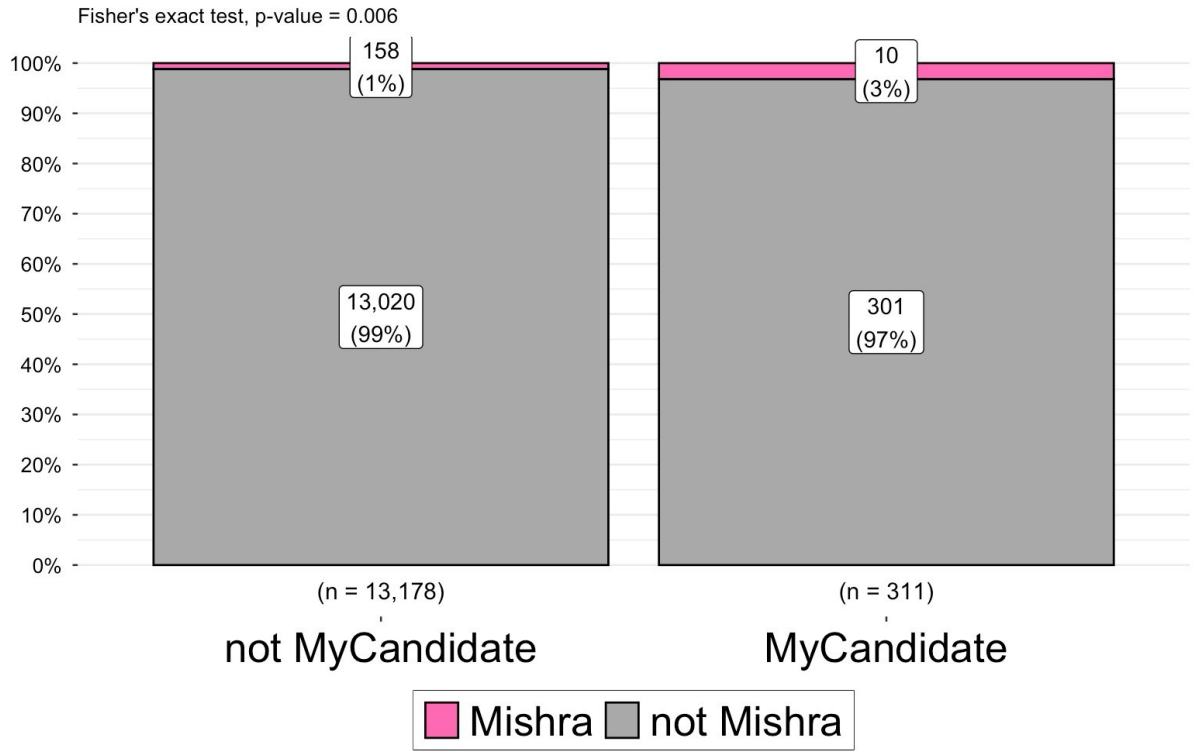

Figure S5: Proportion of candidate genes that overlap with candidate sexually antagonistic genes identified by Mishra *et al.* (2024). A total of 10 out of 311 candidate genes (3%) overlapped with Mishra *et al.* (2024) candidate genes, compared to 158 out of 13,178 background genes (1%).

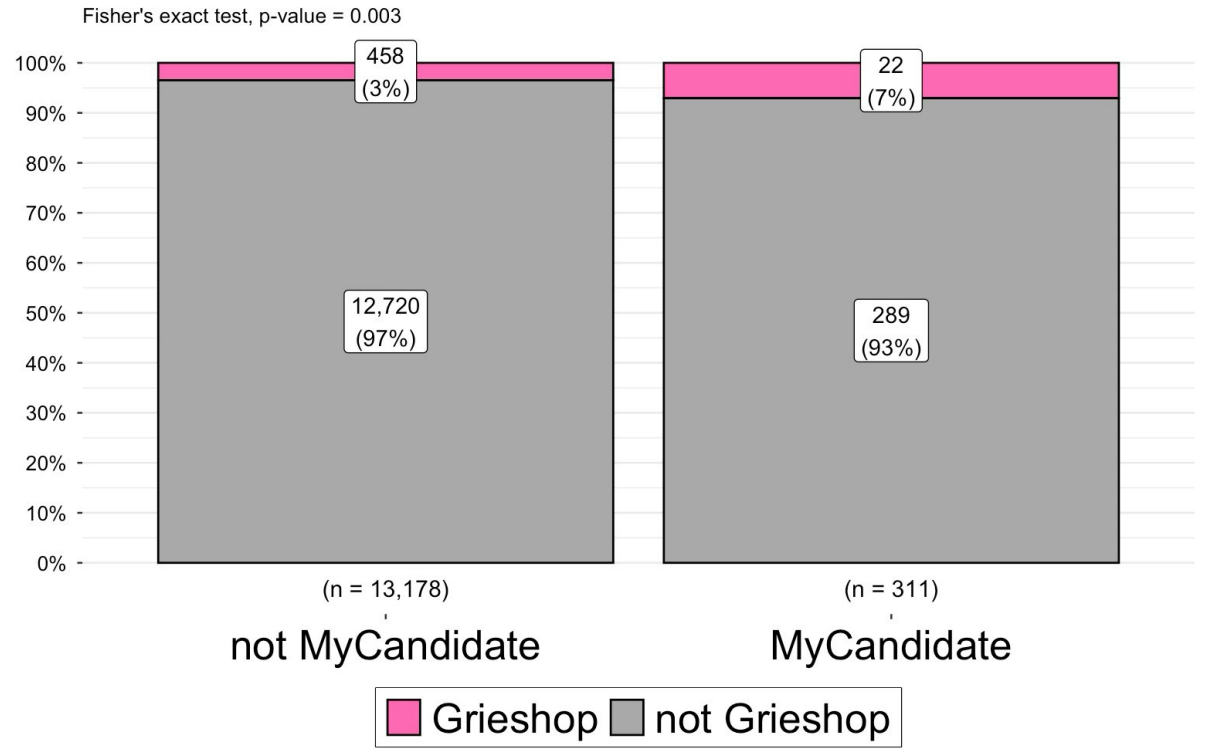

Figure S6: Proportion of candidate genes that overlap with candidate sexually antagonistic genes identified by Grieshop *et al.* (2025). A total of 22 out of 311 candidate genes (7%) overlapped with Grieshop *et al.* (2025) candidate genes, compared to 458 out of 13,178 background genes (3%).

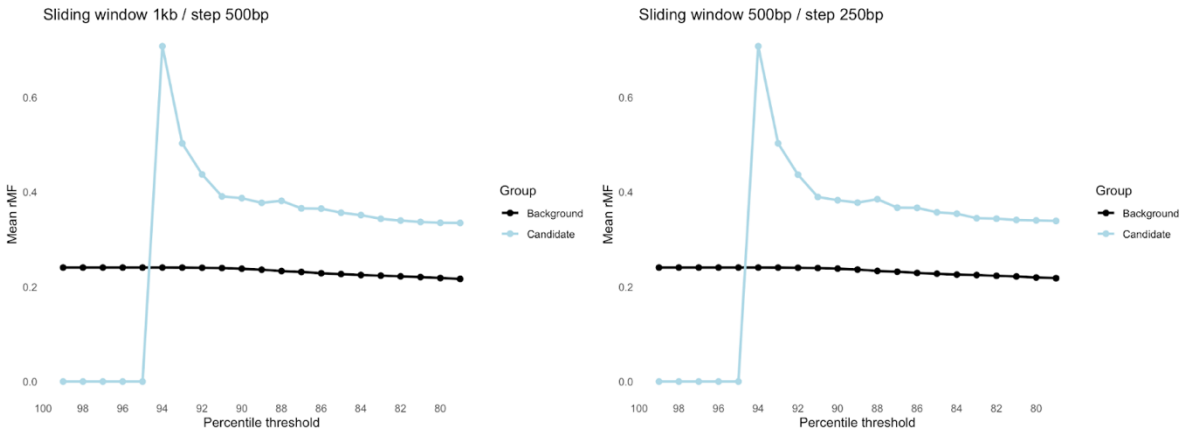

Figure S7: Comparison of 2 sliding window approaches. Left: 1kb window with 500bp step. Right: 500kb window and 250bp step. Comparison of Mean R_{MF} across different percentiles.

Table S1: GO results from cellular compartments category. ‘collagen-containing extracellular matrix’ is significant (P.adjust = 0.003). No other terms were found to be significant.

ID	Description	Gene Ratio	BgRatio	Rich Fact or	FoldEnrichment	zScore	pvalue	p.adjust	qvalue	geneID	Count
GO:0062023	collagencontaining extracellular matrix	8/247	39/8522	0.205128205	7.077338316	6.5717455 8	1.255916477981 96e-05	0.003353 3	0.003331 48	vkg/CG9500/Tig/Tsp/Glt/wb/sca/Pxn	8
GO:0030312	external encapsulating structure	16/247	228/8522	0.070175439	2.421194687	3.7579304 8	0.00094314	0.105821 17	0.105132 8	dpy/vkg/CG9500/Tig/Tsp/Glt/CG13114/wb/Cpr47Ee/Cpr47Ef/sca/lbk/Pxn/Ccn/CG14309/Muc14A	16
GO:0031012	extracellular matrix	15/247	211/8522	0.071090047	2.452750542	3.6916064	0.001189	0.105821 17	0.105132 8	dpy/vkg/CG9500/Tig/Tsp/Glt/wb/Cpr47Ee/Cpr47Ef/sca/lbk/Pxn/Ccn/CG14309/Muc14A	15
GO:0 0.21 4.72 0.12 0.12 0056 basement 4/24 19/8 0526 7.2635 1971 0.00189 6393 5570 04 membrane 7 522 316 84061 69 353 18 99 vkg/Tig/Glt/wb 4											