Sexual Dimorphism through the Lens of Genome Manipulation, Forward Genetics, and Spatiotemporal Sequencing

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Abstract

Sexual reproduction often leads to selection that favors the evolution of sex-limited traits or sex-specific variation for shared traits. These sexual dimorphisms manifest due to sex-specific genetic architectures and sex-biased gene expression across development, yet the molecular mechanisms underlying these patterns are largely unknown. The first step is to understand how sexual dimorphisms arise across the genotype–phenotype–fitness map. The emergence of "4D genome technologies" allows for efficient, high-throughput, and cost-effective manipulation and observations of this process. Studies of sexual dimorphism will benefit from combining these technological advances (e.g., precision genome editing, inducible transgenic systems, and single-cell RNA sequencing) with clever experiments inspired by classic designs (e.g., bulked segregant analysis, experimental evolution, and pedigree tracing). This perspective poses a synthetic view of how manipulative approaches coupled with cutting-edge observational methods and evolutionary theory are poised to uncover the molecular genetic basis of sexual dimorphism with unprecedented resolution. We outline hypothesis-driven experimental paradigms for identifying genetic mechanisms of sexual dimorphism among tissues, across development, and over evolutionary time.

Key words: sexual antagonism, experimental evolution, CRISPR, single-cell sequencing, long-read sequencing.

Significance

Sexual dimorphism is ubiquitous in sexually reproducing species and appears to be driven by widespread patterns of sex-biased gene expression. However, we do not understand how genetic material shared between the sexes creates these patterns or how sex-limited and sexually antagonistic selection shape the evolution of sexual dimorphism in genomes. We propose that this knowledge gap can be addressed by adapting tools from molecular genetics and biomedical research to an evolutionary genetics framework. We highlight experimental paradigms for identifying the genetic architecture of sexually dimorphic phenotypes and 4D genomic technologies for isolating the molecular mechanisms generating sexual dimorphism.

Introduction

A central goal of evolutionary genetics is to understand the genetics of adaptation. This goal requires researchers to probe the genomic response to selection on phenotypes with a known fitness effect in nature (Barrett and Hoekstra 2011).

We can approach this challenging task by studying distinct components of the problem: mapping the genetic basis of the phenotype, measuring selection on the phenotype, and scanning the genome for signatures of this selection. Sexual dimorphism (SD) of phenotypes adds an additional layer of

Box 1.—Molecular Mechanisms That Can Contribute to Sex Differences.
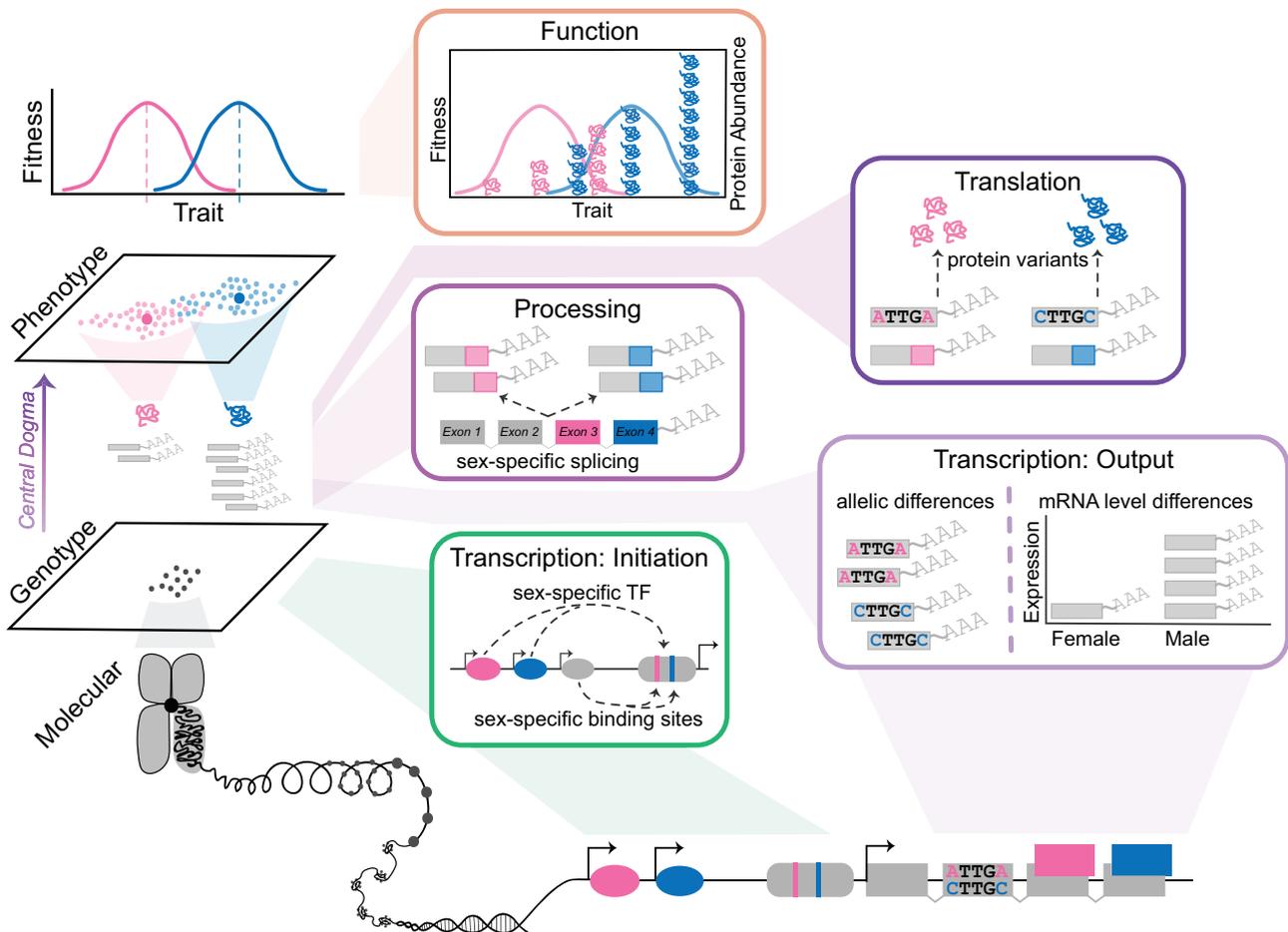
To understand how sex-specific selection drives the evolution of traits in populations, we need some understanding of the underlying genetic basis of the traits, as highlighted in Lewontin's (1974) classic text. A critical component of Lewontin's genotype–phenotype map is the first transformation, which encompasses the central dogma of molecular biology: DNA to RNA to protein (as shown below). These molecular underpinnings are particularly important in the context of SD as the largely, and in some cases completely, shared genetic material is producing distinct phenotypes within each sex.

Phenotypic variation can arise through modifications to coding sequences including gene duplication, changes in gene regulation, and modifications during translation (King and Wilson 1975; Levine and Tjian 2003; Wyman et al. 2012; Grath and Parsch 2016; Mank 2017; Khramtsova et al. 2019). Sex differences can be generated by completely sex-limited genes, often located on sex chromosomes (Mank 2009) or by genetically encoded differences in the initiation of transcription (shown in green). Here, differences in transcription factor (TF) binding frequency between the sexes or sex-specific TF binding sites will drive sex-specific mRNA levels. Additionally, sex-biased deployment of master regulators can initiate a cascade of sexual differentiation.

Transcription can be divided into two stages: the production of mRNA transcripts followed by processing of these transcripts (shown in purple). Differences in transcriptional output between the sexes include overall differences in mRNA expression levels generated through either differential TF binding or sex-specific degradation of mRNA. Alternatively, a sexually antagonistic polymorphism can generate allelic differences in mRNA transcripts between the sexes. Ultimately, this effect is not realized unless the translated protein variants differ in form and function between the sexes (shown in dark purple). During the posttranscriptional regulation stage, sex-specific alternative splicing (Chang et al. 2011; Hartmann et al. 2011; Li et al. 2013) and small RNA regulators (Warnefors et al. 2017; Bezler et al. 2019) generate sex-specific mRNA (as shown in purple). As with a sexually antagonistic polymorphism, this effect is only realized if the sex-specific isoforms have protein variants that differ in form or function (shown in orange).

Although not exhaustive, this list includes the major mechanisms that have been identified or hypothesized to contribute to sexual dimorphism. However, more work is needed to determine if one mechanism is more common than others or if the molecular mechanisms contributing to SD differ for simple versus complex traits.

complexity, because the sexes maximize fitness differently and are subject to different selective pressures (Trivers 1972; Parker 1979; Arnqvist and Rowe 2005). Yet, the sexes share the majority of their genetic material and, thus, SD is a function of shared, and of sex-specific and sex-biased genetic architecture, gene regulation, and gene expression. Therefore, to truly understand how sex-specific selection shapes the evolution of SD in genomes, it is essential to identify the molecular biology processes linking the genome with the phenome (box 1).

Identifying the genetic variants and sex-biased networks underlying SD has proved challenging. In the last decade, research has centered on patterns of sex-biased gene expression, which has led to the identification of strong, consistent sex-biases coupled with rapid molecular evolution and genomic organization of sex-biased genes (Reinke et al. 2000; Jin et al. 2001; Ranz et al. 2003; Cutter and Ward 2005; Yang 2006; Innocenti and Morrow 2010; Bohne et al. 2014; Harrison et al. 2015). Although informative, these global patterns mask the underlying molecular mechanisms and often do not directly provide spatial resolution within the organism. These limitations hinder our understanding of how SD is cued within tissues and across developmental time. Finally, the focus on transcriptional patterns alone excludes other sources of phenotypic variation such as translation.

Combining molecular genetics with classic evolutionary approaches and genome technology, provides an opportunity to uncover the molecular mechanisms linking a sexually dimorphic phenotype with its underlying genetic basis. Such integration is feasible, efficient, and cost-effective in the emerging era of "4D genome technologies" and can provide high-resolution analyses of biological features in distinct physiological and tissue systems, and across developmental and evolutionary time. Using this integrated evolutionary framework, we can begin to address long-standing questions in the field, such as: What is the genetic architecture of sexually dimorphic traits? What are the genetic constraints on sexual dimorphism? What is the relationship between sex chromosome evolution and sexual dimorphism? And, when, where, and how are sex-biased networks formed and how are they sustained across an organism's lifecycle? This perspective aims to provide a synthetic view of how 4D genome technologies integrated into evolutionary frameworks can uncover the mechanistic basis and genomic manifestation of SD with unprecedented detail. We suggest that these new paradigms will overcome an emerging recognition of limitations to existing approaches for deciphering signals of SD and sexually antagonistic selection.

Approaching Outstanding Questions

To map the genetic basis and molecular mechanisms of a sexually dimorphic phenotype, we can manipulate selection, correlate genomic patterns with SD and genetic sex, and verify the functional importance of genes through genomic manipulation. We briefly explore five complementary experimental paradigms and highlight how they can link genotype, phenotype, and fitness across the lifecycle of each sex to provide the maximum temporal resolution of the genetic basis and molecular mechanisms of SD.

Evolve and Resequence

The evolve and resequence (E&R) approach (Schlötterer et al. 2015) combines experimental evolution with whole-genome sequencing to trace allele frequencies over tens or hundreds of generations (fig. 1A–C). Examining allele frequency changes permits an estimation of the strength of selection on regions of the genome that contribute to the SD of interest. E&R is a powerful approach to manipulate selection in a sex-specific manner to examine sex-biased genetic architecture and determine: if there are genomic hotspots of SD, the relative contribution of coding versus regulatory sequence, and the number of contributing loci. Experimental evolution approaches have successfully isolated sex-specific selection (Rice 1996) and sexual selection (Chenoweth et al. 2008; Maklakov et al. 2009; Edward et al. 2010; Snook et al. 2013; Rostant et al. 2020), though few studies have examined the genomic response (see Hsu et al. 2020). New transgenic technology will expand the potential of E&R to identify sex-biased elements of genetic architecture by creating high-throughput mechanisms for altering the variance in or manipulating the developmental timing of a sexually dimorphic phenotype, or isolating selection to a given sex. For example, introducing inducible knockdown technology (supplementary table 1, Supplementary Material online) into the genome prior to E&R can provide a fine-scale experimental tool to alter gene expression in a sex-specific manner. By manipulating gene expression, the phenotypic mean can be shifted toward more or less SD, which will affect the response to selection. Inducible technology can aid in altering gene expression or the timing of gene expression, both of which will affect the sex-specific response to selection. Limiting selection to act within one sex during E&R will also be aided by tools that remove a phenotype in one sex, such as inducible sterility (Kasimatis et al. 2018), or generate progeny of a single sex (Douglas et al. 2020).
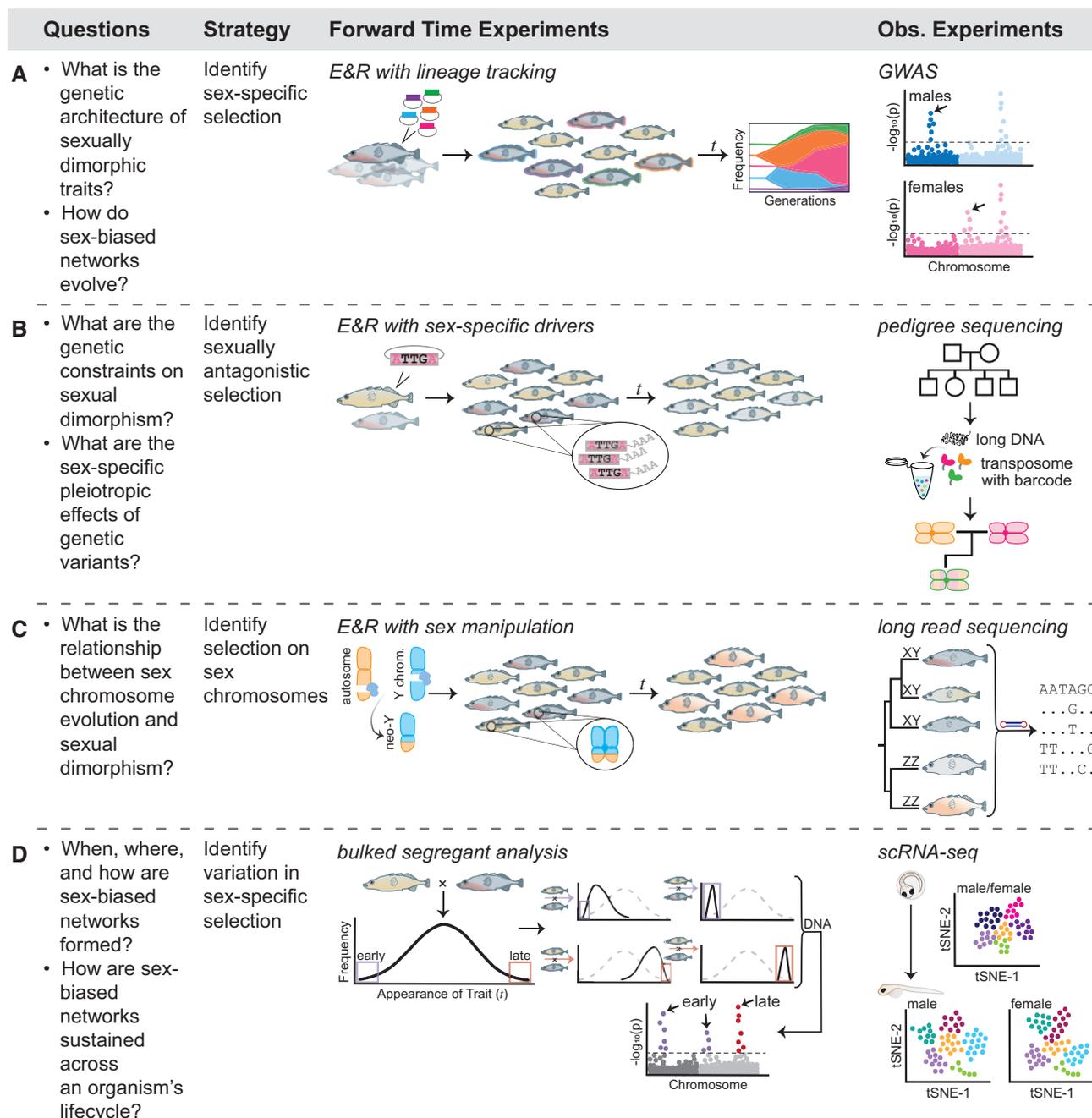
FIG. 1.—Evolutionary frameworks for identifying the genetic basis of SD. Strategies for identifying: (*A*) the genetic basis of SD through experimental evolution and between-sex comparative genomics, (*B*) sexual antagonism and SD through experimental evolution and pedigree tracing, (*C*) sex chromosome directed SD through experimental evolution and comparative genomics, and (*D*) developmental SD through bulked segregant analysis and single-cell sequencing.

Alternatively, transgenic technology can expand our understanding of the genomic response during E&R through haplotype barcoding. This tool would provide a method to track the frequency of individual haplotypes (fig. 1*A*; see Genetic Manipulation). Haplotype barcoding is ideal for tracking fitness differences between genetic variants in a competitive setting and could be applied to sexually dimorphic variation

within and between the sexes. For example, haplotype barcodes could be integrated into multiple genetic backgrounds, crossed to form an ancestral population, and evolved under sex-specific selection or intersexual competition. Unlike traditional E&R experiments which use whole-genome sequencing of the ancestral and evolved population, haplotype barcoding allows for individuals to be sequenced in the smaller barcode

at high coverage across many time points throughout evolution. This repeated sequencing provides high-resolution allele frequency traces of different haplotypes to understand the dynamics of genetic variants and how they relate to fitness changes over time. Haplotype barcoding can also be adapted to study differences in recombination rate between the sexes by integrating multiple barcodes at known positions. Differences in recombination rate between the sexes can influence sex-specific genetic architecture (Trivers 1988; Sardell and Kirkpatrick 2020), which could link sex-biased elements contributing to SD. Barcoding at known genomic regions will allow us to follow this process in forward-time experiments and compare recombination rates between the sexes under different environmental conditions.

E&R can also be used to address the relationship between sexually antagonistic selection and SD by generating a negative genetic correlation between female and male fitness (Bondurianksy and Chenoweth 2009) through manipulating sexual selection (Rice 1996; Pitnick, Brown, et al. 2001; Pitnick, Miller, et al. 2001). Again, genetic manipulations introduced before E&R, such as inducible knockdowns or altered expression through CRISPRi (supplementary table 1, Supplementary Material online; box 2), will facilitate understanding the pleiotropic effects of genetic variants in a sex-specific manner. Manipulating expression during E&R can also provide information on how a gene's interactions are structured within each sex and how these interactions evolve. Additionally, genomic editing can be used to introduce a sex-biased regulatory sequence or genetic variant into the mismatched sex, which will relax the degree of sexual antagonism during E&R and thus reduce the genetic constraints on SD (fig. 1B and box 2). Genomic editing also provides the ability to manipulate the sex-determining region and fuse chromosomes together (Shao et al. 2019) to study neo-sex chromosome formation. When used in an E&R framework, this approach will provide insight in real-time on how neo-sex chromosomes evolve and the role of sex chromosomes in resolving sexual conflict (fig. 1C).

Despite their power, E&R studies are still sensitive to population size, biological replication, and ancestral haplotype structure (Kofler and Schloetterer 2014; Otte et al. 2020). Importantly, the molecular integration of transgenic elements before E&R homogenizes the genome. To create standing genetic variation for selection to act upon during E&R will require either crossing multiple transgenic strains together or mutagenesis. In the case of crossing, haplotype structure must be carefully considered during experimental design as it can greatly impact the sex-specific response to selection. This approach to E&R relies on manipulating the genome of particular isolates or strains rather than following the genomic response of segregating genetic variants and therefore may not represent all possible evolutionary pathways observed in natural populations. These limitations can be avoided by using transgenics tools only after E&R to verify candidate genes. For

example, CRISPR (box 2) can replace a haplotype in the ancestral background with the evolved haplotype (Perli et al. 2020). However, the full benefits of transgenics during E&R will be realized when used as an integrated tool. This goal can be met as transgenics become more efficient and feasible in a range of taxa (supplementary table 1, Supplementary Material online), allowing for multiple strains to be genetically manipulated and crossed.

Bulked-Segregant Analysis

An alternative approach to E&R is bulked segregant analysis (Brauer et al. 2006), which uses selection on the tails of a phenotypic distribution to map the genetic basis of extreme phenotypes. By repeating over multiple rounds of selection, the variance in the trait can be reduced, which facilitates mapping. Bulked segregant analysis could be a powerful approach to mapping genetic variants of SD and understanding the role of dominance in sexually dimorphic traits, both of which will benefit from existing introgression lines between strains or species. This approach may also be particularly useful for selecting on sex-specific variation during development to map the genetic basis of when and how SD is generated (fig. 1D). This approach can be coupled with CRISPR transgenics to validate the function of candidate genes (see Genetic Manipulation). Alternatively, RNA-sequencing and particularly single-cell sequencing can be used to identify expression differences between bulk populations (Ben-David et al. 2020).

Pedigree Tracing

Pedigree and parent–offspring trio sequencing offer an alternative to E&R for studying sexual dimorphism in populations that are not conducive to experimental evolution, such as in natural populations or organisms with a long generation time (fig. 1B; Johnston et al. 2017; Bates et al. 2020; Lucotte et al. 2020). These approaches explicitly correlate haplotype structure with genetic sex and identify recombination events within a population. Additionally, pedigree and trio sequencing approaches explicitly take into account population structure, providing an advantage over genome-wide association studies (GWAS) (Bates et al. 2020). Pedigree tracing has already proved powerful for identifying signatures of selection in wild populations (Johnston et al. 2017; Chen, Juric, et al. 2019). Advances in long-read genomic sequencing and reduced sequencing costs are making these approaches more feasible across taxa. A promising emerging framework being adapted from human genomics is the use of linked-read sequencing to gain insight on phased genomes (Lutgen et al. 2020). Specifically, phased genomic information within a pedigree framework can be used to correlate haplotype structure and local genetic architecture with genetic sex. Additionally, phased genomes gained through linked-sequencing or parent–offspring trio sequencing can be used to study

Box 2.—Genetic Manipulation through CRISPR/Cas.
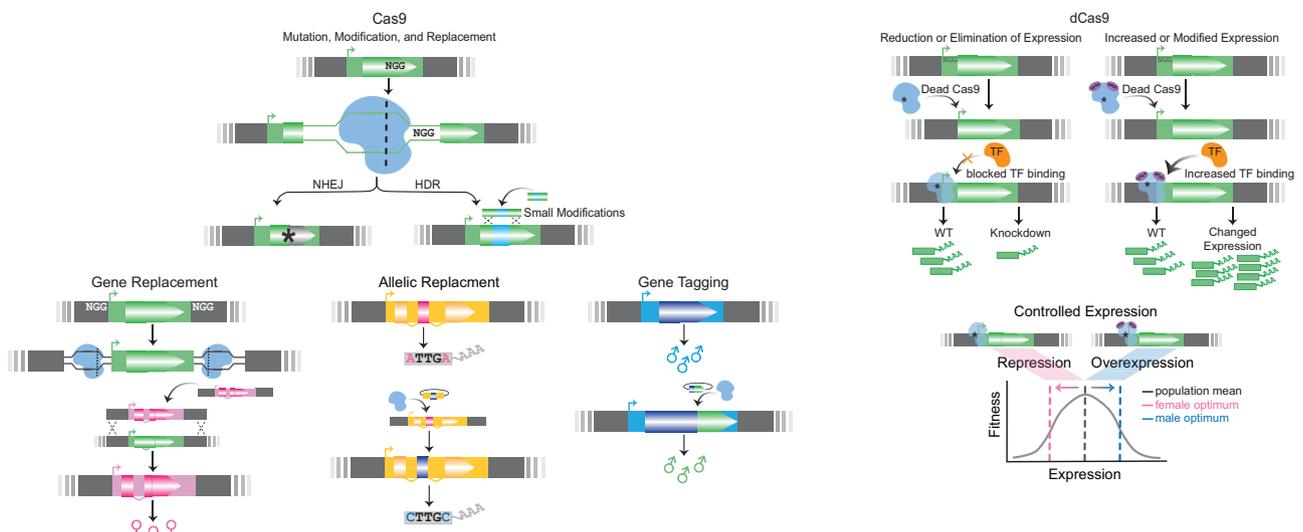
CRISPR (clustered regularly interspaced short palindromic repeats) has become the premier method for mutating and editing the genome with precision (Jinek et al. 2012; Doudna and Charpentier 2014; Pickar-Oliver and Gersbach 2019). Although the vast scope of CRISPR utilities cannot be represented here, we outline three mechanisms and their application to SD. Cas9, the most common nuclease associated with the CRISPR system, is a targetable nuclease, which provides experimental control over the location of the nuclease activity. Cas9 and other CRISPR-associated nucleases are "guided" by specific single-stranded RNA encoding the sequence of interest. The PAM (Protospacer Adjacent Motif) sequence restricts the locations of Cas9 targeting. Cas9 targets guide site locations with "NGG" PAM sequence and cleaves double-strand DNA (shown above). When Cas9 creates a break, the cell will attempt to repair the break by nonhomologous end joining (NHEJ) or homology directed repair (HDR). NHEJ is error-prone and thus a simple way to create mutations in the desired gene. However, depending on the mutation's location, the protein may still fold and function properly. HDR modifies a gene in a specific manner through a "donor template" to repair the DSB (reviewed in Doudna and Charpentier 2014). This process can use the homologous chromosome as a template or non-native DNA template sequence can be introduced to the genome. The synthetic construct (e.g., plasmid, PCR product, or single-stranded oligo) must contain sequences homologous to the genome on both the 5′ and 3′ sides of the desired insert to co-opt the genome repair machinery for integration. HDR has many applications in the study of SD (as shown above). For example, whole genes can be deleted to determine the gene's sex-specific function. Alternatively, whole genes or promoter regions can be replaced with a sex-biased version to test sex-specific function. A more focused approach would directly edit alleles to verify the significance of sexually antagonistic polymorphisms. Beyond verifying the function of genetic elements in generating SD, HDR can also be used to add epitopes or fluorescent tags to aid during experimentation (see fig. 1 and Genetic Manipulations).

Catalytically inactive variants of Cas9, dead Cas9 (dCas9), target and bind to the sequence without cutting the genome (shown above). In doing so, the dCas9 protein physically blocks the binding of transcription factors and creates a knock-down phenotype (Larson et al. 2016; Pickar-Oliver and Gersbach 2019). This utility, termed CRISPRi, is analogous to RNAi, and may complement the knockdown phenotype or be an adequate replacement (Stojic et al. 2018). Alternatively, dCas9 can be fused to various transcription activators, such as VP64, to change the target genes' expression level and specificity (reviewed in Pickar-Oliver and Gersbach 2019). By changing the regulatory sequences driving dCas9 expression, specific genes can be experimentally altered in a tissue-specific or cell-specific manner. Experimentally, this manipulation of expression opens many possibilities to directly manipulate genetic architecture in a sex-biased way.

Although CRISPR's successes have been widespread (supplementary table 1, Supplementary Material online), unique challenges exist for species-specific utility. Targeting the genome requires knowing the DNA sequence and the genomic location of sequences. Adapting CRISPR for new species or highly divergent strains may be complicated if this information is lacking. Additionally, gene duplications and pseudogenes which arose from duplications can also pose a challenge since they likely share much of their sequence in common. Again, high-quality genome assemblies can help

to control for this problem, though off-target CRISPR effects can still occur. Finally, delivery of Cas9 and the guide RNA is unique to each species and will require optimizing the method with that species to achieve targeted genome-editing. Nevertheless, CRISPR has been widely adopted among may species and nonmodels with great success; recent protocols developed for nonmodel organisms, including firebug *Pyrrhocoris apterus* (Kotwica-Rolinska et al. 2019), malaria mosquitos (Hammond et al. 2017), and lizards (Rasys et al. 2019). This continued methodological progress, coupled with advances in sequencing technology, will expand the potential applications of CRISPR across taxa.

segregation distortion and sexually antagonistic variants within the genome (Lucotte et al. 2020).

GWAS and High-Resolution Transcriptomics

An alternative approach to manipulating selection is to observe the genomic footprint of selection in natural populations through GWAS or expression association approaches. GWAS provides a powerful approach to associate sex-specific variation in a phenotype with its underlying genetic basis (fig. 1*A*). Taking such a sex-stratified approach will distinguish sex-specific allelic effects (Khramtsova et al. 2019). Although GWAS is sensitive to population demographics, these confounding effects can be controlled for in a logistic regression framework. An association framework also can be used for gene expression data with the potential to reveal how *cis* and *trans* genomic variants influence transcription on a genome-wide scale (Sun and Hu 2013). This framework will be especially powerful when coupled with cell or tissue-specific transcriptomes (see 4D Transcriptomics). The human Genotype-Tissue Expression project (GTEx) is revolutionizing this area of research, identifying over one-third of genes to have a sex-biased expression profile in at least one of the 44 tissues sampled (Oliva et al. 2020). For both data types, sample size will impact the ability to accurately detect signatures of selection and may be a limiting factor for some natural population studies.

Comparative transcriptomic studies provide network-level information about sex-specific node connectivity and redundancy. By coupling classic evolution-development frameworks, particularly during early development, with single-cell sequencing technology (see 4D Transcriptomics), we can begin to create a continuous understanding of SD through time (fig. 1*D*). Spatial transcriptomics has already transformed developmental biology (Farrell et al. 2018; Zhou et al. 2019) and sex-stratified approaches with these methods will only further our knowledge.

Comparative Genomics and Long-Read Sequencing

Comparative genomic studies focusing on sex chromosomes isolate the genotype space of the genotype–phenotype–fitness map (fig. 1*C*). The relationship between the origin of sex chromosomes and SD is a long-standing area of research (Rice 1984; Charlesworth 1991; Mank 2009; Bachtrog et al. 2011), however, the quality of genome assemblies has been a major limiting factor, especially for nonmodel organisms. Traditional

methods for studying sex chromosomes are also benefiting from new technology. Specifically, long-read PacBio and Oxford Nanopore sequencing (Amarasinghe et al. 2020) are providing chromosome length scaffolds for assembling short-read data. These methods generate high-quality assemblies that extend through repetitive regions and tandem duplications, which are problematic for short-read data, but may be common and potentially important components of sex chromosomes (Bachtrog et al. 2019; Peichel et al. 2019; Bracewell and Bachtrog 2020). Similarly, gene duplication and sex-specific functionalization is viewed as an important mechanism leading to the resolution of sexual conflict and the evolution of SD (Gallach et al. 2010; Connallon and Clark 2011; Gallach and Betrán 2011; Wyman et al. 2012). Long-read sequencing can help identify and disentangle recent duplication events more accurately than standard short-read data. Finally, long-read RNA sequencing technologies, such as Iso-seq, are proving to be powerful in identifying sex-specific alternative splicing and the role of this mechanism in the development of sexually dimorphic traits (Zhao et al. 2019).

Spatial and Temporal Patterns of Sex-Biased Expression and Regulation

Recent advances in sequencing technologies, such as tomographic or spatial transcriptomics (Tomo-seq) and single-cell RNA sequencing (scRNA-seq), allow us to track transcriptomic dynamics across different cell types and tissues, and across development to provide fine-scale resolution of SD in gene expression and regulation. We discuss four methods, which can be used independently or coupled with experimental manipulation to observe the patterns of SD.

4D Transcriptomics of SD: scRNA-Seq and Tomo-Seq

Single-cell sequencing expands the feasibility of quantitative gene expression methods across taxa and biological samples. Specifically, single-cell RNA amplification techniques coupled with cell sorting devices offer a major advantage over bulk-cell RNAseq by providing transcript-level expression for thousands or even millions of cells (supplementary table 1, Supplementary Material online; Tang et al. 2009; Islam et al. 2011; Hashimshony et al. 2016; Haque et al. 2017). More recent analytical improvements are enabling postsequencing identification of cell populations by

applying advanced clustering and unsupervised learning techniques, such as t-distributed stochastic neighbor embedding (t-SNE), greatly improving the spatial resolution in scRNA-seq data (Kobak and Berens 2019). Although these techniques have largely been used to distinguish gene expression profiles between cell populations within a single individual, scRNA-seq comparisons between the sexes in humans is beginning to unveil the mechanisms of SD (Tukiainen et al. 2017). Additionally, new analytical approaches are enabling differential single-cell expression contrasts between individuals (Butler et al. 2018; Ntranos et al. 2019; Becht et al. 2020), which will facilitate contrasts between cell populations of females and males. Although complexity and expense can build up for an experiment with female and male treatments and multiple developmental time points, a cost-effective, although less high-throughput alternative, is quantitative PCR to monitor pivotal genes on specific cell populations that may have been identified in coarser scans (VanInsberghe et al. 2018). Importantly, scRNA-seq will not only provide an understanding of sex-biased differential expression through development (fig. 1D), but can also be used to understand the sex determination cues from sex chromosomes (fig. 1B) with spatial and cellular resolution.

Alternatively, Tomo-seq avoids the cell sorting and classification required for scRNA-seq by providing genome-wide gene expression quantification in contiguously cryo-sliced whole-body segments (supplementary table 1, Supplementary Material online; Combs and Eisen 2013; Junker et al. 2014; Kruse et al. 2016). Organisms and developmental stages with low-dimensional bodies, such as embryos, larvae, and worms, are emerging as ideal systems to examine gene expression along anteroposterior, dorsoventral, and lateral dimensions (Combs and Eisen 2013; Junker et al. 2014; Ebbing et al. 2018). Recent Tomo-seq work in *Caenorhabditis elegans* comparing hermaphrodite and male expression patterns identified the location of genes with sex-biased expression outside of reproductive tissues (Ebbing et al. 2018). Although size remains a limitation for larger-bodied organisms, this technique could be applied, in some cases, to distinguish spatial and functional differences between organs or other low-dimensional structures of females and males (see Wu et al. 2016).

Measuring Sex-Specific Transcription Binding Activity: ATAC-Seq

Sex-specific regulation can arise in part from transcription factors binding to open chromatin (box 1), yet most of the evidence we have about sex-specific regulation comes from indirect studies of *cis*- and *trans*-regulatory changes in interspecies and interpopulation hybrids (Meiklejohn et al. 2014; Turner et al. 2014; Coolon et al. 2018). To directly address the role of regulation variation in SD, chromatin

immunoprecipitation and sequencing (ChIP-seq) and related methods (see Naqvi et al. 2019) can be used to quantify DNA–protein interactions in a high-throughput manner. However, they require a priori knowledge of specific protein targets and large amounts of starting material (Jiang and Mortazavi 2018). Alternatively, the assay for transposase-accessible chromatin sequencing (ATAC-seq, supplementary table 1, Supplementary Material online) is the next iteration of genome-wide DNA–protein interaction assays and overcomes some of these shortcomings by: directly accessing open chromatin enzymatically with the hyperactive Tn5 transposase, not requiring protein-specific markers, allowing for low amounts of starting material, and being time- and cost-efficient (Buenrostro et al. 2013; Yan et al. 2020). Additionally, ATAC-seq is more sensitive, which decreases the signal-to-noise ratio seen in ChIP-seq, and can be integrated into a single-cell sequencing framework (e.g., scATAC-seq). In *C. elegans*, novel regulators have been uncovered using ATAC-seq, revealing complex regulatory dynamics across developmental stages (Daugherty et al. 2017). Overall, ATAC-seq has potential to examine broadly distributed regulatory regions across the genome, which can help disentangle sex-specific binding activity both spatially within the organism and across development.

Genetic Manipulation for Hypothesis Testing

Many toolkits have been devised to manipulate the genetic architecture and expression of specific genes, allowing for spatiotemporal control and visualization of gene expression to manipulate SD and verify candidate genes. We discuss the feasibility and technical limitations of CRISPR gene engineering (box 2) and highlight four established toolkits, which CRISPR made more accessible.

Expression Control through Gal4/UAS

The Gal4/UAS system allows for spatiotemporal control of gene expression by splitting the regulation and coding sequence to independently investigate the effects of regulation versus transcription levels on gene function (supplementary table 1, Supplementary Material online). Utilizing sex-specific promoters to drive Gal4 expression allows for feminization or masculinization of specific tissues (fig. 1B). For example, sex-specific Gal4 drivers were used to investigate SD in *Drosophila* sleep behaviors (Khericha et al. 2016) and pathology (Regan et al. 2016). Extending these studies to a multigeneration framework will allow for selection to be manipulated in a sex-biased manner to understand the effect of sex-biased regulation on population fitness (fig. 1). Although this system provides a powerful approach to control gene expression, native gene expression is not strictly conserved (see Wang

et al. 2017) and must be considered during experimental design and interpretation.

Expression Control through Cre-Lox

Cre-lox allows for deletion of specific sequences (Gu et al. 1994), translocation of chromosome fragments (Van Deursen et al. 1995), inversion of gene orientation (Grégoire and Kmita 2008), and integration of transgenes (Levy et al. 2015) to manipulate genetic architecture in a controlled manner. Two lox sites are integrated for genetic deletions, flanking the desired sequence to be deleted (supplementary table 1, Supplementary Material online). The expression of Cre induces recombination of the two lox sequences, excising the intermediate stretch of DNA between them. Other utilities simply rely on changing the orientation or location of the lox sites. Under tissue-specific promoters, Cre expression can be controlled spatially and temporally to manipulate sex-specific constraints on SD or alter developmental cues (fig. 1*B* and *D*). Cre-lox has been used to investigate sexually dimorphic behavior and delete the testosterone androgen receptor in mice (Juntti et al. 2008). Although Cre-lox can provide precision control over the desired genetic manipulation, several Cre drivers have transient expression and can lead to the Cre recombinase activity in undesired cells and tissue types (Song and Palmiter 2018). To overcome this obstacle, several "split-Cre" systems can drive portions of the Cre recombinase protein under different drivers, allowing for higher specificity (Hirrlinger et al. 2009).

Expression Control through Targeted Knockdowns

We can learn about the molecular function underlying SD through controlled and targeted depletion of gene products in both permanent and inducible contexts. Knockdown methodologies, such as RNA interference (RNAi, supplementary table 1, Supplementary Material online) can be used to suppress expression, which provides a powerful tool for examining expression variation between the sexes (Fire et al. 1998). RNAi causes the knockdown of a gene by eliminating the genes' mRNA by injecting double-strand RNA or in vivo expression (Dzitoyeva et al. 2001; Crotty and Pipkin 2015), and has been adopted in a wide variety of organisms, including humans (Setten et al. 2019). RNAi can be used to verify the necessity and sufficiency of candidate sexually dimorphic genes. For example, RNAi was used to identify the molecular basis of a color SD in the queenless ant, *Diacamma* sp. (Miyazaki et al. 2014), to examine the function of water strider male antennae during mating (Khila et al. 2012), and to test female and male fertility genes in *Drosophila* (Chen et al. 2012; VanKuren and Long 2018; Chen, Delbare, et al. 2019). Although RNAi is a powerful and widely applicable technology across taxa, the effect can be weak and nonspecific degradation can occur (Boutros and Ahringer 2008). In

some cases, CRISPRi can overcome these limitations and provide a substitute for RNAi (box 2).

The auxin-inducible degradation (AID, supplementary table 1, Supplementary Material online) system has recently been utilized for targeted gene knockdown (Nishimura et al. 2009). AID uses a transgenic plant protein, TIR1, which recognizes a small specific degron tag on a protein of interest and degrades this protein in the presence of auxin. The degron tag can be added to native genes by CRISPR (box 2), or transgenic integrations of genes with the degron tag can be introduced into a wild-type or mutant background. Importantly, AID has higher specificity compared with RNAi and temporal control is simpler to achieve through the addition of auxin. Despite its power, AID is sensitive to the concentration of auxin, less permeable in some tissues, and auxin-independent degradation has been observed (Zhang et al. 2015; Papagiannakis et al. 2017; Schiksnis et al. 2020). AID has successfully been used for protein depletion in cell culture and animal models (Kanke et al. 2011; Holland et al. 2012; Zhang et al. 2015; Kasimatis et al. 2018), except in zebrafish where the current form of the AID system has a limited effect (Yamaguchi et al. 2019). To the best of our knowledge, AID has not been specifically applied to questions of SD, however, this method is ideal for manipulating sex-limited selection in an E&R framework (fig. 1*A*).

Haplotype Tracking through Fluorescent Reporters and Barcoding

The ability to visually mark when and where a gene gets expressed is arguably the most basic and essential tool utilized by molecular genetics to investigate genetic architecture and can provide a visual context for expression differences between the sexes. Fluorescent reporters can be tagged to a native protein or act as an independent transgene (box 2) and have been developed in many color variants for a wide range of utilities (Rodriguez et al. 2017), including competition experiments to identify adaptive lineages (Hegreness et al. 2006; Crombie et al. 2018) and sex-stratified experiments to parse sexually dimorphic gene expression (Serrano-Saiz et al. 2017). However, some fluorescent reporters are very dim depending on the transcriptional activity, and translational reporters, in some cases, can disrupt protein activity, which prevents the incorporation of a fluorescent tag.

Although fluorescent reporters allow for simple identification, the total number of reporters are significantly limited. High-throughput approaches that include neutral genomic-integrations—namely barcodes—have recently been adapted to study adaptive lineages in yeast and bacteria (Blundell and Levy 2014; Levy et al. 2015; Jasinska et al. 2020). Although barcoded lineage-tracking has not been explicitly adopted in animal systems, unique lineage identification has been implemented in competitive

experiments utilizing reporters (Marie-Orleach et al. 2016). Expanding on fluorescent reporter marked lineages, various sex-specific lineages could be created and marked for competition experiments (fig. 1A). After overcoming the technical limitation of genomic barcoding, high-throughput lineage tracking will be the next great breakthrough in experimental evolution in animal systems.

Conclusions

SD constitutes much of the diversity observed between organisms and is integrated across the genotype–phenotype–fitness map. By harnessing cutting-edge methods developed for molecular biology and biomedical research, we can design explicit experiments to address how this remarkable diversity evolved from a shared genome. With few exceptions, the technological advancements discussed here will allow us to increase the spatial, temporal, and molecular resolution of the underpinnings of SD, and expand our ability to implement molecular and genetic studies in nonmodel organisms. The field is poised to synergize advances in molecular biology and sequencing technology within evolutionary frameworks, promising novel insights on the creation and maintenance of SD in the near future.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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Associate editor: Betran Esther