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Comptes Rendus
Biologies

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Volume 348 (2025), p. 89-105

Online since: 4 June 2025

https://doi.org/10.5802/crbiol.177

The Comptes Rendus. Biologies *are a member of the Mersenne Center for open scientific publishing*
www.centre-mersenne.org — e-ISSN : 1768-3238

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INSTITUT DE FRANCE

Comptes Rendus. Biologies
2025, Vol. 348, p. 89-105
https://doi.org/10.5802/crbiol.177

Review article

Uncovering the mechanisms of sexual differentiation: insights from *Drosophila* research

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Abstract. The differences between males and females represent the largest phenotypic dimorphism observed in most species. In humans, this variation contributes to disparities in the risk, incidence, and treatment responses for numerous diseases, with many of these significant differences remaining unexplained. While hormones derived from sex organs play critical roles in shaping and maintaining certain sex differences, recent research using the *Drosophila* model underscores the significance of cell-intrinsic mechanisms linked to the sex chromosomes.

Keywords. Sex determination, Sex chromosomes, Drosophila, Development, Physiology.

Funding. Université Côte d'Azur, CNRS (ATIP-Avenir program), INSERM, European Research Council (ERC starting grant CellSex, ERC-2019-STG#850934), French Government (National Research Agency, ANR) through the "Investments for the Future" programs LABEX SIGNALIFE ANR-11-LABX-0028-01 and IDEX UCAJedi ANR-15-IDEX-01.

Note. Bruno Hudry is the recipient of the 2022 Dr. and Mme Henri Labbé prize.

Manuscript received 28 September 2024, revised 21 April 2025, accepted 3 May 2025.

1. Introduction

Living organisms use two main biological mechanisms to generate sex differences [1–3]. The first mechanism involves the development of male- and female-specific sex organs that produce systemic signals. These gonadal hormones can shape and maintain somatic sex differences over time, regardless of the sex of the receiving cells. The second mechanism relies on cell-intrinsic mechanisms, where sex-chromosomal genes create distinctions in equivalent differentiated cells in both males and females. Sex chromosome functions have traditionally been explored using invertebrate model systems. This review will outline how research on the *Drosophila* model has uncovered fundamental principles of sexual differentiation that are broadly applicable across various species.

2. *Drosophila* as a model for the study of sexual differentiation

2.1. *X-Chromosome dose determines sexual fate*

In many species, the development of testes or ovaries is determined by specific genetic elements known as sex chromosomes, which carry the loci responsible for sex determination [4]. In *Drosophila melanogaster*, females possess two X chromosomes, while males have one X and one Y chromosome [5]. The number of X chromosomes acts as the primary sex-determination signal in this species (Figure 1). Individuals with two X chromosomes develop as females, whereas those with a single X chromosome develop as males. This mechanism is referred to as dose-dependent X sex determination. Notably, the

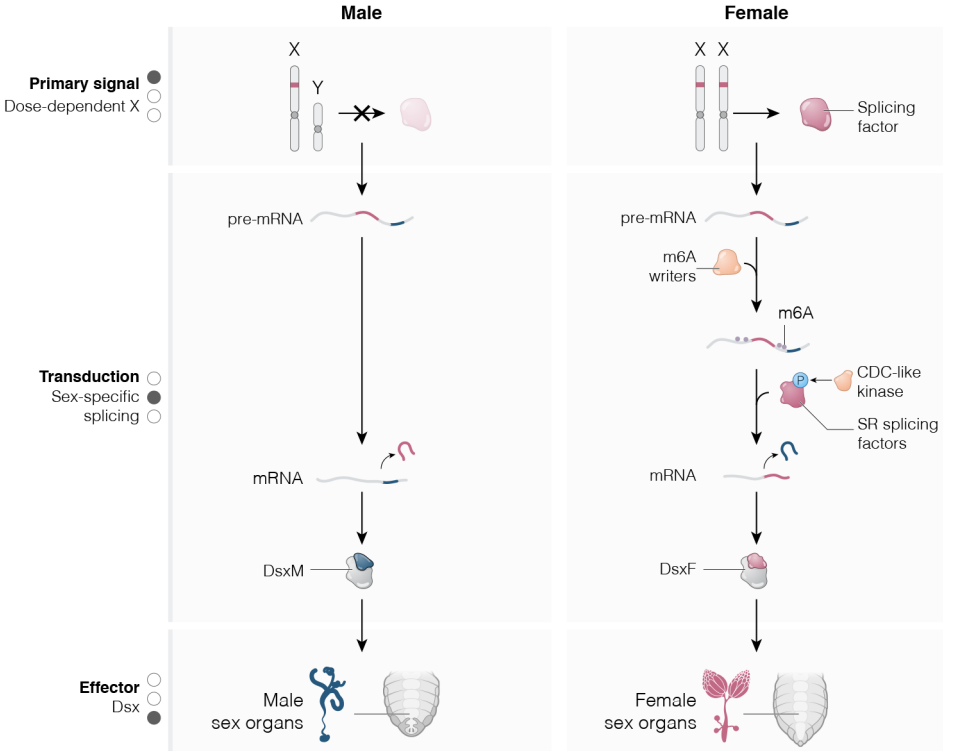

Figure 1. The sex determination process in *Drosophila melanogaster*.

fly's Y chromosome is irrelevant and does not play a role in sex determination. The immediate target of the X chromosome dosage is the X-linked switch gene, *Sex-lethal* (*Sxl*), which directs female differentiation [6]. Sxl functions as a female determinant, produced exclusively in female cells; its absence leads to male developmental commitment. Cells determine whether to express *Sxl* by counting their X chromosomes through a small set of specific X-linked numerator genes that convey the X-chromosome dose to an *Sxl* promoter [7]. A diplo-X dose, involving four genes—*scute*, *sisterless A*, *unpaired*, and *runt*—activates *Sxl* transcription, initiating female development. Conversely, a haplo-X dose is insufficient to activate *Sxl*, resulting in male development. This X chromosome-counting mechanism occurs during a brief 30–40 min period just before the onset of cellularisation. Afterward, Sxl engages in a positive autoregulatory loop that sustains its protein production in female cells. Ultimately, a transient twofold concentration difference in these four X-linked proteins determines sex, leading to distinct sexual fates.

2.2. *Sex-specific splicing mediates sexual determination*

Sxl is an RNA-binding protein that regulates pre-mRNA splicing to drive female development [6]. It controls the alternative splicing of its own gene, as well as those of the SR splicing factor *transformer* (*tra*) and *male-specific-lethal 2* (*msl-2*) [5]. These three female-specific splicing events are crucial for governing all aspects of sexual differentiation. Sxl sustains its own expression by preventing the inclusion of a poison cassette exon that contains a premature stop codon. For its targets *tra* and *msl-2*, Sxl interferes with U2 snRNP auxiliary factor (U2AF) binding, redirecting splicing to an alternative 3′ splice site or promoting intron retention. As a result, the female *tra* isoform contains an extended open reading frame that bypasses the introduction of a premature stop codon. In the case of *msl-2*, intron retention leads to translational repression in the cytoplasm. Sexual fate in *Drosophila* is mainly controlled by alternative splicing, but two

additional biological processes—RNA methylation and splicing factor phosphorylation—also play critical roles in sex determination (Figure 1). Specifically, N6-methyladenosine (m6A) RNA methylation near the poison cassette exon is important for the female-specific alternative splicing of *Sxl*, as demonstrated by the knockout of several subunits of the m6A methylosome complex [8–14]. It is hypothesised that m6A readers bind to these methylated sites, and modulate the splicing machinery to promote skipping of the poison exon in females. Although m6A writers and readers are not absolutely required for sex determination—since null mutants are viable and fertile—multiple components of the m6A pathway genetically interact with *Sxl* in sensitised backgrounds. These findings suggest that RNA methylation contributes to the robustness of *Sxl* alternative splicing, highlighting its role in fine-tuning the sex determination process. Phosphorylation of the female-specific splicing factor Tra is essential for its activity and for sex determination [15]. The fly CDC-like kinase (CLK), known as Darkener of apricot (Doa), mediates this phosphorylation [16]. Mutations in *Doa* lead to somatic female-to-male sex transformations, underscoring its critical role in establishing female fate [15,16].

2.3. *Doublesex is the terminal effector of sexual differentiation*

Sxl regulates the sex-specific splicing of its target *tra*, which in turn influences the splicing of two critical genes: *doublesex* (*dsx*) [17–19] and *fruitless* (*fru*) [20–28]. These two key factors are the terminal effectors of sexual differentiation and shape most of the sex differences in flies [5]. The male-specific isoform of *fru* (*fruM*) is essential for producing transcription factors that govern specific neuronal circuits involved in sexual orientation and aggression [20–28]. Both male (*dsxM*) and female (*dsxF*) isoforms of *dsx* encode transcription factors, though only a few direct targets of Dsx are well characterised [29]. DsxM plays a crucial role in regulating sexual behaviours by controlling the development of male-specific neurons [30,31]. DsxF supports egg growth by activating *yolk protein* (*Yp*) genes in fat body cells [32] and promotes pheromone production by regulating the desaturase DesatF [33]. Sex-specific gonadal development is the last and critical aspect of sexual differentiation regulated by *dsx* (Figure 1) [5,34]. Although *dsx* is expressed in only a subset of cells and required at specific developmental stages to form dimorphic structures [35–37], its continued expression can be essential for maintaining sex differences throughout adulthood. For instance, thermosensitive alleles have highlighted the importance of the sex determination cascade in sustaining sex-specific *Yp* synthesis [38,39] and *desatF* expression [29]. In the adult gonads, sexual plasticity is also evident; continuous expression of DsxM in testis cyst cells is necessary to preserve their male identity, while ectopic expression of DsxF can feminise these cells into a follicle-like phenotype [34,40]. Additionally, *dsx* plays a role in adulthood by inhibiting male-male courtship behaviours [41–43].

3. Sex-determining mechanisms are diverse and can evolve rapidly

Dose-dependent X or Z chromosome sex determination has been observed in two other model organisms following its discovery in *Drosophila*: the invertebrate *Caenorhabditis elegans* [44] and the chicken [45]. In nematodes, males have an X0 genotype, while hermaphrodites are XX. The X-chromosome counting mechanism in *C. elegans* regulates a male-specific switch gene called *xol-1*. Interestingly, the effect of X-chromosome dosage on *xol-1* is the opposite of that on *Sxl* in *Drosophila*: a single X chromosome (haplo dose) leads to high levels of *xol-1* transcripts, while two X chromosomes (diplo dose) result in low levels. X-linked numerator genes convey the X-chromosome dose by repressing *xol-1* in a cumulative, dose-dependent manner at both transcriptional and post-transcriptional levels in XX embryos. Xol-1 encodes a GHMP kinase that promotes male development by repressing the hermaphrodite fate.

In chickens, as in most birds, males are the homogametic sex (ZZ), and females are heterogametic (ZW). Avian gonadal sex determination primarily depends on the dosage of a single Z-linked gene, *Dmrt1*, which is homologous to *dsx* in flies [45]. During the critical period of gonadal development, males (ZZ) express Dmrt1 at significantly higher levels than females (ZW), with Dmrt1 functioning as the primary testis-determining factor. Deleting one copy of *Dmrt1* in chromosomally male (ZZ) chickens leads

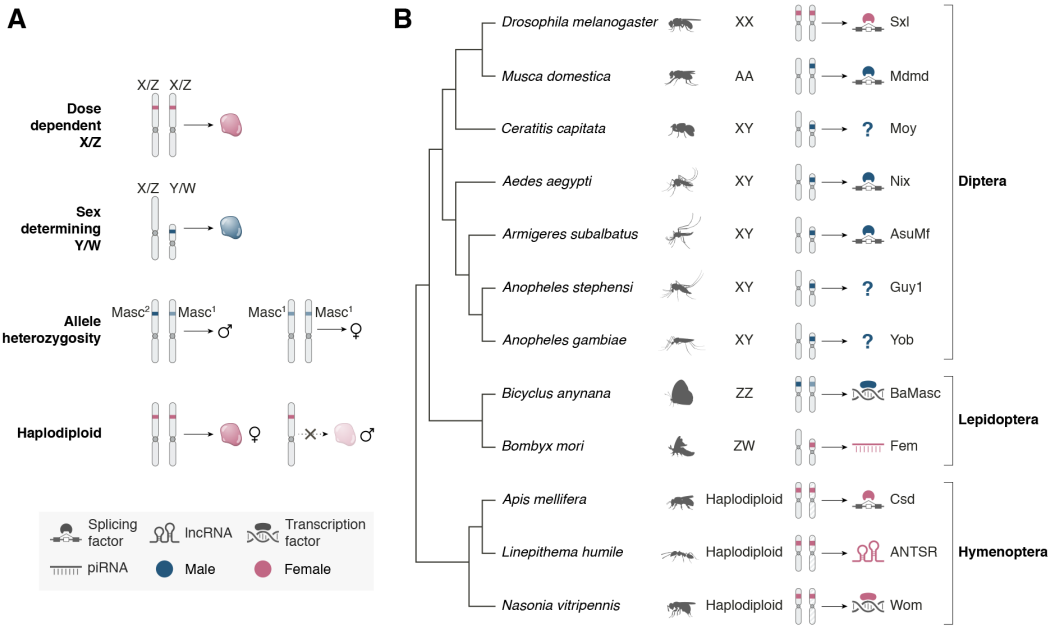

Figure 2. Sex-determining mechanisms are diverse and can evolve rapidly. (A) Beyond dose-dependent X chromosome systems, sex determination can also involve dominant Y/W-linked factors, haplodiploid systems, and allele heterozygosity at a single locus. (B) In many non-Drosophilid Diptera, the primary signal for sex determination is a dominant male-determining factor located on the Y chromosome. While many primary sex determinants encode proteins, in *Bombyx mori*, sex is determined by a single female-specific piRNA, *Fem*, and in the Argentine ant (*Linepithema humile*), the primary sex determinant is a long noncoding RNA (*lncRNA*).

to ovarian development [46,47], while overexpression of *Dmrt1* in genetically female (ZW) gonads results in their masculinisation [48]. Unlike the ubiquitous expression of sex-determining genes, Sxl and xol-1, Dmrt1 expression is specifically restricted to the sex organs. Thus, *Dmrt1* dosage is the central switch in avian gonadal sex determination.

Accurate differentiation into fertile males and females is a fundamental developmental process in animals, yet the primary signals for sex determination are not conserved across species. Diverse mechanisms are employed to determine sex (Figure 2A) [4]. Another common mechanism for sex determination, besides dose-dependent X chromosome systems, involves dominant Y-linked factors that confer maleness (Figure 2A). In many non-Drosophilid Diptera, the primary signal for sex determination is a dominant male-determining factor (often called the M factor) located either on the Y chromosome or at a male-determining locus on a homomorphic sex chromosome. Examples include the male determiner Mdmd in *Musca domestica* [49,50], Maleness-on-the-Y (MoY) in *Ceratitis capitata* [51], Yob in *Anopheles gambiae* [52,53], Nix in *Aedes aegypti* [54–60], AsuMf in *Armigeres subalbatus* [61] and Guy1 in *Anopheles stephensi* [62,63] (Figure 2B). These Y-linked dominant M factors are both necessary and sufficient to initiate male development during embryogenesis, primarily by inhibiting the activity of Tra homologs, thereby suppressing female fate. Similarly, in therian mammals (placental mammals and marsupials), male sex determination is controlled by a Y-linked testis-determining factor, SRY [64]. Teleost fish represent another prime example of a taxonomic group characterised by frequent turnovers of Y-linked factors that govern maleness [65,66] (Figure 3). Within this group, various components of the gonadal TGF-β signalling pathway—including ligands (Amhy [67–71], Gsdfy [72], Gdf6 [73,74]), receptors (Amhr [75–77], Bmpr [78]), and regulators (Id2bby [79])—have

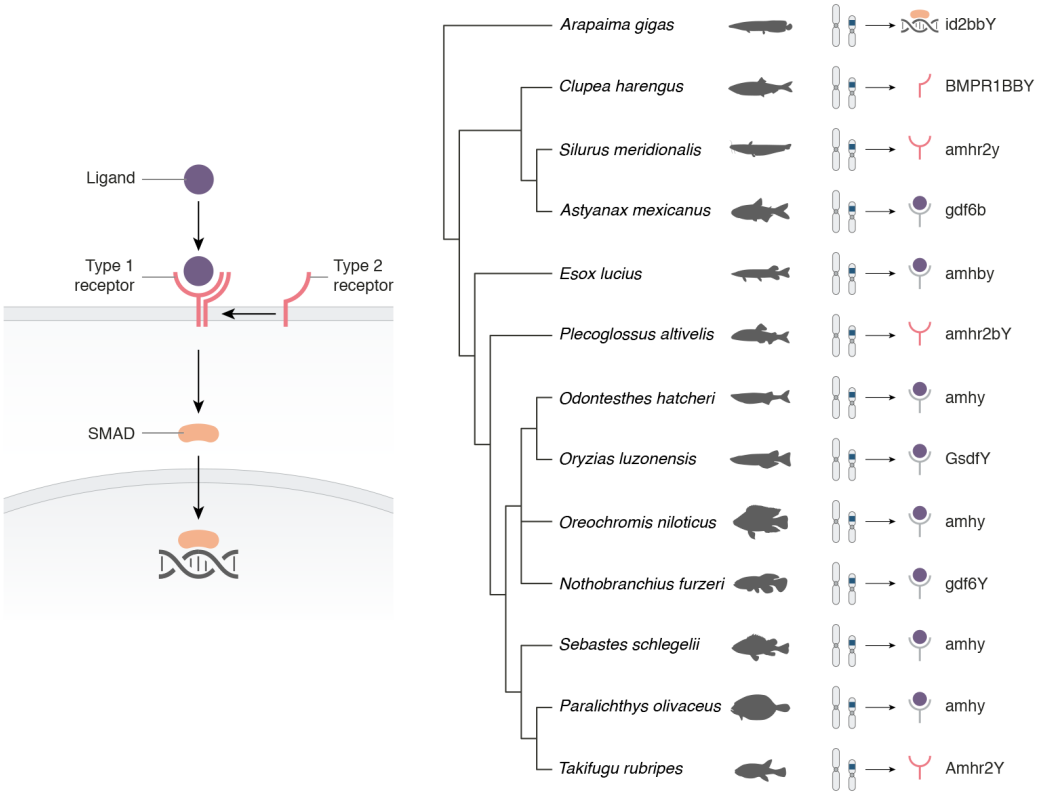

Figure 3. The TGF-β signalling pathway as a hotspot for the evolution of new master sex-determining genes in teleosts. Teleost fish represent a prime example of a taxonomic group characterised by frequent turnovers of Y-linked factors that govern maleness. Within this group, various components of the gonadal TGF-β signalling pathway—including ligands (*Amhy, Gsdfy, Gdf6*), receptors (*Amhr, Bmpr*), and regulators (*Id2bby*)—have been independently recruited to the top of the sex determination cascade.

been independently recruited to the top of the sex determination cascade (Figure 3). This underscores the TGF-β signalling pathway as a hotspot for the evolution of new master sex-determining genes in teleosts [65].

A third strategy for sex determination is the haplodiploid mechanism observed in many hymenopteran insects [80]. In this system, males are haploid and develop from unfertilised eggs, while females are diploid and develop from fertilised eggs. In other words, a paternal genome is essential for initiating female development; in its absence, uniparental haploid males arise. At the molecular level, the master switch gene for sex determination displays a parent-of-origin effect, being maternally silenced and transcribed only from the paternal allele in fertilised eggs to induce female development. This mechanism is exemplified by the *wasp overruler of masculinisation* (*wom*) gene in *Nasonia vitripennis* [80].

Lastly, a recently discovered mechanism of sex determination in the butterfly *Bicyclus anynana* involves allele heterozygosity at a single gene called *Masculinizer* (*Masc*), which acts as the primary sex-determining switch [81]. Embryos with either one or two identical alleles (monoallelic) develop into females, while those with two distinct alleles (polyallelic) develop into males. Heterozygosity activates the Masc factor, triggering male development, whereas hemizygosity or homozygosity result in an inactive Masc state, leading to the default female developmental pathway.

The remarkable diversity of sex determination mechanisms is accompanied by the rapid evolution

of the primary sex determinants, which often vary even among closely related species. This phenomenon is exemplified in Diptera, where none of the M factors share a common origin. For instance, Nix is an SR splicing factor distantly related to the *transformer2* gene in flies [60], *Mdmd* originated from a duplication of the spliceosomal factor gene *CWC22* [49], *AsuMf* from a duplication of an autosomal *Drosophila* behaviour/human splicing (DBHS) gene [61] and MoY bears no resemblance to any known proteins [51]. This evolutionary plasticity is further highlighted by the variety in the molecular nature of master switch genes. While many primary sex determinants encode proteins, in *Bombyx mori*, sex is determined by a single female-specific piRNA, *Fem* [82]. The silkworm utilises a WZ sex determination system similar to birds, where males have two Z chromosomes and females have one Z and one W chromosome. The W-linked *Fem* piRNA dominantly silences *Masc*, which is crucial for male development. In the Argentine ant (*Linepithema humile*), which follows a haplodiploid system, the primary sex determinant is a long noncoding RNA (lncRNA) [83] (Figure 2B).

4. Sex-specific splicing is a widespread mechanism for sex determination

While master sex determinants often evolve rapidly, the phenomenon of sex-specific alternative splicing may serve as a more conserved transduction mechanism of sex determination. The sex-specific splicing of *dsx* is observed across most insects and predates the last common ancestor of hexapods (Figure 4) [84–92]. In many insect species, male and female sexual development is governed by this splicing process. Notably, in early-branching insect orders, *dsx* primarily functions as a male-determining gene, crucial for male-specific sexual differentiation but not for female development [86,93]. Over time, the female-specific *dsx* isoforms evolved additional functions essential for female differentiation.

Splicing has also been linked to sex determination in vertebrates, a process that can be influenced by a number of different factors in this taxonomic group, ranging from genes on sex chromosomes, as seen in flies, to environmental factors such as temperature. In the red-eared slider turtle (*Trachemys scripta*), which exhibits temperature-dependent sex determination (TSD), functional studies have shown that the histone demethylase Kdm6b, an epigenetic modifier, plays a crucial role [94,95]. Kdm6b binds to and demethylates the promoter of the dominant male determinant gene, *Dmrt1* (a *dsx* homologue), thereby activating male development. Remarkably, loss of *Kdm6b* function at the male-producing temperature leads to male-to-female sex reversal [94]. Interestingly, in this species, *Kdm6b* undergoes temperature-dependent alternative splicing (Figure 5A) [96], a phenomenon also observed in other reptilian species with TSD, such as the American alligator (*Alligator mississippiensis*) [97] and the central bearded dragon (*Pogona vitticeps*) [97]. While *P. vitticeps* has a ZZ/ZW chromosomal sex determination system, high temperatures can induce sex reversal from the ZZ genotype to a female phenotype. In sex-reversed individuals, high temperature incubation eliminates an intron retention event in *Kdm6b* gene, and products a specific *Kdm6b* isoform [97]. The functional significance of these alternative splicing events in triggering the male sex determination cascade remains to be fully understood.

What factors regulate the temperature-dependent splicing of *Kdm6b*? Notably, homologs of the fly kinase Doa, such as CLK1, have been implicated in linking temperature changes to alternative splicing through the phosphorylation of SR splicing factors [98]. The temperature-activity profile of alligator CLK4 and turtle CLK1 correspond closely with the critical temperature ranges for temperature-dependent sex determination (TSD) in these species. For instance, CLK1 is active below 26 °C, the male-producing temperature, but its activity significantly decreases at the female-producing temperature above 31 °C. Moreover, inhibition of Clk1-4 prevents intron retention at lower temperatures, providing evidence that temperature-dependent CLK activity controls TSD-associated alternative splicing events. CLK kinases may act as molecular thermometers, functioning as an on-off switch for sex determination by regulating SR factor phosphorylation in response to temperature changes (Figure 5A). However, the specific splicing factors involved in this process have yet to be identified.

Although functional studies in reptiles are still lacking, the role of Kdm6b alternative splicing has been validated in a fish species with temperature-induced sex reversal, the *Nile tilapia* [99]. In many

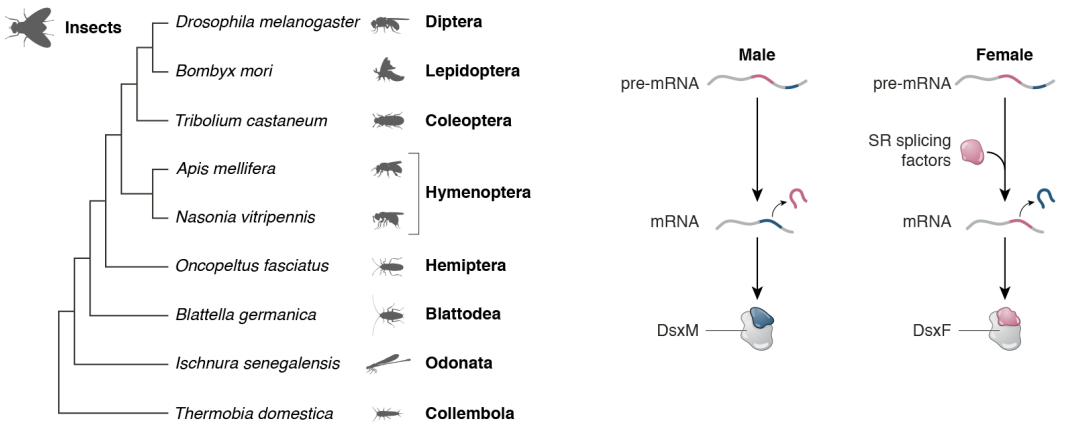

Figure 4. The sex-specific splicing of *dsx* governs sexual differentiation across most insects and predates the last common ancestor of hexapods.

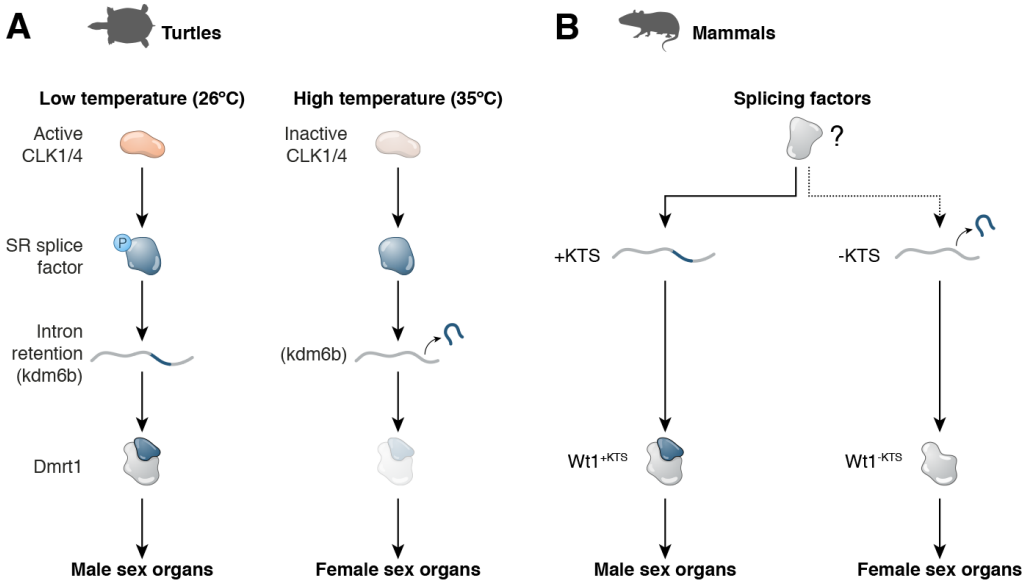

Figure 5. Splicing and its role in vertebrate sex determination. (A) In the red-eared slider turtle (*Trachemys scripta*), which exhibits temperature-dependent sex determination (TSD), *kdm6b* undergoes temperature-sensitive alternative splicing. Kdm6b binds to and demethylates the promoter of the dominant male determinant gene *dmrt1* (a homologue of *doublesex*), thereby promoting male development. In this species, temperature-dependent CLK kinase activity regulates TSD-associated alternative splicing of *kdm6b*. (B) In mammals, the ovarian-determining factor has recently been identified as an alternatively spliced isoform of the Wilms' tumour suppressor gene *Wt1*, specifically the Wt1-KTS isoform.

fish species, sex determination is notably plastic and temperature-sensitive. *Nile tilapia* have a genetic sex-determination system (XX/XY); however, exposure to high temperatures during a critical thermosensitive period can induce XX females to develop as pseudo-males. In this species, alternative

splicing of *Kdm6bb*, a paralogue of *Kdm6b* in *P. vitticeps*, produces two isoforms: *Kdm6bb_tv1*, lacking intron 5, and *Kdm6bb_tv2*, which includes intron 5. Notably, overexpression of *Kdm6bb_tv1*, but not *Kdm6bb_tv2*, induces sex reversal in XX fish. Furthermore, high temperatures specifically upregulate *Kdm6bb_tv1*, directly linking elevated temperatures to the activation of *Dmrt1* expression by demethylating H3K27me3, ultimately leading to male sex determination.

In mammals, while the Y-linked testis-determining gene *Sry* has been known for some time, the ovarian-determining factor was only recently identified as an alternatively spliced isoform of the Wilms' tumour suppressor gene, *Wt1* (specifically the *Wt1-KTS* isoform) (Figure 5B) [100]. Elevated expression of this variant, as observed in Frasier syndrome, can lead to male-to-female sex reversal by promoting ovarian development independently of chromosomal sex. Interestingly, in the turtle *Chelydra serpentina*, which exhibits temperature-dependent sex determination, a rapid increase in *Wt1-KTS* isoform expression is triggered when eggs are shifted from a male- to a female-promoting temperature [101]. Although the exact mechanism by which Wt1-KTS induces ovarian development remains unclear, it is known that Wt1 interacts with Wilms' tumor 1-associated protein (WTAP) to form a splicing complex. The *Drosophila* homolog of WTAP, Fl(2)d, is a key component of the m6A writer complex, which is implicated in the female-specific splicing of *Sxl* and *tra* [12,13]. In mammals, WTAP acts also as a regulatory subunit of the m6A methylome complex, guiding the complex to nuclear speckles and target mRNAs [102]. Therefore, Wt1-KTS may drive ovarian differentiation by influencing sex-specific splicing or by modulating m6A modifications, either directly or indirectly, through its interaction with WTAP.

Additionally, sex-specific splicing has been proposed as a crucial mechanism for sex determination in another phylum, the Platyhelminthes. In schistosomes, which possess ZW sex chromosomes, the splicing factor *u2af2* has been identified as a potential W-linked candidate for primary sex determination [103]. This conserved factor may initiate a sex-specific splicing cascade, akin to the mechanisms that govern sex determination in *Drosophila*.

5. Sex determination: it all evolves around Doublesex

In addition to the conservation of the transduction mechanism, the terminal effectors of sex determination are also highly conserved across the animal kingdom. *dsx* and its homologs have emerged as evolutionarily conserved downstream genes in the sex determination pathway, regulated by a variety of upstream factors [104,105]. In many dioecious species, these transcription factors play crucial roles in sex-specific development through their DNA-binding motif, known as the DM domain (named after *doublesex* and *male-abnormal-3*), suggesting that DM domain genes and sexual reproduction may have coevolved in metazoans.

dsx homologs consistently occupy a key position at the base of the sex-determination cascade in virtually all species studied (Figure 6). In the closest insect relative analysed to date, the branchiopod crustacean *Daphnia magna*, *Dsx1* is transcribed specifically in males, orchestrating male trait formation in both somatic and gonadal tissues [106,107]. Knockdown of *DapmaDsx1* in male embryos results in the development of female traits, including ovarian maturation, while ectopic expression of DapmaDsx1 in female embryos leads to the development of male-like phenotypes [106,107].

In molluscs, *Dmrt1* is exclusively expressed in the testes. Functional experiments in the bivalve species *Tridacna crocea* have demonstrated that Dmrt1 primarily regulates spermatogenesis [108]. Similarly, in the planarian *Schmidtea mediterranea*, the predicted *dsx* homolog plays a male-specific role in the maintenance and regeneration of the testes and male accessory reproductive organs [109].

In vertebrates, the loss of *Dmrt1* is linked to XY sex reversal in humans, and knockout studies in mice have shown that this gene is essential for testis development [104]. *Dmrt1* is also critical for male sexual differentiation in the Chinese soft-shelled turtle, *Pelodiscus sinensis* [110]. In this species, *Dmrt1* exhibits early male-specific embryonic expression, and knockdown of *Dmrt1* in ZZ embryos results in male-to-female sex reversal.

In some cases, *dsx* homologs move up the regulatory hierarchy and act as the upstream sex-determining factor, as observed in chickens [45]. In amphibians, the first primary sex determinant

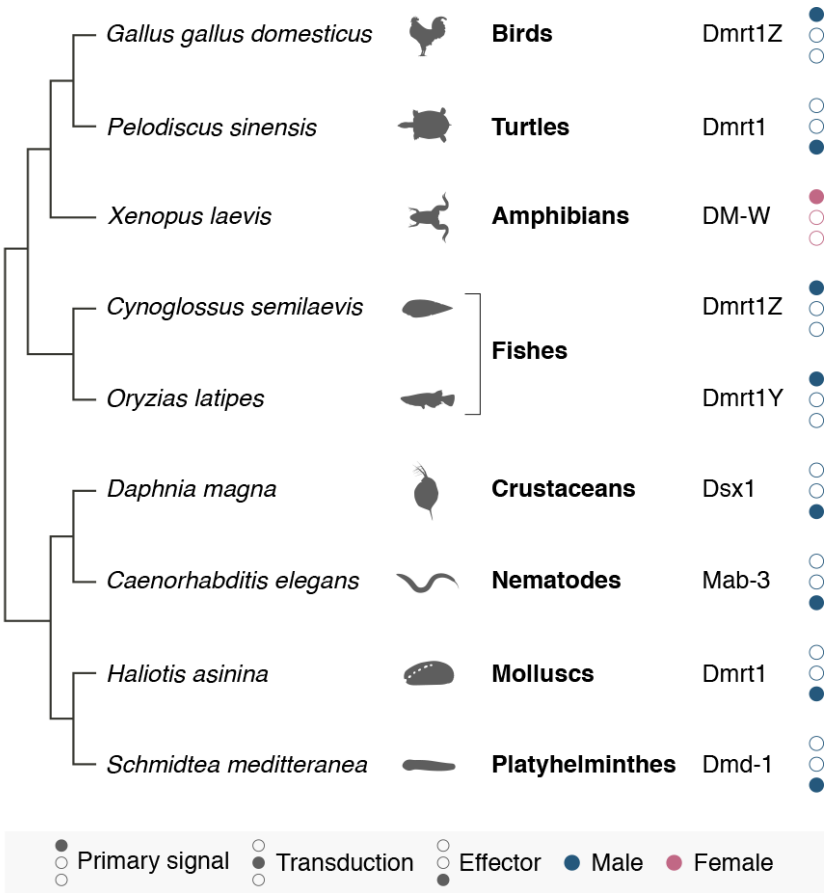

Figure 6. *dsx* homologs occupy a key position at the base of the sex-determination cascade in virtually all species. In some cases, *dsx* homologs move up the regulatory hierarchy and act as the upstream sex-determining factor.

identified is also a DM domain-containing factor: the dominant female-specific gene *Dm-W* in the African clawed frog, *Xenopus laevis* [111,112]. In *Xenopus*, which has a ZZ/ZW sex chromosome system, a duplicated copy of *Dmrt1* on the W chromosome, known as *Dm-W*, acts as a dominant-negative regulator. It interferes with the transcriptional activation of Dmrt1 target genes, functioning as an anti-male factor that promotes ovary formation. Similarly, in some fish species, such as *Cynoglossus semilaevis* (tongue sole) [113,114] and *Oryzias latipes* (medaka) [115,116], a duplicated copy of *Dmrt1* on the sex chromosomes serves as a male-determining factor. In these species, the deficiency of *Dmrt1* results in male-to-female sex reversal phenotypes, underscoring its essential role in male sex determination.

These examples highlight the central and conserved role of *dsx/DMRT1* homologs in sex determination and differentiation across a wide range of animal taxa, emphasising their fundamental importance in the evolution of sexual development. The striking contrast between the strong evolutionary conservation of *dsx/DMRT1* gene functions and the rapid diversification of upstream sex-determining mechanisms has spurred investigation into the evolutionary forces that drive this turnover. A central question is why sex determination remains highly labile in some taxa, yet remarkably stable in others. Although this remains an open area of investigation, mounting evidence suggests that sex chromosomes are frequent hotspots of genetic conflict—driven by processes such as meiotic drive and sexually antagonistic selection. These conflicts may play a key role in

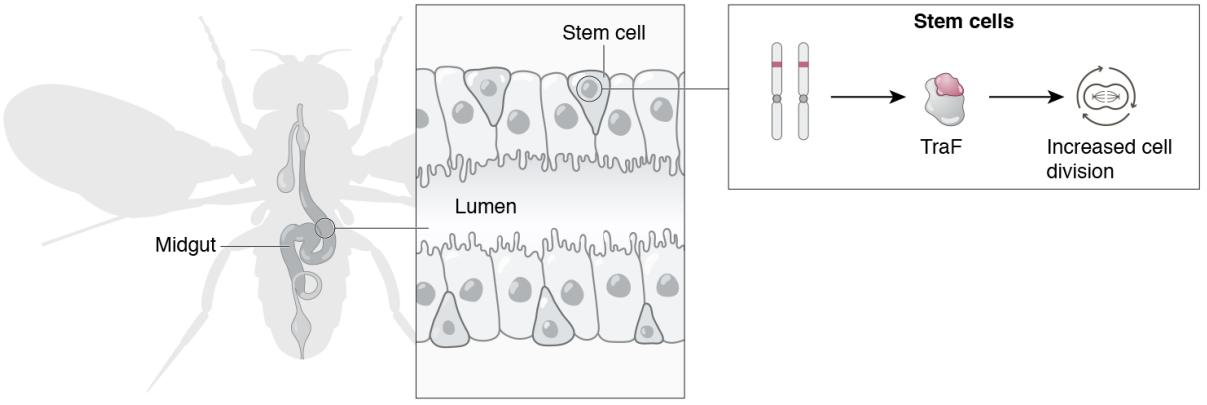

Figure 7. Cell-autonomous sexual differentiation in *Drosophila* intestinal stem cells.

fuelling the rapid evolution and recurrent turnover of both sex chromosomes and their associated master sex-determining loci.

6. Cellular sex and its impact on adult tissue physiology: insights from *Drosophila*

As discussed so far, the process of sex organ determination is increasingly well understood. However, the influence of the intrinsic presence of sex chromosomes, referred to as cellular sex, on dimorphic phenotypes in non-sex organs and physiological traits—especially in adult tissues—remains less explored. Recent studies [117–125] using the *Drosophila* model have begun to shed light on how cellular sex affects organ physiology beyond the gonads.

To investigate this, we used the adult digestive organs of flies as a model system. The intestine, one of the best-characterised adult tissues in *Drosophila*, undergoes self-renewal during adulthood due to its resident population of adult somatic stem cells (ISCs) [122]. Focusing on ISCs, our work highlights the significant role sex chromosomes play in these cells. We discovered that the sexual identity of ISCs—determined by the presence or absence of female sex determinants, Sxl and Tra—profoundly affects both organ size and the predisposition to genetically induced tumours by modulating the proliferation rate of ISCs (Figure 7).

By generating mosaic *Drosophila* in which only certain adult cells underwent sex reversal, we were able to reduce the size of a female gut to a male-like size in vivo within 20 days of adult-specific sex reversal in ISCs. Additionally, this manipulation revealed that only female guts were susceptible to tumour development. These findings also demonstrated that the sexual identity of adult ISCs is: (i) specified during adulthood, (ii) acts in a cell-autonomous manner, and (iii) is partially reversible. Importantly, ISC sexual identity is maintained by a novel branch of the sex differentiation pathway downstream of Tra, independent of the well-known Dsx and Fru pathways. This shows that even *dsx/fru*-negative cells retain the capacity for sexual differentiation into adulthood.

Using newly developed genetic models, we further demonstrated that every cell possesses an intrinsic sexual identity, governed by a binary and static genetic switch [125]. Through cell-specific sex reversals, we and others have shown that cellular sex significantly influences organ size [117,125], body weight [118,119,125], and lifespan [120,124].

7. Beyond hormones: cell-autonomous roles of sex chromosomes in mammalian physiology

Building on this body of work, the role of cellular sex in shaping mammalian physiology has become an active area of investigation. Over the past five years, multiple studies have identified X-linked and Y-linked genes as key regulators of sexual differentiation, acting in a cell-autonomous manner. For decades, it was assumed that Y chromosome genes were largely inactive outside of the testes. However, research from David Page's group demonstrated

that many Y-linked genes are also expressed in non-reproductive tissues in humans [126]. Similarly, a growing body of evidence shows that some X-linked genes are expressed at higher levels in XX cells than in XY cells, due to the presence of two copies of the X chromosome [127]. These findings reveal that sex chromosomes can drive functional differences between male and female cells across the body—not just within the reproductive system.

One such gene is *Kdm5c*, a histone demethylase that removes methyl groups from H3K27me3 and is consistently expressed at higher levels in XX than in XY cells in both mice and humans [128,129]. Recent studies implicate *Kdm5c* in the regulation of metabolism in mice. Experimental data show that mice with two copies of *Kdm5c* exhibit greater body weight, a higher proportion of body fat, and increased food intake during the daytime compared to those with a single copy [130]. These sex differences appear to be primarily driven by *Kdm5c* expression in preadipocytes, where it influences both their development and metabolic activity [130]. This example illustrates how the dosage of an X-linked gene can contribute to sex differences in normal mammalian physiology.

8. From *Kdm6a* to *SHOX*: Gene dosage and the cellular foundations of pathological sex differences

Another compelling example is *Kdm6a* (also known as *Utx*), a gene encoding a histone demethylase that targets H3K27me3. *Kdm6a* has emerged as a key agent of sexual differentiation, contributing to sex differences in both autoimmune disease and neurodegeneration [131]. Notably, most autoimmune diseases are more prevalent in women than in men. Multiple sclerosis (MS), a putative T cell-mediated disease, exhibits a threefold higher incidence in females. A similar female bias is observed in Experimental Autoimmune Encephalomyelitis (EAE), a mouse model of MS induced by immunisation with myelin protein autoantigens and adjuvant [132]. *Kdm6a* is expressed at higher levels in XX than XY $CD4^+$ T cells. Conditional deletion of *Kdm6a* specifically in $CD4^+$ T cells confers strong protection from EAE and reduces neuroinflammation [133]. Transcriptomic analysis revealed that *Kdm6a* loss upregulates T helper cell–related pathways while downregulating genes involved in neuroinflammation [133]. The gene dosage of *Kdm6a*—two copies in XX cells versus one in XY—has also been implicated in sex-biased vulnerability in neurodegenerative diseases. In Alzheimer's disease (AD), men tend to experience earlier mortality, more severe cognitive decline, and greater neurodegeneration than women [134]. In vitro, treatment of neurons with the neurotoxin Aβ42 leads to greater cell death in XY than XX neurons, correlating with higher *Kdm6a* expression in XX neurons. Reducing *Kdm6a* levels in XX neurons to match XY levels increases their vulnerability to Aβ42-induced neurotoxicity. Conversely, elevating *Kdm6a* expression in XY neurons attenuates this toxicity. In vivo, overexpression of Kdm6a in the hippocampal dentate gyrus of XY male mice enhances learning and memory performance.

While lifestyle factors and sex hormones have long been recognised as contributors to sex differences in cancer incidence and outcomes, three recent independent studies have uncovered additional cell-intrinsic, non-hormonal mechanisms involving Y chromosome genes as additional contributors [135–137]. In the first study, conducted in a mouse model of colorectal cancer, male mice showed significantly worse survival than females, mirroring human sex disparities [135]. The authors found that the intrinsic presence of the Y chromosome in cancer cells acted as a risk factor, driven by upregulation of a Y-linked histone demethylase. In contrast, the other two studies revealed a protective role for the Y chromosome in different cancer types. These studies showed that loss of the Y chromosome (LOY) is extremely common across multiple tumour types, often occurring at frequencies higher than those of canonical driver mutations [137]. While LOY may arise in the context of genomic instability and might be dismissed as a passenger event due to the chromosome's small size and low gene density, the authors demonstrated that Y loss can be a driver event—notably in uveal melanoma [137] and bladder cancer [136]. In bladder cancer, experimental removal of the Y chromosome led to increased tumour burden. Further analyses identified *Uty*, a gene located in the male-specific region of the Y chromosome, as partly responsible for this tumour-suppressive effect.

Direct effects of sex chromosome genes on phenotype are also evident in conditions where sex chromosome dosage is altered. For example, a hallmark

feature of Turner syndrome—caused by the complete or partial absence of one X chromosome (typically resulting in an XO karyotype)—is short stature. This phenotype is largely driven by the dosage of an X-linked gene called *short stature homeobox* (*SHOX*). *SHOX* is expressed in the developing long bones of the forearms and lower limbs [138]. Human studies have shown that individuals with a deletion of one copy of *SHOX* exhibit short stature [139,140], whereas individuals with an extra sex chromosome—and therefore an additional copy of *SHOX*—tend to be taller than average [141]. This exemplifies the dose-sensitive role of *SHOX* in skeletal growth.

Together, these studies—all conducted within the past five years—provide compelling evidence in mammals, building on foundational work in *Drosophila*, that the intrinsic expression of sex chromosome-linked genes can drive sex differences. They also highlight the dynamism and rapid growth of this field. Crucially, the manifestation of sex differences in cells, organs, or behaviours varies greatly across species. This diversity reflects the influence of each species' unique ecology and evolutionary pressures. There are no universal or fixed male or female traits—what is sexually dimorphic in one species may be monomorphic in another. While every cell carries the potential to harness sex chromosome genes to generate sex-specific traits, this potential is not always realised. The presence of sex chromosomes does not inherently lead to overt physiological differences. This underscores the importance of studying cellular sex: to reveal when and where it matters, and to understand how it shapes organ development, function, and disease within specific biological contexts.

Declaration of interests

The authors do not work for, advise, own shares in, or receive funds from any organization that could benefit from this article, and have declared no affiliations other than their research organizations.

Acknowledgments

I thank all the members of the BH laboratory for fruitful discussions and comments. This work was supported by the Université Côte d'Azur, CNRS (ATIP-Avenir program), INSERM, European Research Council (ERC starting grant CellSex, Grant number: ERC-2019-STG#850934), and the French Government (National Research Agency, ANR) through the "Investments for the Future" programs LABEX SIGNALIFE ANR-11-LABX-0028-01 and IDEX UCAJedi ANR-15-IDEX-01.

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