Regulation of Doublesex1 Expression for Environmental Sex Determination in the Cladoceran Crustacean Daphnia

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The cladoceran crustacean Daphnia produces only females by parthenogenesis in a healthy population. However, in response to environmental declines such as crowding and lack of foods, it produces eggs destined to become males that are genetically identical to females. During the development of the sexually committed eggs, DM domain-containing transcription factor Doublesex1 (Dsx1) orchestrates male trait formation globally both in somatic and gonadal tissues. Recent studies have revealed that Dsx1 expression is tightly controlled at transcriptional, post-transcriptional, and epigenetic levels to avoid sexual ambiguity. In this review, together with basic information on Dsx1 structure and expression, we introduce the multi-layered Dsx1 regulation and discuss how each regulation is interconnected for controlling male development in environmental sex-determining Daphnia.

Keywords: Doublesex1, environmental sex determination, Daphnia magna, transcriptional regulation, post-transcriptional regulation, epigenetic regulation, male development

INTRODUCTION

Sex determination is a fundamental biological process that regulates differentiation not only of somatic tissues but also of gonads, resulting in the change of physiology and behavior in animals. It is broadly divided into two categories: genetic and environmental. Genetic sex determination is more spread in animals from invertebrates to vertebrates and relies on the difference of genetic elements between males and females (Bachtrog et al., 2014). In environmental sex determination, environmental cues trigger expression of the sex-determining gene and lead to any of male and female developmental programs. An environmental sex-determination system is also found in diverse taxa such as rotifers, nematodes, crustaceans, insects, fishes, and reptiles (Korpelainen, 1990). Exactly how the transient environmental cue initiates the sex-determining cascade and changes the developmental program has been of interest, but the regulatory mechanisms have remained unknown because the animals using the environmental sex-determination system have been poor genetic models.

The cladoceran crustacean is one of the models for studying environmental sex determination. In a healthy population, it produces females parthenogenetically. In contrast, when environmental quality declines, it also produces males that are genetically identical to females. The male then mates with a sexual female that also appears with environmental stressors, resulting in the generation of resting eggs that can withstand environmental hardships such as desiccation (Hebert, 1978). The commitment of sex to males occurs in the oocyte of the mother (Banta and Brown, 1929), meaning that the adult female senses the decline of the surrounding environment and transmits its environmental information to the next generation during oogenesis. Exposure of the crustacean sesquiterpenoid, methyl farnesoate, or its analog such as Fenoxycarb, also stimulates the production
FIGURE 1 | Timing of sexual commitment in environmental sex determination of *D. magna*. Under the healthy condition, the sexually bipotential oocyte is committed to be developed into a female. In contrast, by the mother, the information of environmental decline is detected and converted into sesquiterpenoid signaling, which commits the sexual fate of the oocyte to male.

FIGURE 2 | Domain organization of *Dsx1*. (A) Schematic representation of *Dsx1*. DM: DM domain, OD: Oligomerization domain. (B) Amino acid sequence alignment of DM domains from arthropod *Dsx* orthologs. Arrowheads indicate zinc-chelating amino acids. (C) Amino acid sequence alignment of oligomerization domains of arthropod *Dsx* orthologs. Arrowheads indicate three non-polar amino acids involved in the formation of the interface. *Dsx1*, *D. magna* *Dsx1*; *Tc*, *Tribolium castaneum*; *Bm*, *Bombyx mori*; *Aa*, *Aedes aegypti*; *Dm*, *Drosophila melanogaster*. Adapted and modified from Kato et al. (2011).

FIGURE 3 | Genomic structure of *Dsx1* gene. Exons of *Dsx1* are indicated with light blue boxes. The red arrow shows the region and direction of DAPALR. Polyadenylation sites are indicated with arrowheads. The enhancer element that includes the potential Vrille and Dsx binding sites is shown with its sequence. The position of the DAPALR core element is shown with the sequence that is similar to the TRA-2/GLI element (TGE). Adapted and modified from Kato et al. (2018).
of male offspring (Olmstead and Leblanc, 2002; Tatarazako et al., 2003). This sesquiterpenoid-dependent commitment occurs at the late stages of oogenesis (Figure 1) (Olmstead and Leblanc, 2002; Kato et al., 2010). Taken together, in the cladoceran crustacean, the adult female senses the environmental cue and possibly converts it into sesquiterpenoid by the neuroendocrine system. Thereafter, this hormonal signaling induces sexual commitment of the oocytes to males, which in turn leads to the switching of the developmental program from the female-type to the male-type for male trait development.

**Doublesex** (*Dsx*) gene codes for the transcription factor harboring the DM domain and oligomerization domain and was firstly identified in *Drosophila melanogaster* as a sex determination gene (Hildreth, 1965). The DM domain is a DNA-binding domain that consists of an intertwined zinc-binding module followed by an alpha-helical recognition domain (Erdman and Burtis, 1993; Erdman et al., 1996). The oligomerization domain is known as the ubiquitin-associated domain and is required for dimerization that enhances the binding to the target sequence. *Dsx* gene is subjected to the sex-specific splicing, resulting in male- and female-specific isoforms (Baker and Wolfner, 1988; Burtis and Baker, 1989; Nagoshi et al., 1988). Each isoform shares the same DM domain but differs in the C-terminal oligomerization domain, which changes the interacting proteins and contributes to sex-specific gene regulation (Bayer et al., 2005). In *Caenorhabditis elegans*, the male regulator *mab-3* gene was found to have the DM domain (Shen and Hodgkin, 1988). The orthologous genes have been further identified in vertebrates as “Doublesex- and mab-3 regulated transcription factor (DMRT)” and have been proved to function as a sexual regulator (Raymond et al., 2000; Matsuda et al., 2002; Ge et al., 2017; Smith et al., 2009). In contrast to deep conservation of this gene family for sex-related development, the regulators that act upstream of the DMRT genes in the sex-determining cascade are diverse in animals (Wilkins, 1995; Herpin and Schartl, 2015).

Here we review the characterization and regulation of *Dsx* ortholog named *Doublesex1* (*Dsx1*) in the cladoceran crustacean *Daphnia magna*. We will introduce the functional conservation of *Dsx1* in sexual development and describe how this ancestral sexual regulator is controlled by environmental cues and during embryogenesis in *D. magna*.

**Dsx1 Lacks Sex-specific Splicing Variants**

*D. magna* has two *Dsx* orthologs, *Dsx1* and *Dsx2* (Kato et al., 2011). Because *Dsx2* seems to have lost sex-related function (Kato et al., 2011), its characterization and potential function is not focused in this review. *D. magna* *Dsx1* has the DM domain and oligomerization domain at the N-terminus and C-terminus respectively like *Drosophila* and the other insect *Dsx* proteins (Kato et al., 2011) (Figure 2A). The amino acid sequence of *Dsx1* DM domain is well-conserved including zinc-chelating amino acids (Figure 2B). The oligomerization domain shows a more divergent sequence, but three non-polar amino acids involved in the formation of the interface are conserved (Figure 2C), suggesting that modes of transcriptional regulation of the *D. magna* *Dsx1* are similar to those of *Drosophila* *Dsx*.
The *D. magna* *Dsx1* gene is approximately 20 kb in length and consists of four exons (Kato et al., 2011) (Figure 3). The first and second introns are approximately 9 and 8 kb, each of which is considerably longer than the average size of introns (392 bp) on the *D. magna* genome, which may imply that important regulatory sequences are in the introns. Two transcripts, *Dsx1α* and *Dsx1β*, are produced from the *Dsx1* locus by alternative promoter usage: *Dsx1α* utilizes exon 3 and exon 4, and *Dsx1β* is encoded in exons 1, 2, and 4. The *Dsx1* ORF is included in exon 4 and is not spliced in a sex-specific manner, demonstrating that, unlike *Drosophila* and other insects’ *Dsx*, the same polypeptide is produced in both sexes. The 3′ UTR region contains four alternative polyadenylation sites, resulting in the synthesis of mRNAs with different 3′ UTR lengths (Kato et al., 2011). Even though *Dsx1* gene is not regulated by sex-specific splicing, these sequence characteristics suggest that this gene is intricately regulated at transcriptional and post-transcriptional levels.

**Male-Specific Expression of Dsx1**

Temporal *Dsx1* expression is divided into three phases: initiation, upregulation, and maintenance (Figure 4) (Mohamad Ishak et al., 2017). Before 6-hour post ovulation (hpo), *Dsx1α* mRNA is synthesized both in males and females (initiation phase). Around the timing of gastrulation, from 6 to 9 hpo, its expression is upregulated only in males (upregulation phase). In contrast to *Dsx1α* mRNA, *Dsx1β* mRNA is deposited as a maternal RNA in both sexes. After consumption of maternal RNAs, zygotic *Dsx1β* expression begins in both sexes and its male-specific upregulation occurs at 3 h later than the timing of *Dsx1α* upregulation. This difference of timing for male-specific upregulation of each isoform suggests that maternal sesquiterpenoid signaling first influences the *Dsx1α* promoter. Thereafter, in males, expression of both isoforms is steadily maintained. *Dsx1α* is further upregulated at the instar 5 when the secondary sex characteristics appear. In females, the *Dsx1* locus is persistently silenced except for synthesis of the maternal *Dsx1β* mRNA (Nong et al., 2017).

To investigate where the *Dsx1* activation occurs throughout development, the transgenic *D. magna* for visualizing the *Dsx1* expression was generated (Nong et al., 2017). TALENs that introduce double-strand break at the start codon of *Dsx1* in its exon 4 was constructed and used for non-homologous end-joining mediated knock-in of the donor plasmid harboring the
mCherry ORF and Dsx1 3′ UTR (Figure 5). Coinjection of the TALEN mRNAs with the donor plasmid led to the establishment of two transgenic lines (Line A and Line B) showing mCherry fluorescence. Both lines have a single copy of the mCherry reporter on one of the Dsx1 alleles. In Line A, another Dsx1 allele is mutated with a 6-bp deletion at the start codon, which suggests that this line does not produce any intact Dsx1 protein (Nong et al., 2020). In contrast, Line B has a wild-type Dsx1 gene on another allele. This genotype enables Line B males to develop sex-specific morphology such as elongation of first antennae and to have the reproductive ability for producing sexual eggs by copulating with females, even though those male-specific traits are slightly feminized (Nong et al., 2017). Localization of mCherry fluorescence was consistent with Dsx1 mRNA localization that was analyzed by in situ hybridization (Kato et al., 2011). Therefore, Line B has been used as a Dsx1 reporter line that recapitulates Dsx1 expression (Mohamad Ishak et al., 2017; Kato et al., 2018; Perez et al., 2021).

Live imaging of mCherry fluorescence in Line B male embryos revealed the dynamic change of spatial expression patterns in early embryos (Nong et al., 2020) (Figure 6). During the initiation phase of Dsx1, no clear mCherry signal was detected probably due to low Dsx1 expression levels (Figure 6, stage i and ii). In the upregulation phase, two mCherry signals were detected at 11 hpo. One was the cell cumulus appearing around the invagination, and the other was around the blastopore (Figure 6, stage iii). The mCherry-expressing cumulus moved to the vicinity of the blastopore, and the signal appeared to weaken gradually (Figure 6, stage iv). In parallel, another mCherry-expressing population migrated from the blastopore to the posterior region (Figure 6, stage iv). When naupliar segmentation occurred, the population of cells showing strong mCherry fluorescence converged in the posterior growth zone although it is not clear whether the two mCherry-expressing cell populations merged (Figure 6, stage v). In other animals, cell populations showing

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**FIGURE 7** | Spatially and temporally specific expressions of mCherry in the Dsx1 reporter strain. All embryos and daphniids shown in this figure are males of the Line B. hpo, hours post-ovulation; dpo, days post-ovulation. For the 14 hpo panel: ventral view. For the 24 hpo panel: ventral view (left) and dorsal view (right). For the 42 hpo and instar 1 panels: ventral view (left), dorsal view (middle), and side view with the ventral side facing right (right). A1, first antenna; T1, first thoracic leg; Pro, proctodeum; Ge, genital (i.e., penis in this case); Te, testis; AM, antennal muscle; LM, lateral muscle; VM, ventral muscle; CEd, carapace edge; Gp, gonopore. Scale bars = 200 µm. Adapted from Nong et al. (2017).
similar migration behavior are known as a primary organizer that induces differentiation in the adjacent area (Bénazéraf and Pourquié, 2013; Akiyama-Oda and Oda, 2003). This suggests that the migrating cell population may induce sexual differentiation in their surrounding cells. The posterior growth zone is known to supply the progenitor cells for the formation of thoracic appendages (Martin and Kimelman, 2009), suggesting that, in male embryos of *D. magna*, this region distributes masculinized progenitor cells for the development of the male-specific structure of thoracic appendages. Strong mCherry fluorescence was also observed in the buds of the first antennae, and in parallel, strong mCherry fluorescence was detected at the boundary between the head and thoracic segments (Figure 6, stage v). Later, at 18 h, more intense mCherry fluorescence was detected in the first thoracic appendage (Figure 6, stage vi), where hooks for female capture are formed at the later juvenile stage. During these earlier embryonic stages, rather broader mCherry fluorescence patterns were observed, indicating that the embryos are still sexually immature. Localization of mCherry fluorescence of female embryos was similar but its intensity was weaker compared to male embryos until the naupliar segmentation stage. Thereafter, there was no mCherry fluorescence in female embryos (Figure 6).

As embryonic development progressed toward the first instar stage that corresponded to 72 hpo, mCherry signal was localized to organs characteristic of males, first

FIGURE 8 | Dimorphic development of *D. magna*. Eggs induced to become males were obtained from *D. magna*. After injection of the synthesized dsRNA, sexually dimorphic phenotypes were examined at the fifth or sixth instar except for the first antennae (third instar). The first two columns represent normal male and female phenotypes, respectively. The third and fourth columns represent phenotypes of individuals injected with dsRNAs of dsx1 and dsx2, respectively. (A) Lateral view of the head. Arrowheads indicate the first antennae. (B) First thoracic limb. The dotted line shows the outline of the stout chitinized hook. A female-type long filament corresponding to the hook is labeled with an asterisk. (C) Gonad. Daphnids were embedded in paraffin and sectioned, followed by standard hematoxylin and eosin staining. Dorsal is left, ventral is right. Dotted circled lines show gonads at both sides of a gut. T and O indicate testis and ovary, respectively. Arrowheads indicate large lipid droplets lying among the eosinophilic yolk granules. Arrows indicate lumens into which the mature spermatozoa are released. Adapted from Kato et al. (2011).
antennae, first thoracic leg, external genitalia, and testis (Figure 7, instar 1). The expression of Dsx1 in these male-specific traits stably continued until the fourth instar stage (Figure 7, instar 4). After males showed secondary sex characteristics at the instar 5, mCherry fluorescence expanded to the other several male-specific structures, including the carapace edge below the helmet, the tip of the penis, and the skeletal muscle (Figure 7, instar 7) (Nong et al., 2020). These imaging results suggest that Dsx1 is tightly regulated by time and place for controlling male development.

Dsx1 is Essential for Male Trait Development
A previous study reported that knockdown of Dsx1 led to feminization in males (Kato et al., 2011). Long dsRNA targeting Dsx1 mRNA was injected into eggs that were destined to be developed into males. By Dsx1 RNAi, the first antennae of males became short like those of females (Figure 8A) and a copulatory hook for capturing females was lost from the first thoracic leg (Figure 8B). In addition to the...
feminization of somatic tissues, the gonads also showed sex reversal from testes to ovaries (Figure 8C). When the chemically modified Dsx1-targeting siRNA was injected, the effects of Dsx1 silencing in adults became severe enough to allow the knockdown males for laying eggs (Kato et al., 2018), showing that Dsx1 is the key sex determination factor in D. magna.

Phenotyping of the Dsx1 mutants Line A and Line B aforementioned also showed Dsx1 dependency of male trait development (Figure 4) (Nong et al., 2017; Nong et al., 2020). Compared to Line B males, Line A males showed more severe feminization throughout development, which were obvious in the first antennae and carapace (Figure 9A). The first antennae of Line A were as short as those of wild-type females in younger juveniles in contrast to Line B males with rather elongated first antennae. At instar 5 when the secondary sex characteristics appeared, the difference in the degree of feminization between the two mutants became clearer. Line B male showed the transformation of first antennae and carapace into the mature adult-like structures at this instar whereas Line A gradually transformed morphology of those two male traits across the following instar stages. Feminization of Line A males was also observed throughout the body such as larger body size (Figures 9B,C), the modest opening of the ventral carapace (Figure 9C), bulky head (Figure 9D), female-like genital and anus (Figure 9E). In addition, Line A male developed ovary-like darkened gonads but did not produce any eggs (Figures 9B,C). Male-specific traits are developed exclusively dependent on Dsx1 activity in D. magna.

**TRANSCRIPTIONAL, POST-TRANSCRIPTIONAL, EPIGENETIC CONTROL OF Dsx1**

bZip Transcription Factor Vrille Upregulates Dsx1 Expression

On the D. magna genome, upstream of the Dsx1a transcription start site, there is an element that resembles
the consensus sequence of the binding site for the bZip transcription factor Vrille. This element overlaps a potential Dsx binding site (Figure 3) (Mohamad Ishak et al., 2017). A similar element harboring both the bZip transcription factor and Dsx binding sites had been characterized well as an enhancer of yolk protein gene in Drosophila (An and Wensink, 1995). Importantly, in D. magna, male-specific expression of Vrille occurred at 6 hpo before upregulation of Dsx1 begins (Figure 10A) even though Vrille did not show any sexually dimorphic expression in the later stages of embryos (Figure 10B). Vrille knockdown reduced Dsx1 expression in male embryos (Figures 10C,D) whereas its overexpression in females activated Dsx1 expression and in turn led to the development of male trait, elongation of first antennae (Mohamad Ishak et al., 2017). Disruption of the Vrille binding site on the Dsx1α promoter region inhibited Dsx1 activation in male embryos, demonstrating that Vrille is required for Dsx1 activation during the upregulation phase. Interestingly, there was a potential binding site of the sesquiterpenoid receptor
Methoprene-tolerant (Met) in the Vrille promoter, suggesting the possibility that the Met may directly regulate Vrille expression (Mohamad Ishak et al., 2017). In Drosophila, Vrille functions as a sex regulatory factor downstream of Dsx (Lebo et al., 2009). Vrille might be repeatedly co-opted in the sex-determining pathways with the different hierarchical position in animals as reported in the other sex-determining genes (Herpin and Schartl, 2015).

Sex Differences of Histone Modification and DNA Methylation Level on Dsx1 Locus

A comprehensive epigenomic analysis of Daphnia pulex revealed that, on the Dsx1 locus, there were sex differences of active and repressive histone marks, histone H3 trimethylation at lysine4 (H3K4me3), and histone H3 trimethylation at lysine79 (H3K79me3) in addition DNA methylation status (Kvist et al., 2020). The H3K4me3 level was around 300-fold higher but the H3K79me3 level was around 5,000-fold lower in males compared to those in females. CpG methylation level was higher in females. These epigenetic marks suggest opened and closed chromatin states in males and females respectively. Although epigenetic marks on D. magna Dsx1 remain unknown, a previous study found that Dsx1 enhancer element-targeting Crispr/Cas9 could not induce any mutation on the Dsx1 enhancer element in female embryos but resulted in indel formation in males (Mohamad Ishak et al., 2017). CRISPR/Cas9 is known to have weaker activity against the closed chromatin (Chen et al., 2016; Daer et al., 2016; Jensen et al., 2017), supporting the sex difference of the epigenetic state on the Dsx1 locus.

LncRNA DAPALR Antagonizes Shep-Dependent Translational Repression of Dsx1

The RNA binding protein Alan shepard (Shep) ortholog was proved as a post-transcriptional regulator for Dsx1α mRNA in D. magna (Perez et al., 2021). The relationship between Shep and
Dsx1α mRNA was found unexpectedly based on phenotypic analysis of female embryos injected with DsRed2 reporter RNA harboring the Dsx1α or Dsx1β 5′ UTR (Kato et al., 2018). Despite lacking the Dsx1 ORF, the chimeric DsRed2 RNA including the Dsx1α 5′UTR induced development of the male trait and activation of Dsx1 in females. The RNA that coded for only Dsx1α 5′UTR also led to Dsx1 activation. To investigate the molecular mechanism underlying the unexpected function of Dsx1α 5′UTR for Dsx1 regulation, the proteins interacting with Dsx1α 5′UTR were examined by the MS analysis with D. magna lysates and one of the identified proteins was Shep. D. magna Shep gene is expressed both in males and females during embryogenesis. Silencing of Shep in the Dsx1 reporter line increased mCherry expression in male and female embryos (Figures 11A,C). In contrast, overexpression of Shep reduced the mCherry expression in the Dsx1 reporter line (Figures 11B,F). However, both loss- and gain-of-function did not change Dsx1 transcript levels (Figures 11D–F), suggesting that Shep represses Dsx1 expression at a post-transcriptional level. Dsx1α 5′ UTR has a sequence like “tra-2 and GLI element (TGE)” (Figure 3), which has been identified as a binding site of Sup-26, an ortholog of Shep in C. elegans. In vitro reporter assay demonstrated that Shep represses translation of Dsx1α mRNA via TGE (Figure 13A) (Perez et al., 2021).

Dsx1 gene transactivation function of the Dsx1α 5′ UTR is attributed to the inclusion of this 5′ UTR sequence within long noncoding RNA DAPALR (Dsx1 alpha promoter associated long RNA) (Figure 12A). This lncRNA is transcribed from upstream of the transcription start site of Dsx1α mRNA in sense orientation and overlaps Dsx1α 5′ UTR (Figure 3) (Kato et al., 2018). DAPALR is a capped and non-polyadenylated RNA. This lncRNA expression is male-specific as well as Dsx1 but its expression level is around 10 times lower than that of Dsx1. DAPALR is activated by Vrille, which may suggest co-regulation...
of DAPALR with Dsx1. RNAi-mediated knockdown of DAPALR in males led to their feminization both in somatic tissues and germline, which resulted in the production of offspring (Figure 12B). This feminized phenotype was caused by a reduction of the Dsx1 transcript level (Figure 12C). In contrast, overexpression of DAPALR in females induced upregulation of the Dsx1 gene, resulting in the development of the male trait, elongation of the first antennae (Figures 12D–F). DAPALR activates Dsx1 in trans and Dsx1a 5′ UTR functions as a transactivation element of DAPALR (Kato et al., 2018).

In in vitro translation system including the reporter mRNA harboring Dsx1a 5′ UTR, DAPALR addition canceled the Shep-dependent translational suppression (Perez et al., 2021). Similarly, Dsx1a 5′ UTR had the ability for canceling the Shep function (Figure 13A). Thus, Dsx1a 5′ UTR was also designated as a “DAPALR core element” (Figure 3). Importantly, DAPALR and its core element inhibited the function of Shep as a translational repressor in a dose-dependent manner (Figure 13B). DAPALR functions as a decoy of Shep through its core element that corresponds to Dsx1a 5′ UTR, which in turn increases Dsx1 expression.

CONCLUDING REMARKS

Figure 14 shows how each regulation for Dsx1 expression is potentially interconnected. Adult parthenogenetic females detect environmental cues and convert them to sesquiterpenoid signaling that leads to the sexual commitment of oocytes from females to males. This hormone also leads to the activation of the Vrille gene at the gastrulation stage. Vrille may bind to the Dsx1 enhancer element and triggers activation of Dsx1, a master regulator of male development. Importantly, the active Dsx1 locus also produces lncRNA DAPALR that overlaps with Dsx1a 5′ UTR in the same orientation. With a potential Dsx binding site in the Dsx1 intron 2, Dsx1 possibly activates its own expression via a positive feedback loop, which may contribute to the maintenance of Dsx1 expression throughout development. Loss- and gain-of-function analyses of Dsx1 and its regulators such as Vrille, Shep, and DAPALR provided us evidence that ectopic Dsx1 expression in females resulted in intersex phenotypes. However, the sexual ambiguity of Daphnia is rare in nature, suggesting robust regulation of Dsx1 expression. To achieve stringent suppression of Dsx1 expression in females, Shep might be used for translational repression of unintentional Dsx1a mRNA by promoter leakage that may cause the autoregulation of Dsx1. In contrast, in males, DAPALR evicts Shep and unlocks Dsx1 translation. This post-transcriptional regulation may function as a fail-safe system by which Dsx1 expression is guaranteed and sexual ambiguity is avoided (Perez et al., 2021). It is also possible that the epigenetic state of the Dsx1 locus may be changed from closed to open chromatin as a memory of the environmental information that the mother has experienced.

The mechanistic bases of the unique Dsx1 control system in the upregulation and maintenance phases during development would be an important future direction. Understanding the molecular pathway between the maternal sesquiterpenoid signaling and Dsx1 upregulation could not only provide us evolutionary insights into the diversity of upstream components in the sex determination cascades of animals but also lead to unraveling mechanisms of transgenerational regulation of development in response to environmental stimuli. As described in this review, the masculinized cells that migrate the ventral region in early embryos might be a key cell population that lets its surrounding cells know the sexual fate. For understanding how the male-specific Dsx1 expression is maintained, it would be important to clarify the positive-feedback regulation of Dsx1 and the factors involved in the epigenetic regulations. To prove the decoy model of DAPALR and Shep, the stoichiometry of the post-transcriptional regulators and the target Dsx1 mRNA must be investigated. For these future objectives and challenges, in vivo functional analysis of Dsx1 regulators in Daphnia must be moved from the individual level to tissue and cellular levels. By using CRISPR/Cas or TALEN-mediated knock-in (Nakanishi et al., 2015; Nakanishi et al., 2016; Kumagai et al., 2017), specific cell population needs to be fluorescently labeled, isolated, and subjected to single-cell analysis. This approach will allow us to move away from current analyses using heterogeneous cell populations and to identify Dsx1 regulators that control developmental stage-, tissue- and cell-specific traits at high resolution, which provides us a comprehensive understanding of Dsx1 regulation for environmental sex determination in D. magna.

AUTHOR CONTRIBUTIONS

YK wrote the initial manuscript draft and generated the figures and tables. HW revised the manuscript. Both authors approved the final article.
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