Title	Crosstalk between environmental factors and sex determination pathway: insights from lepidopteran insects and cladoceran crustaceans
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Citation	Current Opinion in Insect Science. 2025, 71, p. 101403
Version Type	VoR
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Review

Crosstalk between environmental factors and sex determination pathway: insights from lepidopteran insects and cladoceran crustaceans

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Insects exhibit a remarkable diversity of sex determination systems. Sex determining mechanisms have been extensively analyzed using the genetic model insects, such as *Drosophila melanogaster*, revealing that insect sex is determined in a cell-autonomous manner. The sexual identity of each cell is governed by the conserved transcription factor Doublesex, while the regulatory mechanisms controlling its expression are species specific. In contrast, our understanding of how environmental factors modulate the sex determination pathway remains limited. In this review, we summarize recent discoveries on the crosstalk between environmental factors and sex determination pathways in the lepidopteran insects and the cladoceran crustaceans, which are closely related to insects. We discuss how the symbiotic bacterium *Wolbachia* hijacks the host WZ/ZZ sex determination pathway in the lepidopteran *Ostrinia furnacalis*. In addition, we highlight how males that are genetically identical to females are produced in response to environmental stimuli in the cladoceran crustacean *Daphnia magna*. Based on these findings, we explore the evolutionary, ecological, and applied implications of the molecular mechanisms underlying environmentally influenced sex determination.

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Introduction

Insects exhibit a remarkable diversity of sex determination systems, including male and female heterogamety as well as haplodiploidy [1,2]. In addition to genetic factors, a variety of environmental influences — both abiotic (e.g. temperature, photoperiod) and biotic (e.g. symbiotic microbes) — can modulate sex in arthropods [3]. While classical environmental sex determination, in which environmental cues directly determine sex, is rare in insects, sex manipulation by environmental factors is widespread in this clade [4]. We use the term 'environmentally influenced sex determination', including direct and indirect modulation of sex.

In insects, sex determination generally occurs in a cell-autonomous manner, where each cell establishes its sexual identity through an intrinsic genetic pathway [5]. This sex determination pathway has been extensively studied in insects with sex chromosomes, revealing that the conserved key factor is the transcription factor Doublesex (DSX) [6•]. However, the regulatory mechanisms controlling DSX expression have diversified throughout evolution [2,7•]. In contrast, our understanding of how environmental factors modulate the sex determination pathway remains limited. This review first provides a brief overview of sex determination pathways in insects. We then summarize recent discoveries on the crosstalk between environmental factors and sex determination pathways in lepidopteran insects and cladoceran crustaceans. Finally, we discuss the evolutionary and ecological implications of environmental factor–mediated sex determination, as well as potential applications of these insights.

Sex determination pathways in insects

In insect sex determination pathways, the *dsx* gene undergoes sex-specific splicing through a genetic cascade (Figure 1). The sex-specific splicing of *dsx* was first elucidated in *Drosophila melanogaster*. *sex-lethal* (*sxl*) is activated in XX but not XY embryos due to X-linked transcription factors [8]. In females, *sxl* promotes its own splicing, skipping a premature stop codon. The resulting functional SXL^F regulates the splicing of the *transformer* (*tra*), producing functional TRA^F [9]. TRA^F, together with Transformer-2 (TRA2), directs the female-specific splicing of *doublesex* (*dsx*), leading to female development. In males, the default splicing cascade produces nonfunctional SXL^M and TRA^M, resulting in the male-specific isoform DSX^M,

Figure 1
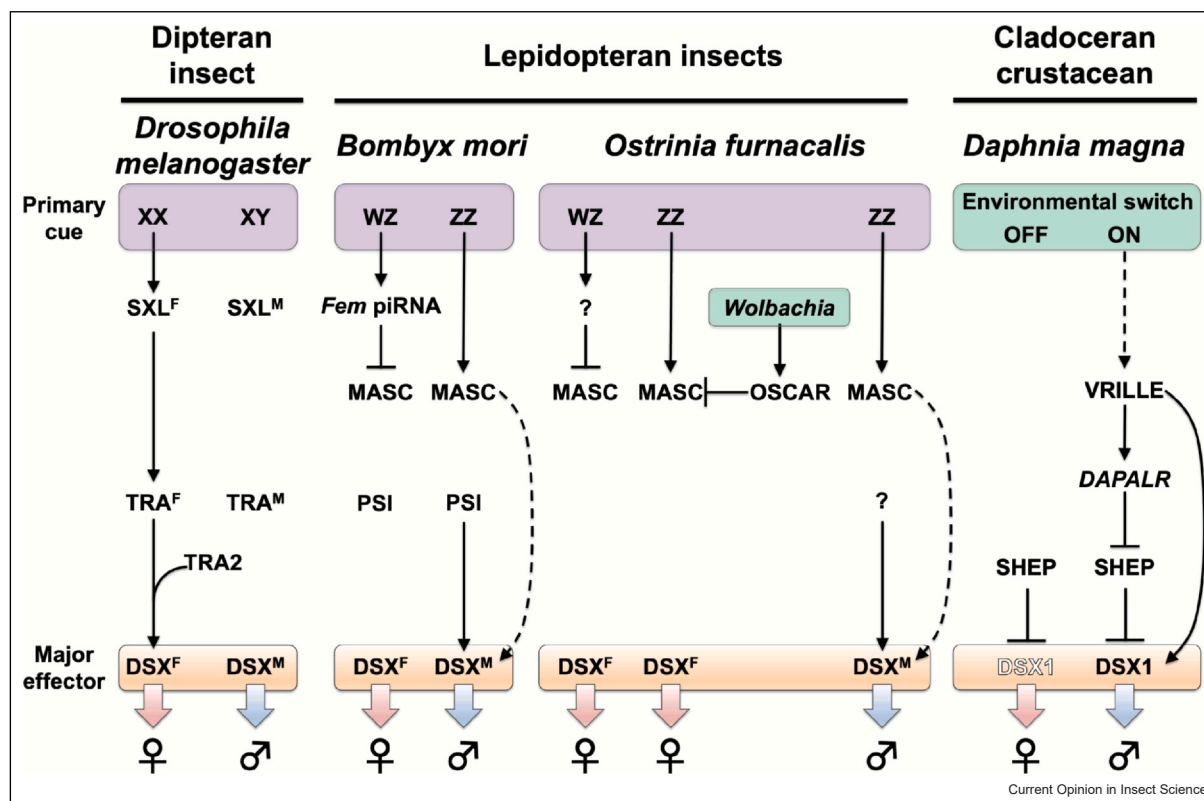
Sex determination pathways and their crosstalk with environmental factors. Regions in purple, green, and orange indicate male and female heterogamety, environmental factors, and conserved DSX transcription factors orchestrating sexual trait development, respectively. Solid and dotted lines represent direct and indirect relationships between factors. Question marks indicate that the corresponding factor has not been identified. The *dsx1* gene, which is normally silenced but may be expressed due to stochastic gene expression in females, is written in white text. Abbreviations: SXL, Sex-lethal; TRA, Transformer; TRA2, Transformer 2; DSX, Doublesex; *Fem*, Feminizer; MASC, Masculinizer; PSI, P-element somatic inhibitor; OSCAR, Osugoroshi protein containing CifB C-terminus-like domain and many Ankyrin Repeats; SHEP, Alan Shepard; *DAPALR*, Dsx1 alpha promoter-associated long RNA.

which drives male differentiation [10] (Figure 1, *D. melanogaster*). The sex-specific splicing of *dsx* is widely conserved across insects [11] and is even found in the apterygote firebrat [6•], suggesting that this regulatory mechanism is ancestral. However, the splicing regulators of *dsx* differ in certain orders, such as Lepidoptera, as discussed later. Outside of insects, sex-specific *dsx* transcripts have not been reported, except in the anostracan crustacean *Artemia franciscana* [12], suggesting that *dsx* regulation in noninsect arthropods is primarily controlled at the transcriptional level. DSX regulates the transcription of numerous genes involved in oogenesis, spermatogenesis, and sexual dimorphism, ultimately directing the development of sexual traits [13–15].

Symbiotic bacteria modulate a sex determination pathway in the lepidopteran insects

In lepidopteran insects, maternally inherited symbiotic bacteria such as *Wolbachia* can manipulate host sex determination through two distinct modes: male killing and

male-to-female sex reversal (feminization). In male killing, infected male embryos fail to develop and die during early embryogenesis, resulting in strongly female-biased sex ratios. This phenomenon has been documented in several species, including *Homona magnanima* [16], *Acraea encedon* [17], and *Ostrinia furnacalis* [18]. In contrast, feminization refers to the development of genetic males as phenotypic females, as observed in *Eurema mandarina* [19,20] and *Eurema hecabe* [21]. In this review, we focus on male killing because its molecular mechanisms have recently been elucidated in greater detail. This phenomenon has been recognized since the early 1900s [22].

The discovery of the sex determination mechanism in the model lepidopteran *Bombyx mori* has laid the foundation for unraveling the molecular basis of the male killing phenomenon in lepidopteran species. In *B. mori*, sex is genetically determined by the WZ/ZZ system, with females being WZ and males ZZ [23]. The splicing regulation of the *dsx* gene has been reported to

Figure 2
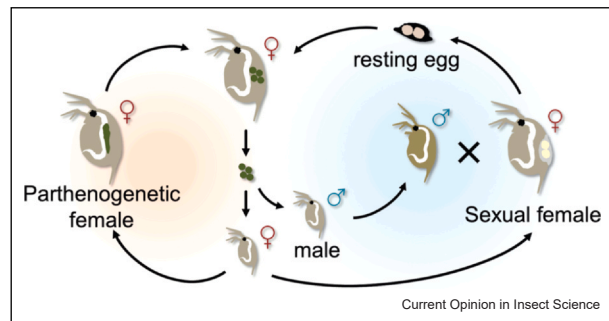
Cyclical parthenogenesis of the cladoceran crustaceans. Parthenogenetic mothers can produce genetically identical females and males. Under favorable conditions, indicated in orange, females reproduce parthenogenetically to produce clonal females. Under deteriorating conditions, indicated in blue, females produce males that mate with sexual females to generate resting eggs. When environmental conditions improve, the diapause of the resting eggs ends, leading to the emergence of parthenogenetic females.

differ from that in *D. melanogaster*. In *B. mori*, PSI, a homolog of P-element somatic inhibitor, promotes male-specific splicing of *dsx* [24,25]. A recent breakthrough in *B. mori* sex determination research was the discovery that PIWI-interacting RNAs (piRNAs) are expressed from the sex determination locus. This finding led to the identification of the *masculinizer* (*masc*) gene on the Z chromosome, which encodes a transcription factor containing a CCCH-tandem zinc finger domain [26]. MASC not only regulates male-specific splicing of *dsx* but also plays a role in dosage compensation, ultimately directing male development [26]. Conversely, the female-specific *Feminizer* (*Fem*) piRNA targets and cleaves *masc* mRNA, initiating female development [26] (Figure 1, *B. mori*).

In the male killing phenomenon of *O. furnacalis*, male offspring infected with *Wolbachia* fail to develop properly and die during early embryogenesis [27,28]. This phenotype closely resembles the embryonic lethality observed in *B. mori* male embryos subjected to *masc* RNAi. Since *O. furnacalis masc* exhibits the same functions as *B. mori masc* [29] (Figure 1, *O. furnacalis*,), researchers hypothesized that *masc* might be targeted by factors produced by *Wolbachia*, leading to male-specific lethality. To test this hypothesis, cell lines derived from *O. furnacalis* embryos infected with *Wolbachia* were established and used to identify *Wolbachia* proteins that interact with the MASC protein. This approach led to the discovery of the OSCAR protein, which associates with the CCCH-type zinc finger domain ZF1 of MASC via its ankyrin repeats [30••]. This interaction promotes MASC degradation, likely through the proteasome pathway. The interaction between OSCAR and MASC provides crucial insight into how endosymbiotic bacteria can hijack the lepidopteran genetic sex determination system.
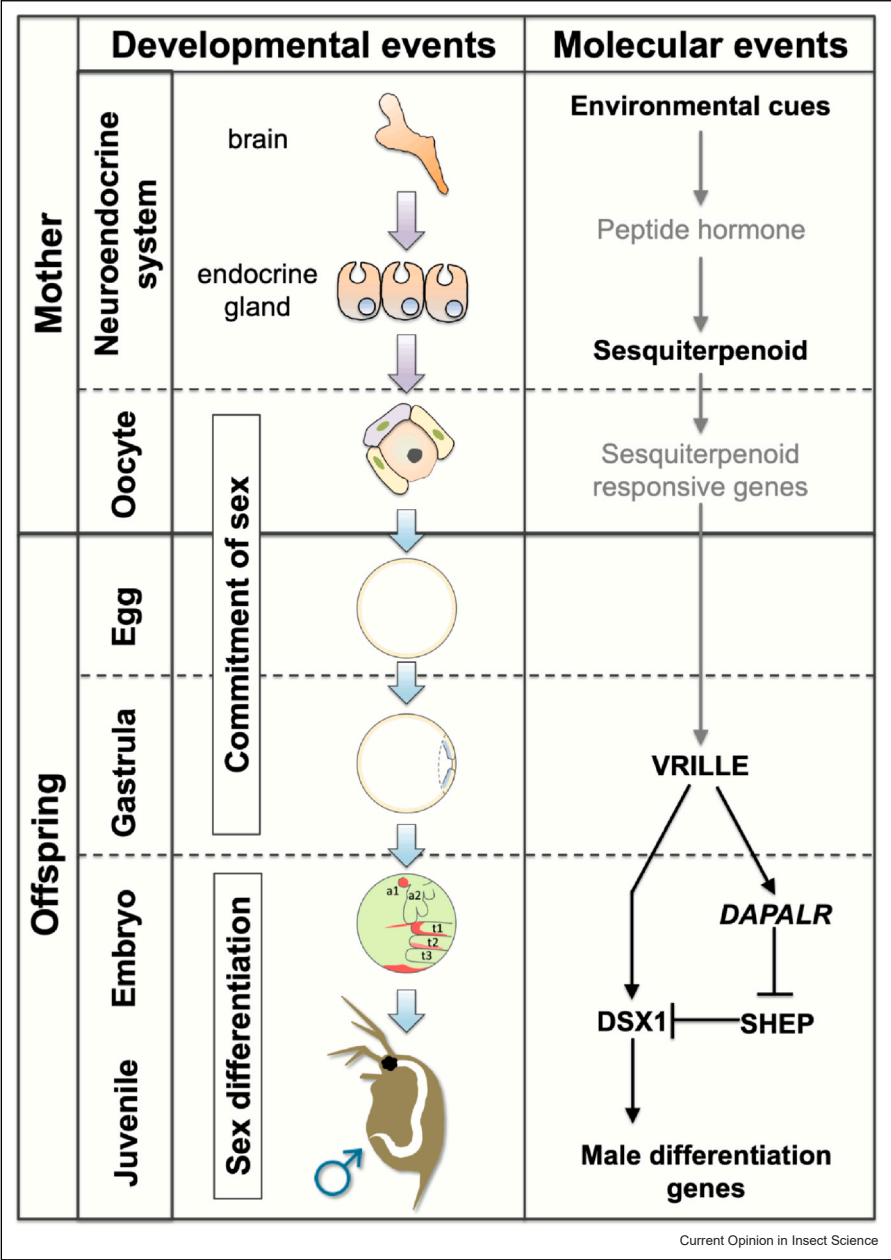
Environmental sex determination in *Daphnia magna*

Daphnia magna is a freshwater crustacean belonging to the order Cladocera within the class Branchiopoda and is the closest relative of insects for which genetic manipulation, including genome editing, has been successfully applied [31,32]. In cladoceran crustaceans, environmental signals serve as the primary cue for sex determination. Adults reproduce by parthenogenesis, producing genetically identical female and male offspring. Male offspring are produced only in response to environmental stimuli, such as crowding and a shortening photoperiod. Under deteriorating environmental conditions, sexual females also appear, mate with males, and produce sexual eggs, known as resting eggs, which are resistant to environmental hardships, such as desiccation and can remain viable for decades. They hatch when conditions improve [33,34] (Figure 2). The cyclical parthenogenesis in *Daphnia* was first documented in 1857 [34].

In the early 2000s, juvenile hormone agonists were shown to stimulate male production [35,36••], suggesting that sesquiterpenoids act as mediators between environmental signals and sex determination. The commitment of sex by environmental factors and sesquiterpenoids occurs during ovarian maturation [36••,37], suggesting that mothers detect environmental cues in the brain and produce sesquiterpenoid hormones via the neuroendocrine system. These signals may reach oocytes, inducing male-destined eggs [36••] (Figure 3). During the development of male-destined embryos, the insect *dsx* ortholog *dsx1* is activated and expressed in male-specific traits, leading to male development [38]. Unlike insect *dsx* genes, *dsx1* does not produce sex-specific splice variants [38], indicating that sesquiterpenoids activate *dsx1* transcription (Figure 1, *D. magna*). However, there is a time lag of more than 12 hours between the critical period for sesquiterpenoid-induced male commitment and the activation of *dsx1* during embryonic development, suggesting that *dsx1* is not a direct target of sesquiterpenoids. Interestingly, in male-destined embryos, *vrille* is activated around the gastrulation stage, before the initiation of *dsx1* expression (*D. magna*, Figure 1) [39]. *vrille* encodes a bZIP transcription factor and activates *dsx1* expression (Figures 1 and 3). Since *vrille* does not exhibit sexually dimorphic expression after *dsx1* activation, it may function as a transcriptional driver of *dsx1* [39].

In *D. magna* females, *dsx1* remains repressed throughout life. Ectopic expression of *dsx1* in females or reduction of its activity in males results in intersex phenotypes [38,40], suggesting strict regulation of *dsx1* expression for establishing sexual identity. A previous study proposed that female development is ensured by the translational repression of unintended *dsx1* transcripts arising from stochastic gene expression. In this model, the RNA-

Figure 3

Developmental and molecular events during male determination in *D. magna.* Male determination in response to environmental cues occurs intergenerationally. A mother detects environmental cues and may release a sesquiterpenoid hormone via the neuroendocrine system. The oocyte receives this sesquiterpenoid signal and develops into male-destined eggs. Commitment to the male sex results in the activation of *vrille* in the gastrula, leading to the derepression of *dsx1*, which orchestrates male differentiation. VRILLE also activates the long noncoding RNA *DAPALR*, which functions as a decoy for the translational repressor SHEP, thereby lowering the threshold for *dsx1* expression. Words written in gray indicate that the responsible gene(s) have not yet been identified. The red-colored regions of the embryo indicate tissues expressing *dsx1*. Relationships represented by gray arrows have not been experimentally elucidated. Abbreviations: a1, first antenna; a2, second antenna; t1, first thoracopod; t2, second thoracopod; t3, third thoracopod.

binding protein SHEP binds to the 5′ UTR of *dsx1* and represses its translation, raising the threshold for *dsx1* expression and thereby preventing sexual ambiguity [41]. Although SHEP is also expressed in males, it may be sequestered away from the *dsx1* 5′ UTR by the long noncoding RNA *DAPALR*, which overlaps in the sense direction with the *dsx1* 5′ UTR [42]. This suggests that *DAPALR* counteracts SHEP-dependent translational

repression of *dsx1* in males [41•]. Like *dsx1*, *DAPALR* expression is triggered by VRILLE and is activated only in male-destined embryos [42] (Figure 1, *D. magna*). In other *Daphnia* species, epigenetic silencing of *dsx1* in females has been suggested [43]. Identifying the silencing factors and how environmental signals affect epigenetic regulators is a key future direction.

Evolutionary and ecological perspectives
Studies on sex determination in lepidopteran insects and cladoceran crustaceans illustrate how environmental factors can modulate a sex determination pathway. It has long been hypothesized that natural selection influences the transition between genetic and environmental triggers of sex determination [44]. A laboratory experiment using *D. melanogaster* supported this hypothesis by demonstrating that a mutation in a sex determination pathway gene can shift the primary cue for sex determination from genetic factors to temperature [45]. Interactions between environmental factors and the sex determination pathway may be acquired and fine-tuned throughout evolution.

The evolutionary arms race between *Wolbachia* and its lepidopteran hosts involves continuous adaptations and counter-adaptations. Hosts can evolve suppressor mutations to counteract male killing [46]. In response, *Wolbachia* may evolve new male killing mechanisms [47]. The male killing mechanism mediated by the OSCAR protein is conserved across multiple lepidopteran species [48•], suggesting that this mechanism provides a molecular framework for understanding the ongoing arms race driven by the complex interplay between endosymbionts and their hosts. This interaction may also play a role in shaping arthropod evolution [47,49].

Species with environmentally influenced sex determination systems are vulnerable to environmental changes, leading to skewed sex ratios and population decline. Insect growth regulators, including juvenile hormone analogs, have been used as insecticides and are considered more environmentally friendly due to their specificity and lower toxicity [50]. However, studies on environmental sex determination in *D. magna* have demonstrated that juvenile hormone analogs can alter sex ratios by modulating the sex determination pathway in nontarget cladoceran crustaceans. Understanding how environmental factors influence sex determination is crucial for predicting ecological consequences and developing conservation strategies for arthropod populations.

Conclusion and future perspectives
Environmentally influenced sex determination has long been recognized [3,22,34]. Recent studies on lepidopteran insects and cladoceran crustaceans have demonstrated that environmental factors can influence sex

determination in at least two distinct ways: by hijacking the sex determination pathway at an intermediate stage or by acting as a primary signal to drive the pathway. Feminization by symbiotic bacteria has also been reported in isopods such as *Armadillidium vulgare*, where genetic males develop as phenotypic females due to early embryonic infection [51]. However, the molecular mechanisms underlying symbiont-induced sex manipulation in isopods remain largely unexplored [52]. Investigating the interaction between environmental factors and the sex determination pathway in other arthropod species is also important, as it will provide deeper insights into both the diversity and commonality of these mechanisms.

To understand how environmental factors modulate sex determination in nonmodel arthropods, identifying key regulators of sex determination is essential. High-throughput transcriptomic approaches, such as short-read RNA-seq and long-read RNA-seq, have proven invaluable for identifying candidate sex-specific transcripts, as demonstrated in studies on sex determination in silkworms and *Daphnia* [26,53]. Furthermore, the establishment of cell culture systems, such as those used to identify the male killing factor OSCAR, enables biochemical analyses that significantly advance our understanding of sex determination mechanisms [30••].

Unraveling these mechanisms opens up possibilities for developing molecular-based environmental control strategies. For instance, analyzing the universality and diversification of male-killing genes like *oscar* could contribute to the development of pest control strategies [48•]. Similarly, investigating species-specific differences in the affinity between the juvenile hormone and its receptor could lead to the development of species-specific insecticides that do not affect sex determination in nontarget organisms [54]. Thus, understanding the regulatory mechanisms of environmentally influenced sex determination is a crucial research priority not only for basic biology fields such as evolution, development, and genetics but also for applied disciplines, including environmental toxicology and agriculture. Further research will yield biological and applied insights.

Data Availability

Data will be made available on request.

Declaration of Competing Interest
We affirm that this manuscript has not been published or submitted elsewhere and that all authors have approved its submission.

The authors declare that they have no known competing financial interests or personal relationships that could

have appeared to influence the work reported in this paper.

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