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Doctoral Dissertation

**Epigenetic suppression of the male-determining
gene *doublesex1* in female *Daphnia magna***

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September 2025

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ABSTRACT

In the microcrustacean *Daphnia magna*, environmental cues determine sex by regulating the male-determining gene *doublesex1* (*dsx1*). Male development is driven by the sustained expression of *dsx1*, meanwhile repression of *dsx1* is essential for female development. Although the transcriptional activation of *dsx1* in males has been described, the mechanisms responsible for its silencing in females remains unclear. To investigate potential chromatin-level regulators, this study examined the roles of Heterochromatin Protein 1 (HP1) and Polycomb (Pc) in *D. magna*. Four HP1 orthologs and one Pc ortholog were identified, all containing conserved chromatin-interacting domains. Expression analyses during embryogenesis revealed no sexual dimorphism, and *HP1-1* consistently showed the highest transcript abundance among the *HP1* orthologs. Functional analyses suggest that HP1-1 contributes to the repression of *dsx1* in females. RNAi-mediated knockdown of *HP1-1* resulted in derepression of *dsx1*, accompanied by male-like traits at embryonic and juvenile stages. However, lethality prior to adulthood prevented confirmation of full sex reversal. Overexpression of *HP1-1* in males did not significantly reduce *dsx1* expression, indicating that additional factors may be required for repression in that context. In contrast, *Pc* knockdown did not alter *dsx1* expression under the conditions tested, although it caused embryonic defects and lethality, consistent with a general developmental role. This suggests that *dsx1* repression in females may be maintained through Pc-independent pathways, possibly relying more on HP1-mediated heterochromatin.

CHAPTER 1

General Introduction

1.1 Overview of sex determination mechanisms

Sex determination is a fundamental biological process that governs the development of sexual phenotype in multicellular organisms. This process exhibits considerable diversity across taxa and is regulated by a range of mechanisms that determine whether an individual will develop as male, female, or, in some cases, hermaphrodites such as snails, earthworms and some species of fish. Broadly, sex determination systems can be categorized into genetic sex determination (GSD) and environmental sex determination (ESD) (Bachtrog et al., 2014). While the former relies on intrinsic genetic factors, such as sex chromosomes or specific sex-determining genes (Zarkower, 2001), the latter is mediated by external environmental cues that influence developmental pathways (Korpelainen, 1990).

In many vertebrates and a large number of invertebrates, GSD systems are prevalent. These often involve sex chromosomes—such as the XX/XY system in mammals (Sinclair et al., 1990) or the ZZ/ZW system in birds (Smith et al., 2009)—where the presence or absence of a dominant sex-determining factor initiates a cascade of gene regulation resulting in sexual differentiation. For instance, the *SRY* gene on the Y chromosome in mammals acts as a master regulator initiating male development (Koopman et al., 1991).

In contrast, ESD mechanisms are found in various reptiles, fish, and crustaceans, where environmental conditions such as temperature, photoperiod, population density, or chemical exposure determine sex during critical developmental windows (Ge et al., 2017; Kato, Kobayashi, et al., 2011; Pieau et al., 1999). Unlike GSD, ESD lacks fixed genetic triggers and instead relies on the organism's ability to interpret and respond to environmental cues through epigenetic and hormonal regulation (Ge et al., 2018; Matsumoto et al., 2016).

1.2 *Daphnia magna* as a model organism

Daphniids are freshwater microcrustaceans with a distinctive life cycle that relies on environmental stimuli, including levels of nutrients and light/dark photoperiod. The genetically cloned female offspring is produced through parthenogenesis or asexual reproduction under optimal conditions. Furthermore, genetic recombination during mitotic division has not been proven (Hebert & Ward, 1972). Under stressful environments, parthenogenetically produced males are generated, and they are genetically identical to parthenogenetically produced females (Kleiven et al., 1992). Subsequently, reproduction will transition from parthenogenesis to sexual reproduction (Figure 1). The distinctive life cycle of *Daphnia* is significant for research in various areas, including the evolution of environmental and genetic sex determination, as well as epigenetic regulation.

Daphnia species serves as a significant model organism for toxicological research due to its role as a major consumer, connecting producers and consumers within the food chain, and its sensitivity to environmental cues (Stollewerk, 2010). Consequently, numerous studies about their evolution, ecology, and reproduction have been documented. *Daphnia* offers numerous advantages as an epigenetic model, including a rapid life cycle (Harris et al., 2012), ease of handling, genetically identical offspring (Hebert & Ward, 1972) and availability of complete genome sequencing (Colbourne et al., 2005). Furthermore, this information has facilitated genetic manipulation techniques. RNA interference (Kato, Shiga, et al., 2011), ectopic expression (Kato et al., 2012), targeted mutagenesis (Naitou et al., 2015; Nakanishi et al., 2014), and targeted genome modification (Kumagai et al., 2017; Nakanishi et al., 2015, 2016) constitute essential gene manipulation methodologies that have facilitated extensive gene functional analyses, contributing to the comprehension of molecular mechanisms, including those related to sex determination.

Dark-light period, population density, and temperature have been identified as environmental factors influencing sex determination in *D. magna* (Kleiven et al., 1992). Furthermore, the presence of juvenile hormone (JH) and its analogs was observed to influence sex differentiation (Oda et al., 2005; Tatarazako et al., 2003). JH is a vital hormone in invertebrates, important for development and reproduction. Exposure to JH analog during ovarian development resulted in the production of male progeny (Olmstead & Leblanc, 2002). Notably, male offspring exposed to JH are genetically identical to unexposed female offspring.

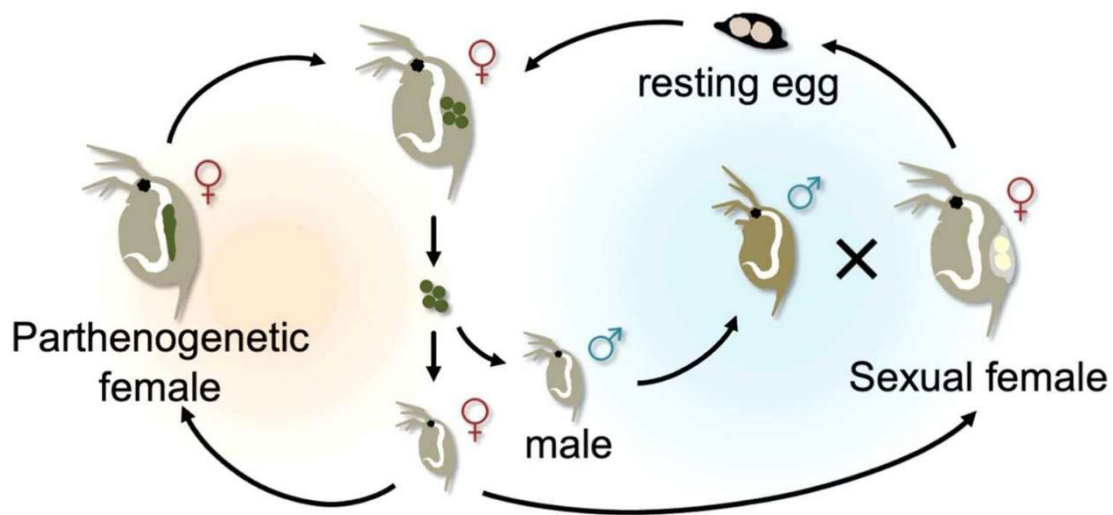


Figure 1 Parthenogenetic and sexual life cycle of *D. magna*

The diagram demonstrates the parthenogenetic (asexual) and sexual cycles of *D. magna*. Under typical circumstances, female *Daphnia* produce diploid eggs that mature into female offspring via parthenogenesis. When subjected to environmental stressors such as reduced photoperiod, food scarcity, and population density, *Daphnia* females generate male progeny, followed by haploid eggs that require fertilization by males. The fertilized egg, known as the resting egg, is covered in a protective hard shell referred to as the ephippium. The dormant eggs can withstand adverse environments and undergo diapause for an extended duration.

1.3 Regulation of *dsx1* expression

The *doublesex1* (*dsx1*) gene has been identified as the key regulator of environmental sex determination in *D. magna*. It encodes a DM-domain-containing transcription factor that directs male-specific development in somatic and gonadal tissues (Kato, Kobayashi, et al., 2011). Unlike in many insects where *dsx* genes generate sex-specific isoforms through

alternative splicing (Baker & Wolfner, 1988; Burtis & Baker, 1989; Nagoshi et al., 1988), *D. magna* produces a single Dsx1 protein in both sexes, making its sex-specific expression critical for determining sexual fate (Kato, Kobayashi, et al., 2011). The regulation of *dsx1* expression is orchestrated through transcriptional, post-transcriptional, and epigenetic mechanisms (Figure 2). At the transcriptional level, the bZIP transcription factor Vrille, expressed specifically in males, activates *dsx1* by binding to a male-specific enhancer upstream of the *dsx1a* promoter, thereby translating environmental cues into gene activation (Mohamad Ishak et al., 2017). *DAPALR* is long noncoding RNA which has function on transactivation and maintain *dsx1* expression (Kato et al., 2018). Post-transcriptionally, the RNA-binding protein SHEP suppresses *dsx1a* translation in both sexes, but this repression is lifted in males through the action of the lncRNA *DAPALR*, which sequesters SHEP and enables Dsx1 protein synthesis (Perez et al., 2021). Moreover, epigenetic profiling in *D. pulex* has shown that *dsx1* locus is marked with different histone modification (Kvist et al., 2020). The chromatin mark is consistent with an open, transcriptionally active configuration in males and a repressive, closed chromatin state in females. Such observations suggest that epigenetic mechanisms, particularly histone modifications may play a critical role in establishing and maintaining the sex-specific expression of *dsx1* and thus serve as a gateway for further investigation into chromatin-based regulation of environmental sex determination in *Daphnia*.

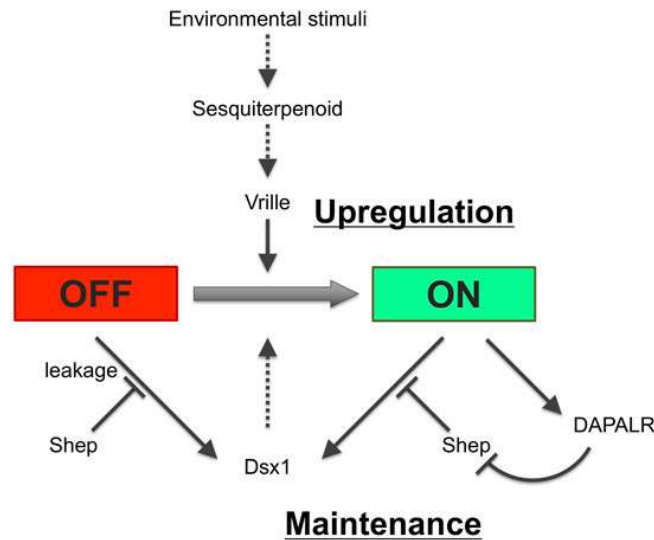


Figure 2 Potential regulatory network for *dsx1* expression.

In response to environmental stimuli, juvenile hormone or sesquiterpenoid hormone signaling activates the bZIP transcription factor gene *vrille*, leading to *dsx1* transcription. *dsx1* mRNA is further regulated at the translational level: SHEP represses leaky translation in females, while in males, the lncRNA *DAPALR* inhibits SHEP to permit Dsx1 protein synthesis. Black arrows indicate activation, black bars indicate repression. The red and green background colors of the OFF and ON boxes represent repressed and active transcriptional states, respectively. Solid and dotted lines indicate validated and non-validated relationships by experiments

1.4 Epigenetic regulation in *Daphnia* spp.

Epigenetics refers to heritable changes in gene expression that occur without alterations to the underlying DNA sequence (Bird, 2007). These regulatory mechanisms include DNA methylation, histone modifications, and non-coding RNA-mediated gene silencing, all of which contribute to the dynamic control of gene activity in response to developmental and environmental signals (Trerotola et al., 2015). Although *D. magna* genome encodes homologs of the three major DNA methyltransferases (Dnmt1, Dnmt3a, Dnmt3b), global CpG methylation levels are reported to be extremely low (< 1%) (Liu et al., 2025; Robichaud et al., 2012). Thus, DNA methylation is unlikely to serve as a predominant epigenetic mechanism in *Daphnia*, and histone modifications are considered the dominant chromatin-based regulators relevant to this study.

1.5 Histone modification and chromatin states

Histone modifications play a critical role in the regulation of gene expression by modulating chromatin structure and accessibility. The presence of modified histones including H3K4me3, H3K9me3, H3K14ac, H4K20me2, and H3K27me3 in *Daphnia* has been detected (Kvist et al., 2020; Robichaud et al., 2012), highlighting histone-based mechanisms as dominant epigenetic regulators in this genus. Given their capacity to mediate dynamic changes in gene expression in response to environmental stimuli, histone-based mechanisms are particularly relevant to environmentally sensitive processes such as sex determination.

These modifications influence transcription in different ways. For example, H3K4me3 is a well-established marker of transcriptional activation and is typically enriched at promoter regions of actively transcribed genes (Wang et al., 2023). This modification is associated with open chromatin conformation, allowing access to the transcriptional machinery (Igolkina et al., 2019). Meanwhile, H3K9me3 and H3K27me3 are associated with transcriptional silencing and heterochromatin formation. The coexistence of H3K4me3 and repressive marks such as H3K27me3 at the same genomic locus can form a bivalent chromatin state, keeping developmental genes in a poised state, particularly during early embryogenesis (Akmammedov et al., 2019; Bernstein et al., 2006; De Gobbi et al., 2011; Schuettengruber et al., 2009).

Importantly, repressive histone marks serve as binding platforms for chromatin-associated proteins such as Heterochromatin Protein 1 (HP1) and Polycomb group (PcG) proteins, respectively. HP1 recognizes H3K9me3 and contributes to the formation of constitutive heterochromatin, thereby maintaining a stable and heritable silenced state (Fischle et al., 2003; Jacobs & Khorasanizadeh, 2002; Liu et al., 2005; Studencka et al., 2012). In contrast, PcG proteins bind to H3K27me3 to establish facultative heterochromatin, enabling dynamic and reversible gene repression during development and cell differentiation (Fischle et al., 2003; Yang et al., 2021; Zhang et al., 2017).

Together, these histone modifications provide a mechanistic framework for understanding how chromatin-based repression can regulate key developmental genes in *D. magna*, including the sex-determining gene *dsx1*. This sets the stage for investigating the specific contributions of HP1 and Polycomb proteins to *dsx1* silencing, which are the focus of the present study.

1.5.1 Heterochromatin protein 1

Heterochromatin Protein 1 (HP1) encoded by *Su(var)2-5* gene was initially discovered in *D. melanogaster* through genetic screenings for modifiers of position effect variegation (PEV) (James & Elgin, 1986). HP1 is evolutionally conserved and plays crucial roles in chromatin organization and gene regulation. HP1 contains conserved chromodomain (CD) on N-terminus which is responsible for H3K9me3 binding and chromoshadow domain (CSD) on C-terminus for facilitating homodimerization (Fischle et al., 2003). The functions of HP1 related sex determination have been reported in several organisms as follows. HPL2 depletion affected male development in *C. elegans* (Studencka et al., 2012). In *D. melanogaster*, a reduction of *HP1a* reduced male viability (Liu et al., 2005). In addition, null mutation of *HP1a* revealed an aberrant splicing pattern of *Sxl_{Pm}* in both male and female in *D. melanogaster* (Li et al., 2011). Conditional germline knockout of *HP1a* caused an ectopic expression of male specific *Phf7* isoform in ovary of *D. melanogaster* (Smolko et al., 2018). Knockdown of *HP1a* in female *D. melanogaster* induced an alteration of ovarian morphology and completely agametic, resulting in sterile female (Casale et al., 2019).

1.5.2 Polycomb

Polycomb (Pc) was initially identified as a transcriptional repressor regulating the expression of homeotic genes in *D. melanogaster* (Lewis, 1978). While Pc in *D. melanogaster* has not been reported as a direct contributor to sex determination, its homologs in mice (M33 or Cbx2) and humans (CBX2) possess roles crucial to sex determination. The function of the

M33 mutant is not only conserved in its ability to cause homeotic transformations in *D. melanogaster*, but it also demonstrates a novel role in male-to-female sex reversal in XY mice (Katoh-Fukui et al., 1998). *M33* knockout additionally affected the expression of genes related to gonadal development, including *Sry* and *Sox9* (Katoh-Fukui et al., 2012). Recently, *Cbx2* was identified as a repressor of ovary-promoting genes through the Wnt signaling pathway (Garcia-Moreno et al., 2019). Research on Disorders of Sex Development (DSD) in humans has demonstrated the conserved role of *CBX2* in sex determination. A heterozygous mutation in *CBX2* in a 46, XY individual resulted in male-to-female sex reversal with the presence of ovaries (Biason-Lauber et al., 2009).

1.6 Objective of this study

While HP1 and Pc proteins are well known for their gene-silencing roles in GSD, their contribution to ESD remains unclear. In *D. magna*, *dsx1* is believed to be repressed in females via a heterochromatic chromatin state (Kvist et al., 2020; Mohamad Ishak et al., 2017). However, the specific chromatin regulators responsible for this repression remain largely unknown.

To address this knowledge gap, the present study aimed to investigate the functional roles of HP1 and Pc in the transcriptional regulation of *dsx1* in female *D. magna*. The identification and characterization of HP1 orthologs, followed by functional analyses of HP1-1 through knockdown and overexpression experiments, are described in Chapter 2. Chapter 3 focuses on the identification of the Pc ortholog and its functional analysis during embryonic development.

CHAPTER 2

HP1-1 silencing derepressed *dsx1*

and caused male-like traits in female embryos

2.1 Introduction

Daphnia magna is an established model organism in ecotoxicology and developmental biology due to its rapid parthenogenetic reproduction and sensitivity to environmental cues (Hebert & Ward, 1972). Under favorable conditions, it produces clonal female offspring, whereas environmental stress or exposure to juvenile hormone analogs triggers the production of genetically identical males (Kleiven et al., 1992). This reproductive plasticity offers a powerful system for studying environmental sex determination (ESD) without the confounding effects of genetic variation (Hebert & Ward, 1972; Vandegehuchte et al., 2010).

DM-domain transcription factors are evolutionarily conserved and play central roles in both genetic sex determination (GSD) and ESD systems across taxa. Their shared function lies in directing the development of sex-specific traits by regulating downstream gene networks. A key regulator of sex-specific development in *D. magna* is the DM-domain transcription factor *doublesex1* (*dsx1*) gene (Kato, Kobayashi, et al., 2011), homologous to *dsx* in insects (Baker & Wolfner, 1988), *mab-3* in nematodes (Inoue & Nishida, 2010), and *Dmrt1* in mammals (Raymond et al., 2000).

Proper sex determination in *D. magna* requires precise control of *dsx1* expression: it is activated in males and silenced in females. This regulation occurs through multiple mechanisms, including the transcription factor Vrille (Mohamad Ishak et al., 2017) and post-transcriptional repression by SHEP (Perez et al., 2021) and the lncRNA *DAPALR* (Kato et al., 2018).

Recent epigenomic analyses in *D. pulex* show that the *dsx1* locus is marked by low levels of H3K4me3 and high levels of H3K27me3 in females, suggesting transcriptional silencing through chromatin-based repression (Kvist et al., 2020). Supporting this, enhancer mutations at the *dsx1* locus could be introduced in male but not female embryos, consistent with a closed chromatin state in females (Mohamad Ishak et al., 2017).

Based on these findings, it has been hypothesized that Heterochromatin Protein 1 (HP1), a well-known chromatin-associated silencer, may contribute to the repression of *dsx1* in females. To test this hypothesis, this chapter investigates the role of HP1—particularly HP1-1—in regulating *dsx1* expression. The study involves identification of HP1 orthologs, analysis of their temporal expression, and functional assays using RNA interference and overexpression in a *dsx1*-reporter strain.

2.2 Materials and methods

2.2.1 *Daphnia* stains and culturing conditions

The wild-type *D. magna* strain (NIES clone) was used for total RNA extraction to assess *HP1* expression in male and female embryos during development. This strain was obtained from the National Institute of Environmental Studies (NIES, Tsukuba, Japan) and has been maintained through many generations.

The main transgenic line used was the *dsx1*-reporter strain, which carries the *mCherry* gene inserted upstream of one allele of the *dsx1* coding region. As a result, mCherry fluorescence serves as a readout for *dsx1* activation. This strain also expresses *eGFP* fused to the histone *H2B* gene, driven by the *elongation factor 1 α 1* (*EF1 α 1*) promoter/enhancer, which facilitates the monitoring of embryonic development (Nong et al., 2017).

Daphniids were cultured in Aachener Daphnien Medium (ADaM) (Klüttgen et al., 1994) at 22–24 °C with a 16 h light / 8 h dark photoperiod. They were fed daily with 120 μ L of

Chlorella vulgaris (8×10^9 cells/mL; Nikkai Center, Tokyo, Japan) and 10 μ L of 0.15 g/mL baker's yeast solution (Marusan Pantry, Ehime, Japan).

2.2.2 Artificial male production by fenoxycarb exposure

Male embryos were required to examine *HPI* expression levels and perform functional analyses. To induce male production, 2- to 3-week-old adult daphniids carrying 42-hour-old embryos in their brood chambers were exposed to fenoxycarb (Wako Pure Chemical Industries, Japan), a synthetic juvenile hormone agonist, at a critical developmental time point previously described (Tatarazako et al., 2003).

A stock solution of 1 mg/L fenoxycarb was prepared using *N,N*-dimethylformamide (Nacalai Tesque Inc., Kyoto, Japan) as the solvent and then diluted in ADaM medium to a final concentration of 1 μ g/L. Daphniids were incubated in this solution for 16-18 hours, until ovulation occurred. The freshly ovulated eggs were then collected from the brood chamber for downstream applications.

2.2.3 Identification of HP1 orthologs in *D. magna*

The genomic locations of *HPI* orthologs in *D. magna* were identified using tblastn searches against the genome assembly ASM2063170v1.1 from the NCBI database (<http://www.ncbi.nlm.nih.gov/>). Amino acid sequences of HP1 orthologs from *D. melanogaster* (listed in Table 1) were retrieved from NCBI and used as queries.

Table 1 Accession number of proteins used as query

Species	Name in NCBI	Accession number
<i>D. melanogaster</i>	suppressor of variegation 205, isoform A	NP_476755.1
	heterochromatin protein 1b, isoform A	NP_572521.2
	heterochromatin protein 1c	NP_651093.1

To validate orthology, multiple sequence alignment and phylogenetic analysis were performed using protein sequences obtained from various species (Table 2). Multiple alignments were generated using ClustalW with the following parameters: pairwise

alignment—gap opening penalty of 6.00, gap extension penalty of 0.21; multiple alignment—gap opening penalty of 10.00, gap extension penalty of 0.24, delay divergent cutoff of 30%, and gap separation distance of 4.

A phylogenetic tree was constructed using the *p*-distance method and the neighbor-joining algorithm, implemented in MEGA version X (Kumar et al., 2018). The tree was rooted using *Schizosaccharomyces pombe* Swi6 as an outgroup.

Table 2 Accession number of proteins used for HP1 multiple sequence alignment

Common name	Scientific name	Gene name (Definition in NCBI)	Accession number
Water flees	<i>D. magna</i>	chromobox protein homolog 1	XP_032787128.1
		chromobox protein homolog 3	XP_032790926.1
		chromobox protein homolog 1	XP_032796193.1
Fruit fly	<i>D. melanogaster</i>	suppressor of variegation 205, isoform A	NP_476755.1
		heterochromatin protein 1b, isoform A	NP_572521.2
		heterochromatin protein 1c	NP_651093.1
Chicken	<i>G. gallus</i>	chromobox protein homolog 1	NP_001383115.1
		chromobox protein homolog 3	NP_989974.1
Human	<i>H. sapiens</i>	chromobox protein homolog 5	NP_036249.1
		chromobox protein homolog 1	NP_001120700.1
		chromobox protein homolog 3 isoform a	NP_057671.2
Fission yeast	<i>Schizosaccharomyces pombe</i>	heterochromatin (HP1) family chromodomain protein Swi6	NP_593449.1

2.2.4 Total RNA extraction

Total RNA was extracted from collected samples using a combination of mechanical disruption and chemical purification. Samples were placed in 2.0 mL tubes (TOMY, TM-625S) containing zirconia beads (1.0 mm and 3.0 mm in diameter) and homogenized in Sepasol-RNA I Super G reagent (Nacalai Tesque Inc., Kyoto, Japan) using a Micro Smash (TOMY, MS-100).

RNA isolation followed the Sepasol manufacturer's protocol, including phenol/chloroform extraction and ethanol precipitation. To enhance RNA recovery, 10 µg of yeast tRNA (Invitrogen, Carlsbad, CA, USA) was added as a carrier to each sample during

purification. The final RNA pellet was dissolved in 18 μ L of DNase/RNase-free water (Life Technologies, Grand Island, NY, USA), and RNA concentration was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

2.2.5 First-strand cDNA synthesis by reverse transcription

First-strand cDNA was synthesized from 1 μ g of purified total RNA using PrimeScript III Reverse Transcriptase (Takara Bio, Shiga, Japan). The RNA sample was mixed with 25 ng of random primers (Invitrogen, Carlsbad, CA, USA), 4 μ L of 2.5 mM dNTPs (Takara Bio, Shiga, Japan), and ultrapure water, then heated to 65 °C for 5 minutes and immediately chilled on ice. Subsequently, 4 μ L of 5 $\times$ First-Strand Buffer (Takara Bio, Shiga, Japan), 1 μ L of RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, Carlsbad, CA, USA), and 200 units of PrimeScript III Reverse Transcriptase were added. As a negative control for genomic DNA contamination, a parallel reaction was prepared for each sample by omitting the reverse transcriptase and replacing it with ultrapure water.

Reactions were incubated in a Takara thermal cycler using the following program: 30 °C for 10 minutes, 42 °C for 60 minutes, and 70 °C for 15 minutes. The resulting cDNA was validated by PCR amplification of the *β -actin* gene using forward (5'-GGCAAGGAATAGTTCGATAC-3') and reverse (5'-CACCGACGTACGAATCCTTCTGACC-3') primers, followed by gel electrophoresis.

2.2.6 Quantitative RT-PCR

To analyze the temporal expression of the four HP1 orthologs during embryogenesis, male and female daphniids were collected at 0, 12, 24, 48, and 72 hours post-ovulation. cDNA synthesized from total RNA at each developmental stage (see Section 2.2.5) was subjected to quantitative reverse transcription PCR (RT-qPCR).

RT-qPCR reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and gene-specific primers (Table 3) designed to produce amplicons shorter than 200 bp. Amplification was conducted on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following cycling conditions: 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Relative expressions were calculated based on Ct values, normalized to the ribosomal protein *L32* gene.

To evaluate *HP1-1* and *dsx1* expression in RNAi and overexpression experiments, two to three injected embryos were pooled per replicate. Total RNA extraction (Section 2.2.4) and cDNA synthesis (Section 2.2.5) were performed as previously described. RT-qPCR analysis was then conducted on the resulting cDNAs. Gel electrophoresis and melting curve analysis were used to confirm correct amplicon size and the absence of non-specific products.

Table 3 Primer sequences for RT-qPCR using in this study

Gene name	Primer sequence (5'- 3')
<i>L32</i>	Forward: GACCAAAGGGTATTGACAACAGA
	Reverse: CCAACTTTTGGCATAAGGTACTG
<i>HP1-1</i>	Forward: GGAAAGGCTATGACGACGAA
	Reverse: TCTTCAGTAGGTAGTGACCGTTT
<i>HP1-2</i>	Forward: GGATTGGCTATGGTGATGATG
	Reverse: CTTTTCTTTGGTTTCAGCTTGC
<i>HP1-3</i>	Forward: AGCAATCAAAGCGGGACAA
	Reverse: GGGAAAGGAATGGTAAAGTGGA
<i>HP1cd</i>	Forward: CCTGCTCCCATGTGTTTTCA
	Reverse: AGTCGCTCCACCAGAAGCA
<i>dsx1</i>	Forward: CCATTCATCATTACCAAATCCCTTC
	Reverse: AAGTTTGGTGTAGGGGAGGATGAG

2.2.7 Microinjection

Microinjection was performed according to the established protocol of Kato, Shiga, et al. (2011). Eggs were collected from 2–3-week-old *D. magna* individuals immediately after

ovulation and transferred to ice-chilled M4 medium (Elendt & Bias, 1990) supplemented with 80 mM sucrose (M4-sucrose).

A 2 mM solution of Lucifer Yellow dye (Invitrogen, Carlsbad, CA, USA) was added to the injection mixture (siRNA or plasmid) as a marker to confirm successful injection. Injections were carried out on ice. Surviving embryos were transferred to a 96-well plate containing 100 μ L of M4-sucrose and incubated at 23 °C.

2.2.8 Gene knockdown by RNAi

To knockdown the *HPI-1* gene, its coding sequence was retrieved from the *D. magna* genome assembly ASM2063170v1.1 (NCBI). Target sequences for siRNA design were identified using the Block-iT RNAi Designer tool (<https://rnaidesigner.thermofisher.com/rnaiexpress/>).

The siRNA sequences used in this study are listed in Table 4. Two dTdT overhangs were added to the 3' ends of each siRNA strand. The oligonucleotides were dissolved in DNase/RNase-free water (Life Technologies, Grand Island, NY, USA). A final concentration of 100 μ M *HPI-1*-targeting siRNA was used for microinjection.

Table 4 siRNA sequences used in this study

Name of siRNA	Sequence (5'-3')
siCtrl	CGGUCUCGCUGAAGUCGGUAAGAAA
siHP1-1a	GAAAGUCCUGCUGAGGAAGAGUACU
siHP1-1b	AAGAAACGGUCGAGUGGAAUAUCUU

2.2.9 Ectopic expression of *HPI-1* using plasmid

To obtain the coding sequence (CDS) of *HPI-1*, PCR was performed using KOD-plus DNA polymerase (Toyobo, Osaka, Japan) with cDNA derived from male-destined embryos of the *dsx1*-reporter strain at 18 hours post-ovulation as the template. The primers used are listed in Table 5, and PCR reaction components and conditions are detailed in Tables 6 and 7. The

expected amplicon size was 618 bp. The PCR product was purified and concentrated using the QIAGEN MinElute PCR Purification Kit (Qiagen, Germantown, MD, USA).

Table 5 Primers for *HP1-1* CDS amplification

Oligo	Sequence (5'-3')
HP1-1 CDS	Forward: ATGGGTCGAAGCACAAAAG
	Reverse: GTCCGTTCCATCTTTCTCCT

Table 6 Reaction mixture for PCR using KOD-plus polymerase

Reagent	Volume
10X KOD-Plus-Buffer	2.5 μ L
dNTP mix (2 mM)	2.5 μ L
25 mM MgSO ₄	1 μ L
5 μ M HP1-1 CDS Forward primer	1.5 μ L
5 μ M HP1-1 CDS Reverse primer	1.5 μ L
Genomic DNA (50 ng/ μ L)	2 μ L
MilliQ water	13.5 μ L
1 U/ μ L KOD-plus	0.5 μ L
Total	25 μL

Table 7 PCR cycle conditions

Temperature ($^{\circ}$ C)	Duration	Cycle no.
94	2 min	1
94	15 sec	25
55	30 sec	
68	45 sec	
68	7 min	1

To construct a plasmid for *HP1-1* expression under the control of the *D. magna* *EF1 α 1* promoter/enhancer, the *H2B* CDS in the pCS-EF1 α 1::H2B-GFP plasmid was replaced with *HP1-1-P2A* using GeneArt Seamless Cloning and Assembly Enzyme Mix (Invitrogen, Carlsbad, CA, USA), generating pCS-EF1 α 1::HP1-1-P2A-GFP.

A control plasmid was created by removing the *HP1-1-P2A* fragment from the expression plasmid using a PCR-based method (Hansson et al., 2008). Both plasmids were transformed into *E. coli* and purified using the PureYield™ Plasmid Miniprep System (Promega, WI, USA),

followed by ethanol precipitation and resuspension in DNase/RNase-free water (Life Technologies, Grand Island, NY, USA).

The plasmids (at concentrations of 50 or 100 ng/ μ L) were microinjected into male-destined embryos of the *dsx1*-reporter strain. Injected embryos were imaged at 30 hours post-injection to assess fluorescence intensity. Additionally, two to three embryos per group were collected at the same time point for total RNA extraction and cDNA synthesis as previously described.

2.2.10 Fluorescence photography and quantification

Samples were imaged using a Leica DC500 CCD digital camera mounted on a Leica M165FC fluorescence microscope (Leica Microsystems, Mannheim, Germany). Embryonic fluorescence imaging was performed using GFP and mCherry filters, with parameters listed in Table 8.

Table 8 Camera's parameters for fluorescence observation.

Filter	GFP	mCherry
Exposure	1.0s	2.0s
Gain	3.0x	8.0x
Saturation	1.0	1.0
Gamma	1.0	1.6

Fluorescence intensity of mCherry and GFP was quantified using ImageJ software following the protocol described by (Törner et al., 2014). Total fluorescence per embryo was normalized to background signal.

Relative Fluorescence Intensity (RFI) was calculated by dividing the total fluorescence of each injected embryo by that of an uninjected sibling from the same clutch, minimizing variability due to maternal autofluorescence. RFIs of treated embryos were then compared with

controls. A minimum of eight embryos per group (control and treated) were analyzed at each time point.

2.3 Results

2.3.1 Characterization of HP1 orthologs in *D. magna*

To identify potential HP1 orthologs in *D. magna*, the genome assembly ASM2063170v1.1 available from the NCBI database was queried. This analysis revealed four *HP1* genes with high sequence similarity to those of *D. melanogaster*. All four genes are composed of five exons (Figure 3) and were designated as *HP1-1* (located on linkage group [LG] 6), *HP1-2* and *HP1cd* (both on LG7), and *HP1-3* (on LG10) based on their chromosomal positions. Predicted amino acid sequences of these orthologs were aligned with HP1 proteins from *D. melanogaster* and *H. sapiens* to identify conserved domains. Three of the HP1 proteins—HP1-1, HP1-2, and HP1-3—possess the characteristic CHROMO (CHROMatin Organization MODifier) domain (CD) at the N-terminus and a chromoshadow domain (CSD) at the C-terminus (Figures 3 and 4). In contrast, HP1CD lacks CSD but contains three tandem CDs. The predicted protein lengths are comparable, comprising 206 amino acids for HP1-1, 221 for HP1-2, 207 for HP1-3, and 218 for HP1CD.

To analyze the evolutionary relationship of *D. magna* HP1, a phylogenetic tree with HP1 proteins from other organisms was constructed through the neighbor-joining method. The phylogenetic analysis revealed that the HP1s exhibit the closest relationship to HP1b in *D. melanogaster* (Figure 5). These results confirmed that the proteins found in *D. magna* genome are HP1 with high similarity to other organisms' HP1, specifically in their conserved domains.

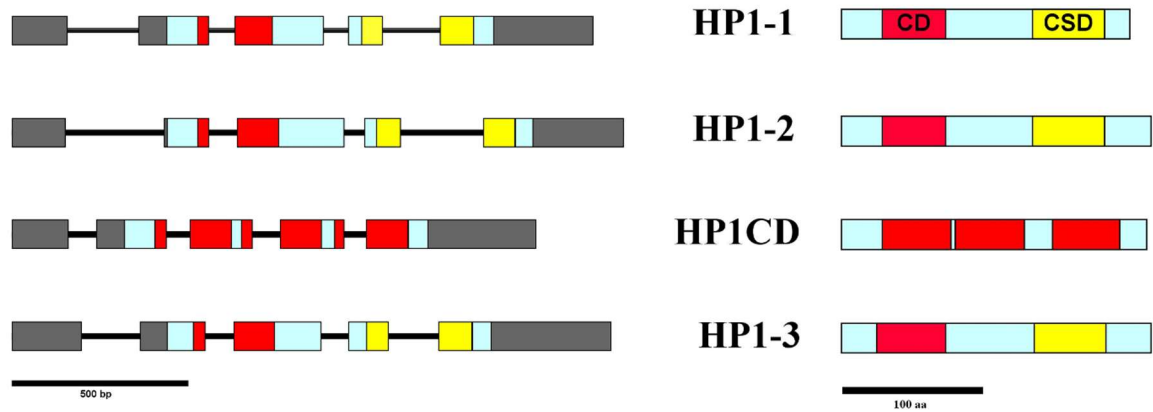


Figure 3 Genomic annotation of *HP1* genes in *D. magna*

The exons are shown in grey boxes and HP1 ORFs are shown in blue boxes. The chromodomain (CD) and chromoshadow domain (CSD) are shown in red and yellow boxes within ORF.

(A) Chromodomain

<i>D. magna</i>	HP1-1	30	EY SVEKVM DRRVRN - GRVEYLLKWKGY DDEDNTWEPEENLDCPALISEFEE	79
	HP1-2	30	EY EVEKVV DRREVN - GKVEYLLKWKGY GDDNTWEPVEALECHELIEEF EK	79
	HP1-3	24	EF SVEKVM DRRERN - GKVEYFLKWKGF GEEDNTWEP EENLDCPALIAEFEN	73
	HP1cd	30	LY TVEQIMDKRIVK - GKVQYLLKWKGY DHSQNTWESHENMECPDLIAAF EK	79
Fruit fly	HP1cd	89	YI VEKIMDKRVRK - GKVQYLLKWKGF DHSTNTWENEENMDCPGLISEFEN	138
	HP1cd	150	KY VVEKVV DKRRK - GAVQYLLKWKGY DESENTWEQAENMDCPDLISEFEN	199
	HP1a	23	EY AVEKIIDRRVRK - GKVEYLLKWKGY PETENTWEP ENLDCQDLIQQYEA	72
	HP1b	3	EF SVERVEDKRTVN - GRTEYLLKWKGY PRSENTWEP VENLDCPDLIANFEE	52
Human	HP1c	7	NF VVERIMDKRITSE GKVEYLLKWKGY TSADNTWEP EENCDCPNLIQKFEE	57
	HP1alpha	19	EY VVEKVLDRRVVK - QVVEYLLKWKGF SEEHNTWEP EKNLDCPELISEFMK	66
	HP1beta	20	EY VVEKVLDRRVVK - GKVEYLLKWKGF SDEDNTWEP EENLDCPDLIAEFLQ	69
	HP1gamma	29	EF VVEKVLDRRVVN - GKVEYFLKWKGF TDADNTWEP EENLDCPELIEAFLN	78

(B) Chromoshadow domain

<i>D. magna</i>	HP1-1	135	LD PEKIIIGATD - - SS GELMFLMKWKSS DEADLVPARQANSKCPQIVIQFY EERL	186
	HP1-2	152	QE PEVIIGATDNF HKGELAFVLKWKSS DTPEFVLSSRANVMWPQMVIKFY ESRL	205
	HP1-3	136	LE PEMIIGASN - - DTGELCFLMKWKSS DEADLVPA SQANRLCPQVVIQFY EERL	187
	HP1a	147	LE AEKILGASD - - NNGRLTFLIQFKGV DQAEMVPSSVAN EKIPRMV IHFY EERL	198
Fruit fly	HP1b	99	LE ASKILGATD - - SS GHLMFLMKWKGS DHADLVPAKLANTRC PQVVIQFY EERL	150
	HP1c	85	LE LAEIVGATD - - VTGDIKYLVRWQFCDEFDLVPSAQIV EKDPQMLIDYFQKMA	136
	HP1alpha	121	LE PEKIIIGATD - - SC GDLMFLMKWKD TDEADLVLAKEANVKCPQIVIAFY EERL	172
	HP1beta	117	LE PERIIGATD - - SS GELMFLMKWKNS DEADLVPAKEANVKCPQVVISFY EERL	168
Human	HP1gamma	121	LD PERIIGATD - - SS GELMFLMKWKDS DEADLVLAKEANMKCPQIVIAFY EERL	172

Figure 4 Multiple sequences alignment of the chromodomain and chromoshadow domain of HP1

Alignment of the (A) Chromodomain and (B) Chromoshadow domain of the different HP1 orthologs from *Drosophila melanogaster* (fruit fly) and *Homo sapiens*. The color is based on the physicochemical property of the amino acid-based on ClustalW.

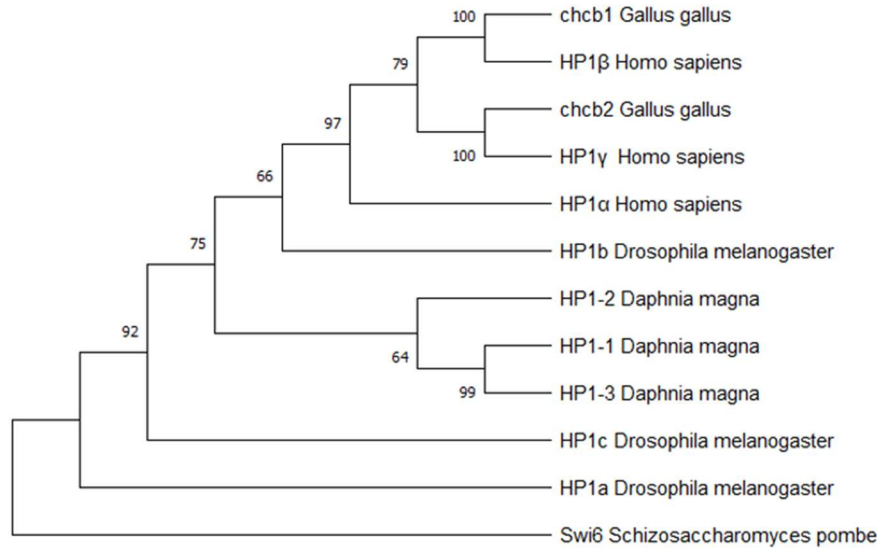


Figure 5 Phylogenetic tree of HP1

A phylogenetic tree of HP1 proteins. Most animal species have several HP1 isoforms, but those in *Drosophila* are not generally orthologous with particular vertebrate isoforms. The percentages of the replicate tree in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary distances were computed using the *p*-distance method.

2.3.2 Asexual dimorphic expression of *HP1* orthologs during embryogenesis

To investigate whether the expression of *HP1* orthologs exhibit sexual dimorphism comparable to *dsx1*, quantitative real-time PCR (qRT-PCR) was performed to assess their temporal expression during embryonic development. All four *HP1* genes were expressed in both male and female embryos, with no detectable differences in expression levels between sexes, indicating a lack of sexual dimorphism (Figure 6). Among them, *HP1-1* exhibited the highest expression levels throughout development, with a peak immediately after ovulation (0 hours post-ovulation, 0 hpo), followed by a gradual decline over time. *HP1-3* showed elevated expression during early embryogenesis (0 and 12 hpo) with a decreasing trend in later stages. In contrast, the transcript levels of *HP1-2* and adjacent gene *HP1cd* were at the highest at 0 hpo, followed by a drastic decrease to consistently low levels. The results of this study suggest that *HP1-1* is predominantly expressed among HP1 genes during embryogenesis. Consequently, *HP1-1* were prioritized for further functional analysis.

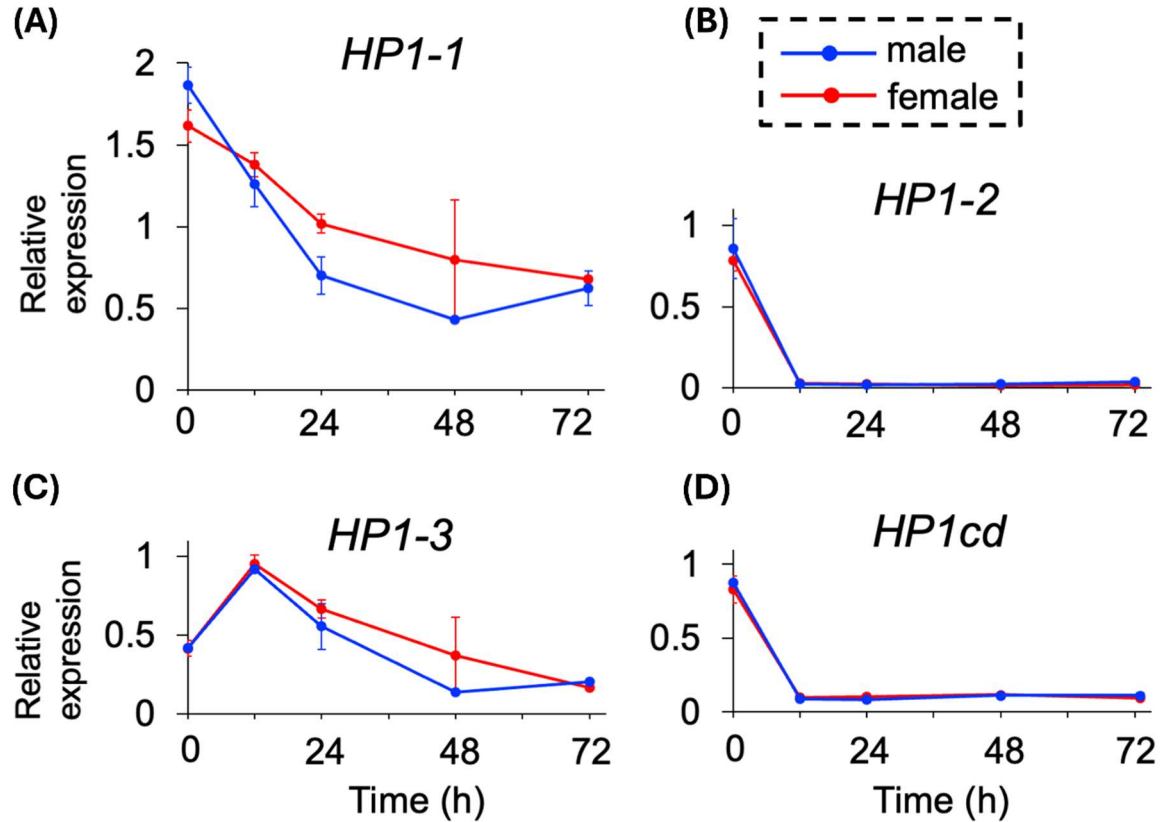


Figure 6 Temporal expression profile of *HPI* orthologs in embryonic developmental stages of *D. magna*.

The expression levels of (A) *HPI-1*, (B) *HPI-2*, (C) *HPI-3* and (D) *HP1cd* in female (red) and male (blue) embryos were determined at 0, 12, 24, 48, and 72 hours post-ovulation (hpo). Results are shown as relative expression normalized with the ribosomal protein *L32*. Error bars indicate the standard error of the mean (n = 3).

2.3.3 *HP1-1* silencing derepresses the expression of *dsx1* in female embryos

To further elucidate the role of *HP1-1* in environmental sex determination (ESD), RNA interference (RNAi) was employed to deplete *HP1-1* expression during embryogenesis. To evaluate the effect of *HP1-1* knockdown on *dsx1* expression, a *dsx1*-reporter transgenic line was utilized. In this strain, *mCherry* gene is expressed under the control of the *dsx1* promoter/enhancer in one allele, enabling direct visualization of *dsx1* expression. Additionally, this strain expresses nucleus-localized eGFP, fused to histone H2B and driven by the *elongation factor 1a1* (*EF1a1*) promoter/enhancer, facilitating precise observation of nuclear morphology and accurate staging of embryonic development.

Gene encoding *Maltose-binding protein (MalE)* in *E. coli* was used for designed negative control siRNA (siCtrl). Injection of *HP1-1*-targeting siRNA (siHP1-1a and siHP1-1b, 100 μ M) into female-destined embryos resulted in severe developmental defects. Most injected individuals became immobile during the juvenile stage and died before completing gonadal development (Table 8), preventing evaluation of adult sexual differentiation. To capture earlier developmental effects, embryos were examined at 48 h post-injection (stage 12), a time point when sexually dimorphic elongation of the first antenna becomes clearly detectable if *dsx1* is derepressed. Moreover, Dsx1-mCherry expression is robust and spatially localized at this stage, enabling reliable assessment of both morphological and molecular phenotypes. At this stage, siHP1-1 injected embryos exhibited widespread mCherry fluorescence, including in male-associated structures such as the first antenna (An1) and thoracic appendages (T1), whereas control females injected with siCtrl showed no such signal (Figure 7A). By the juvenile stage (72 h post-injection), siHP1-1 injected embryos displayed elongation of the first antenna and strong mCherry expression (Figure 7B). Taken together, these results indicate that *HP1-1* knockdown derepresses *dsx1* expression and induces male-like traits at embryonic and juvenile stages, although confirmation of full masculinization at the adult level was not possible due to lethality.

The relative fluorescence intensity (RFI) of mCherry significantly increased in siHP1-1a injected female embryos compared to siCtrl, indicating derepression of *dsx1* (Figure 8A). Quantitative RT-PCR analysis of *HP1-1* confirmed that *HP1-1* knockdown was successful by reduction the transcript level of *HP1-1* to 9.3% and 7.4% compared to siCtrl embryos in HP1-1a and HP1-1b siRNA injected samples, respectively (Figure 8B). Expression of *dsx1* was upregulated following *HP1-1* knockdown. siHP1-1a-injected embryos showed 15-folds increase (SE = 1.58, 95% CI [8.27, 21.82], n = 3), and siHP1-1b-injected embryos showed 17.4-folds increase (SE = 1.75, 95% CI [9.87, 24.94], n = 3), compared to siCtrl (1.0 ± 0.2 ,

95% CI [0.18, 1.9], $n = 3$) (Figure 8B). Both siRNAs induced statistically significant upregulation of *dsx1* relative to controls ($p < 0.05$), and together these consistent results support a role for HP1-1 in female-specific repression.

To confirm the sex-specific function of *HP1-1* on *dsx1* expression, *HP1-1* knockdown was performed in male embryos in the same manner. A depletion of HP1-1 in male embryos caused immobilized juveniles, led to lethality before adult stage (Table 9). To capture earlier developmental effects, embryos were examined at 24 h post-injection (stage 7), a time point when sexually dimorphic elongation of the first antenna becomes clearly detectable. mCherry fluorescence in HP1-1 targeting siRNA-injected male embryos did not differ from siCtrl-injected male embryos (Figure 9). Even though the expression of *HP1-1* significantly reduced in siHP1-1a and siHP1-1b-injected male embryos compared to siCtrl male embryos, *dsx1* transcript level did not affect (Figure 10). Taken together, these findings suggest that HP1-1's repression of *dsx1* expression in females requires a female chromatin context.

Table 8 Effects of *HP1-1* RNAi on *dsx1*-reporter female embryos of the strain

siRNA	Injected	Hatched	Immobile juvenile	Increase of mCherry fluorescence
siCtrl	9	9	0	0% (0/8)
siHP1-1a	23	22	18	100% (18/18)
siHP1-1b	10	10	6	100% (6/6)

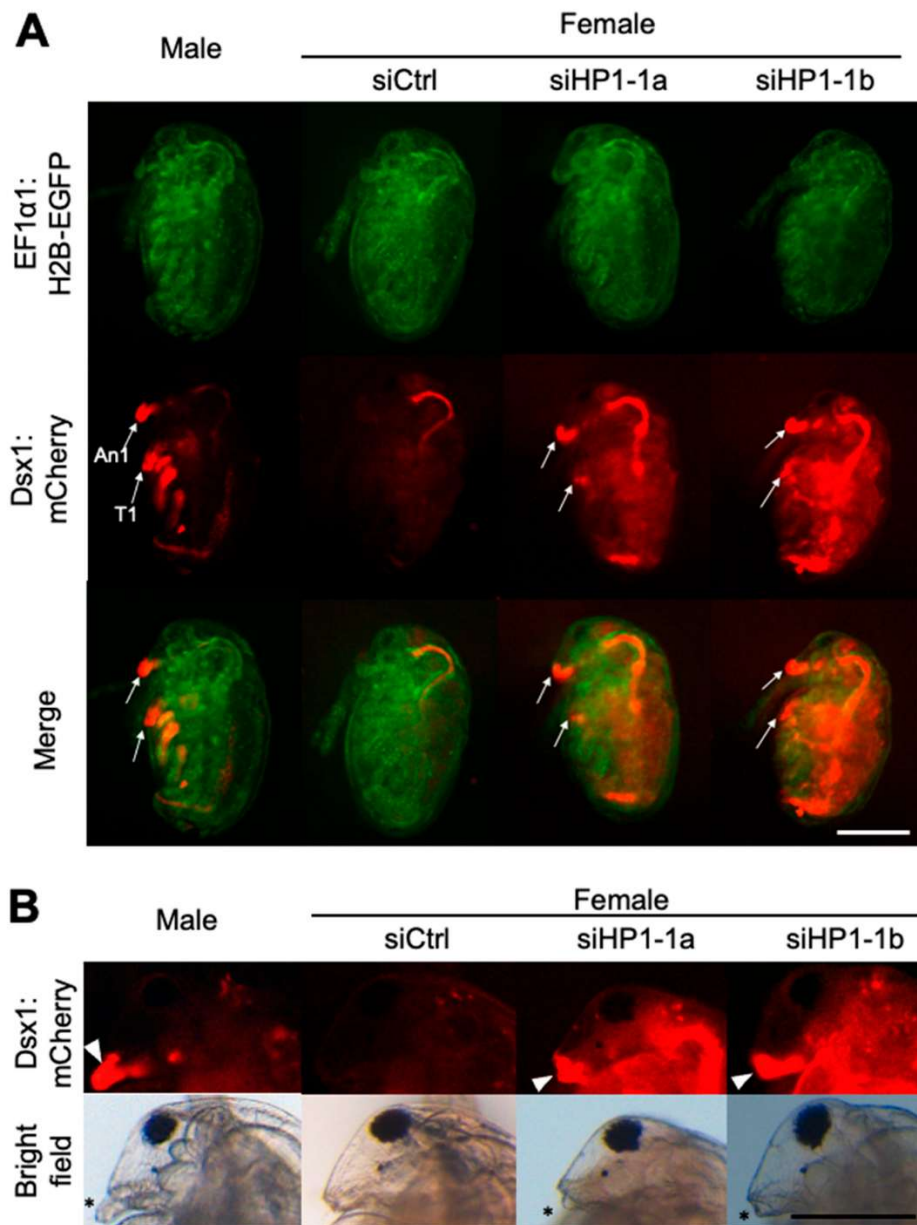


Figure 7 Phenotypes of *HP1-1* RNAi females.

(A) Phenotypes of female embryos injected with *HP1-1* siRNAs. Embryos from the *dsx1*-reporter line were injected with each either control siRNA (siCtrl) or *HP1-1* siRNAs (siHP1-1a, siHP1-1b) just after ovulation and observed at stage 12 (48 h after injection). Fluorescence from EF1 α 1:H2B-EGFP (green) and Dsx1:mCherry (red) is shown. An1: first antenna, T1: first thoracic appendage. The scale bar represents 200 μ m. (B) Phenotypes of female juveniles injected with *HP1-1* siRNAs. The white arrowhead indicates the localization of mCherry at the base of the first antenna. The asterisks mark the position of the first antenna. The scale bar represents 200 μ m.

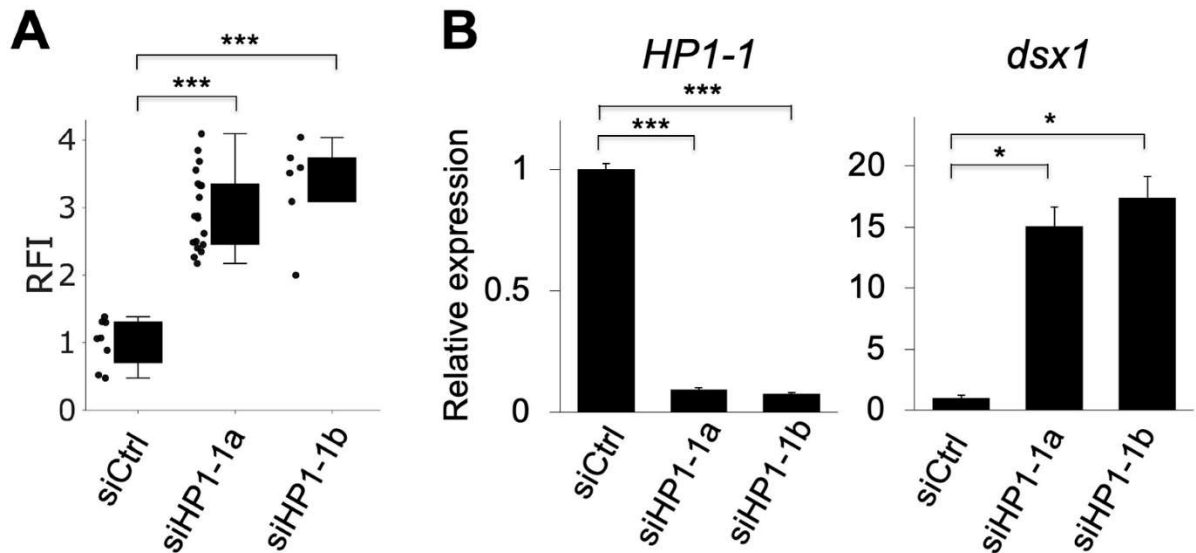


Figure 8 Quantitative analysis of mCherry fluorescence and gene expression following *HP1-1* knockdown in females.

(A) Relative fluorescence intensity (RFI) of mCherry in *dsx1*-reporter female embryos injected with control siRNA (siCtrl), *HP1-1* siRNA-1 (siHP1-1a), or *HP1-1* siRNA-2 (siHP1-1b). Each dot represents one embryo. *** $p < 0.001$ (t -test) (B) Quantitative RT-PCR analysis of *HP1-1* (left) and *dsx1* (right) in embryos injected with siCtrl, siHP1-1a or siHP1-1b. Expression levels were normalized to *ribosomal protein L32*. Error bars indicate S.E.M ($n = 3$). * $p < 0.05$, *** $p < 0.001$ (Student's t -test).

Table 9 Effects of *HP1-1* RNAi on male embryos of the *dsx1*-reporter strain

siRNA	Injected	Hatched	Immobile juvenile	Increase of mCherry fluorescence
siCtrl	7	7	0	0% (0/7)
siHP1-1a	13	13	0	0% (0/13)
siHP1-1b	22	11	0	0% (0/11)

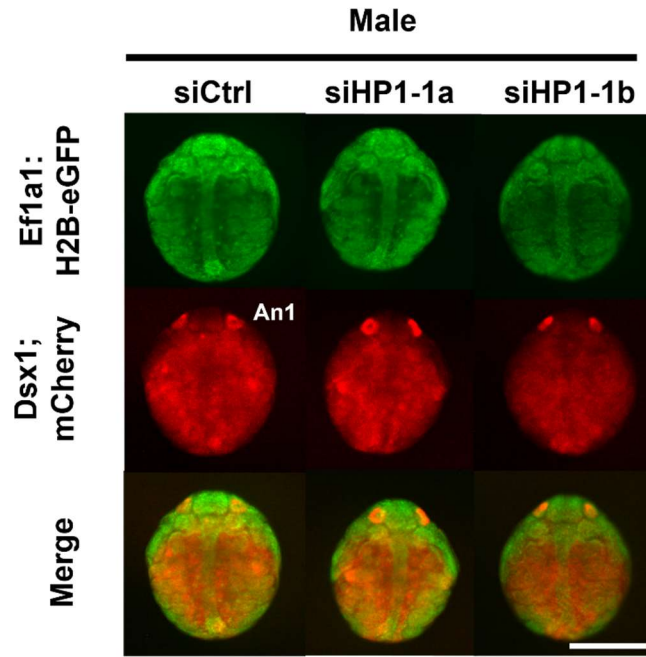


Figure 9 Phenotypes of *HP1-1* RNAi males.

Phenotypes of male embryos injected with *HP1-1* siRNAs. Embryos from the *dsx1*-reporter line were injected with each either control siRNA (siCtrl) or *HP1-1* siRNAs (siHP1-1a, siHP1-1b) just after ovulation and observed at stage 7 (24 h after injection). Fluorescence from EF1 α 1:H2B-EGFP (green) and Dsx1:mCherry (red) is shown. An1: first antenna. The scale bar represents 200 μ m.

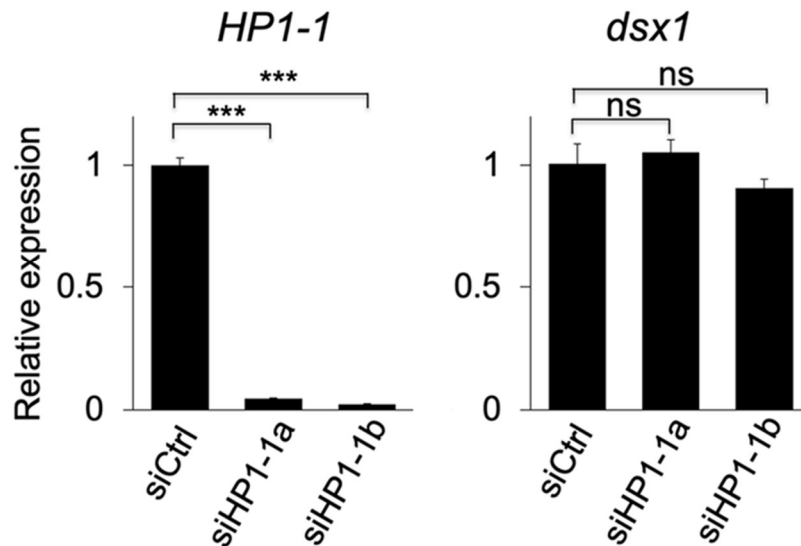


Figure 10 Gene expression following *HP1-1* knockdown in males.

Gene expression profiles of *HP1-1* and *dsx1* in siCtrl and siHP1-1 injected in male embryos. RT-qPCR results are shown as expression levels normalized with *ribosomal protein L32* gene. Error bars indicate S.E.M (n = 3). *** $p < 0.001$, ns: not significant (Student's t -test).

2.3.4 Ectopic expression of *HP1-1* in male embryos is insufficient to repress the expression of *dsx1*

As the findings suggested that the reduction of *HP1-1* in female embryos enhanced the expression of *dsx1*, embryos were microinjected with a plasmid construct expressing HP1-1 under the *Ef1a1* promoter to test whether HP1-1 is sufficient to repress *dsx1* in males. *HP1-1* expressing plasmid harboring *HP1-1* CDS fused with a self-cleaving P2A sequence and eGFP under *Ef1a1* promoter/enhancer (pHP1) was constructed and introduced into destined to be male *dsx1*-reporter embryos. Plasmid harboring eGFP (pGFP) was used as a negative control in this experiment. The phenotypes of injected embryos were observed at 30 h after injection (stage 7) when *dsx1* shows significant sexually dimorphic expression (Kato, Kobayashi, et al., 2011), and because plasmid-driven HP1-1 expression is transient, making early observation critical for detecting regulatory effects.

Injection of the HP1-1 expression plasmid into male embryos affected viability in a dose-dependent manner. At the lower concentration (50 ng/μl), embryos survived to later developmental stages without obvious morphological defects and could develop into swimming juveniles (Table 10, Figure 12). By contrast, a higher concentration (100 ng/μl) caused early embryonic lethality, preventing further assessment of *dsx1* regulation (Table 10, Figure 13). To evaluate the regulatory effect of HP1-1 under viable conditions, embryos injected with 50 ng/μl plasmid were analyzed at 30 h, the developmental stage when sex-specific differences in *dsx1* expression become apparent. At this concentration, *HP1-1*-overexpressing embryos did not show a significant reduction in mCherry fluorescence compared to *GFP* plasmid-injected controls (Figures 11A–B). qPCR confirmed elevated *HP1-1* transcript levels (Figure 11C), and importantly, the plasmid's internal GFP reporter also increased, verifying that the construct was successfully expressed. Despite this, *dsx1* transcript

levels remained unchanged (Figure 11C). These results indicate that *HP1-1* overexpression alone is insufficient to repress *dsx1* in males under the tested conditions.

Table 10 Effects of *HP1-1* overexpression on male embryos of the *dsx1*-reporter strain

Plasmid	Conc. (ng/μl)	Injected	Hatched	Swimming juvenile
pGFP	50	10	10	5
pHP1		7	6	6
pGFP	100	7	7	6
pHP1		19	0	0

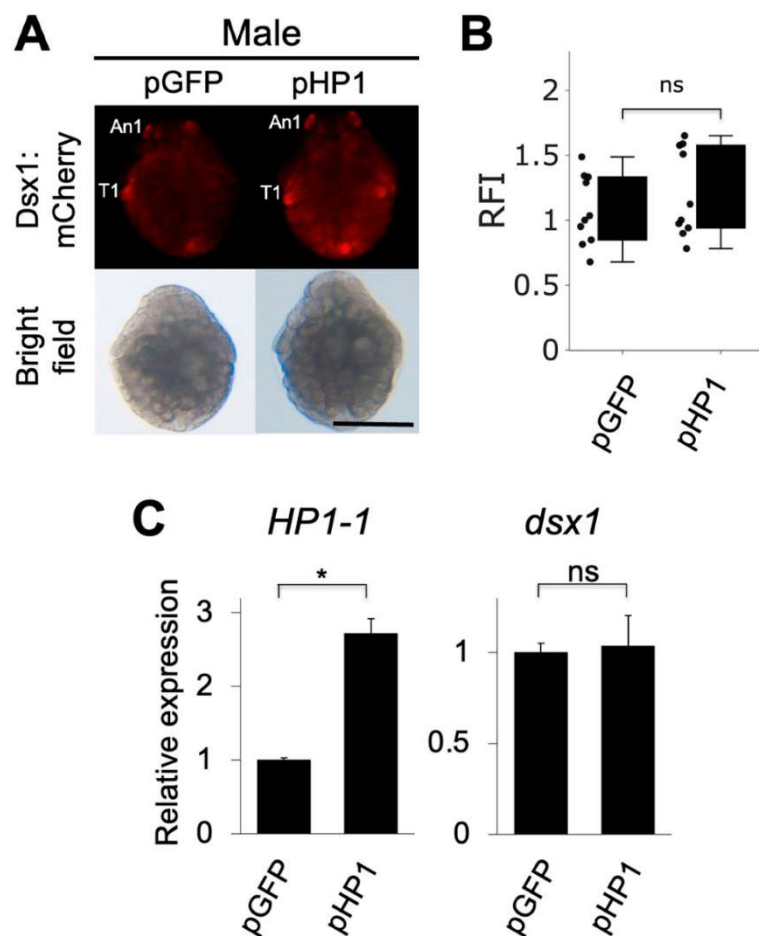


Figure 11 Phenotypes and gene expression of males injected with *HP1-1* expression plasmids

(A) Phenotypes of male embryos injected with *HP1-1* expression plasmid (pHP1). The *GFP* expression plasmid (pGFP) was used as a control. Embryos from the *dsx1*-reporter line were injected just after

ovulation and observed at stage 7 (30 h after injection). The scale bar represents 200 μm . **(B)** Relative fluorescence intensity (RFI) of pHP1-injected embryos, normalized to pGFP-injected controls. Each dot represents one embryo. **(C)** Quantitative RT-PCR analysis of *HP1-1* (left) and *dsx1* (right) transcript levels in embryos injected with each plasmid. RT-qPCR results are shown as expression levels relative to pGFP embryos. Error bars indicate S.E.M (n = 3). * $p < 0.05$, ns: not significant (Student's *t*-test).

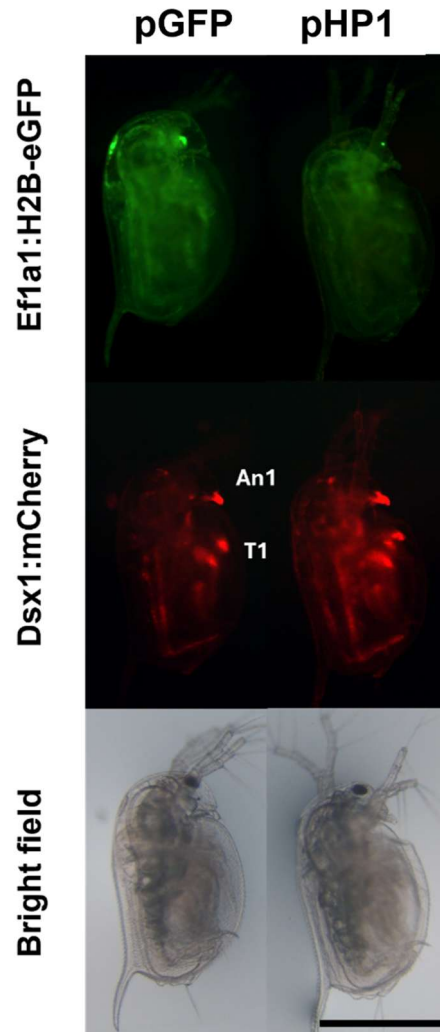


Figure 12 Overexpression of *HP1-1* in male *dsx1*-reporter embryos at a total concentration of 50 ng/ μL

(A) Phenotypes of male juveniles of *dsx1*-reporter strain injected with a total concentration of 50 ng/ μL of either pGFP as control or pHP1 observed at first instar (72 h after injection). An1: first antennae, T1: first thoracic appendage. Bar indicates 200 μm .

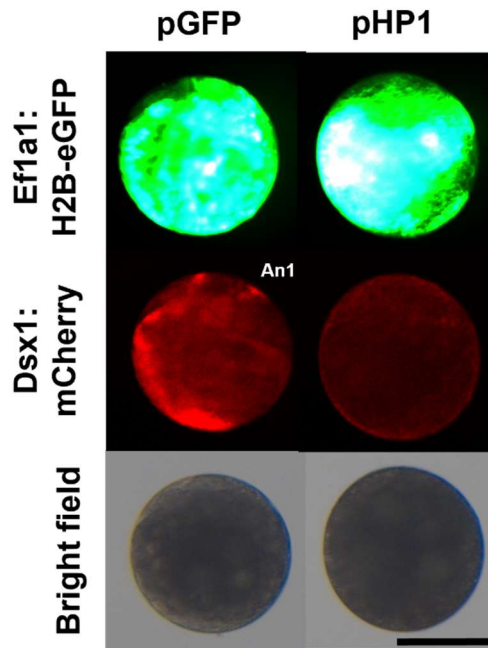


Figure 13 Overexpression of *HP1-1* in male *dsx1*-reporter embryos at a total concentration of 100 ng/ μ L

Phenotypes of male *dsx1*-reporter embryos injected with a total concentration of 100 ng/ μ L of either pGFP as control or pHP1 observed at stage 7 (30 h after injection). An1: first antennae. The scale bar indicates 200 μ m.

2.4 Discussion

In *D. magna*, male production under environmental sex determination (ESD) is triggered by activation of *dsx1*, a DM-domain transcription factor essential for male development (Kato, Kobayashi, et al., 2011). Previous work showed that mutations upstream of *dsx1*, within the Vrilite binding site, could not be recovered in female-producing conditions, suggesting that the *dsx1* locus is protected from Cas9 accessibility in females (Mohamad Ishak et al., 2017). Heterochromatin Protein 1 (HP1) is a well-characterized chromatin-associated factor that promotes heterochromatin formation and transcriptional silencing and has been implicated in sex-specific gene regulation in diverse organisms (Li et al., 2011; Liu et al., 2005; Smolko et al., 2018; Studencka et al., 2012). These findings led us to hypothesize that HP1 plays a role in repressing *dsx1* in females, maintaining the locus in a transcriptionally inactive heterochromatic configuration and thereby preventing male development in the absence of environmental cues. To test this hypothesis, the present study examined the functional role of

HP1 in the *D. magna* sex determination pathway, with a particular focus on its potential involvement in the transcriptional regulation of *dsx1*.

Heterochromatin Protein 1 (HP1) is an evolutionarily conserved protein found across a wide range of eukaryotic organisms. Homologs of HP1 have been identified in species ranging from fission yeast (*Swi6* in *Schizosaccharomyces pombe*) to humans (CBX1, CBX3, and CBX5) (Lomber et al., 2006; Lorentz et al., 1994). Members of the HP1 family are characterized by two conserved domains: the chromodomain (CD) at the N-terminus and the chromoshadow domain (CSD) at the C-terminus, separated by a less conserved hinge region. The CD mediates the recognition and binding of HP1 to H3K9me2/3, a well-established epigenetic mark associated with gene silencing (Fischle et al., 2003). In contrast, the CSD is responsible for facilitating homo- and hetero-dimerization, enabling interactions with other chromatin-associated proteins (Meyer-Nava et al., 2020; Smothers & Henikoff, 2000). In *D. magna*, four HP1 orthologs were identified in this study: HP1-1, HP1-2, HP1-3, and HP1CD. With the exception of HP1CD, all contain the two conserved domains and are closely related to HP1b in *D. melanogaster*. These structural features suggest that the HP1 orthologs in *D. magna* are functionally capable of recognizing methylated histones and forming dimers, supporting their putative roles in heterochromatin formation and transcriptional silencing.

Interestingly, HP1CD, which contains three tandem chromodomains and lacks a CSD, represents a novel structure not previously reported in any other organism. However, a predicted protein with a similar structure can be found in available genome databases of related *Daphnia* species (*D. carinata*, *D. sirensis*, *D. pulicaria*, *D. pulex*) as well as in the crustacean *Meganyctiphanes norvegica* (Northern krill), suggesting a potentially conserved function in specific lineages.

Among the four identified *HP1* orthologs, *HP1-1* was selected for further analysis due to its consistently highest expression levels throughout embryogenesis. The *HP1-1* transcript

exhibited peak expression at 0 hours post-ovulation (hpo), followed by a marked decline to its lowest level at 12 hpo, and subsequently increased and remained stable after 24 hpo. This temporal pattern suggests that *HP1-1* is initially provided as maternally deposited mRNA, with zygotic transcription occurring at later stages of development. The expression of *HP1-1* exhibited no evidence of sexual dimorphism, a pattern consistent with findings in other model organisms such as *C. elegans*, *D. melanogaster*, and mice, where HP1 family proteins are similarly expressed in a sex-independent manner despite participating in sex-specific functions (Brown et al., 2010; Liu et al., 2005; Studencka et al., 2012). This suggests that sex-specific function of *HP1-1* in *D. magna* is not governed by its expression level but rather by its chromatin localization, interaction with other regulatory proteins, or its recruitment to specific loci.

The *doublesex1* (*dsx1*) gene plays a central role in the sex determination pathway of *D. magna* (Kato, Kobayashi, et al., 2011). Its expression is male-specific, initiated by the transcription factor *Vrille* and maintained throughout the male's lifespan (Mohamad Ishak et al., 2017). In contrast, *dsx1* must be actively repressed in females to prevent inappropriate activation of the male developmental program. Results from the present study demonstrate that *HP1-1* contributes to the transcriptional silencing of *dsx1* in females, as RNAi-mediated knockdown of *HP1-1* leads to *dsx1* derepression and induces male-like traits at embryonic and juvenile stages, suggesting a role for *HP1-1* in establishing or maintaining a heritable heterochromatic state at the *dsx1* locus. Notably, *HP1-1* knockdown in females showed a phenocopy with *DAPARL* and *Vrille* overexpression in females (Kato et al., 2018; Mohamad Ishak et al., 2017).

In contrast, overexpression of *HP1-1* in males did not measurably affect *dsx1* expression under the conditions tested. This differs from the knockdown experiments, which showed clear derepression of *dsx1* in females, suggesting that while *HP1-1* is required for repression in

females, it may not be sufficient on its own to silence *dsx1* in males. Several factors could explain this discrepancy. First, plasmid-driven expression may not have achieved sufficiently high protein levels, as higher plasmid concentrations caused early lethality and prevented evaluation of *dsx1* regulation. Second, repression by HP1-1 may depend on specific histone modifications, such as H3K9me3, which may be absent or insufficiently enriched at the actively transcribed *dsx1* locus in males. In *D. pulex*, for example, the activating mark H3K4me3 is enriched at *dsx1* locus in males (Kvist et al., 2020). Although a previous study in *D. melanogaster* reported that HP1 can be recruited to promoters independently of H3K9me3, particularly to regions on chromosome 4 (Figueiredo et al., 2012), this alternative recruitment mechanism likely does not appear to occur at the *dsx1* locus in *D. magna*. To clarify these possibilities, future experiments such as ChIP assays for H3K9me3 enrichment and HP1-1 binding at the *dsx1* locus will be necessary to directly test whether HP1-1 has the capacity to repress *dsx1* in males.

Our findings are consistent with a model in which HP1-1 and SHEP act in parallel to repress *dsx1* in females. In this framework, HP1-1 may establish a repressive chromatin environment at the *dsx1* locus, while SHEP inhibits translation of *dsx1* mRNA, together preventing inappropriate activation of the male developmental program. Support for this model comes from the observation that *Shep* knockdown phenocopies *HP1-1* knockdown, leading to derepression of Dsx1 in females as evidenced by enhanced mCherry fluorescence in *dsx1*-reporter transgenic *Daphnia* (Perez et al., 2021). In males, these two repressive layers are relieved: Vril1 activates transcription, and the lncRNA *DAPALR* antagonizes SHEP-mediated translational inhibition, thereby enabling Dsx1 protein synthesis (Mohamad Ishak et al., 2017; Perez et al., 2021).

While this dual inhibition model provides a useful conceptual framework, it should be regarded as hypothetical. The present study does not directly demonstrate HP1-1 binding to the

dsx1 locus or identify the specific histone modifications mediating its recruitment. Similarly, no direct evidence has yet confirmed a functional interaction between HP1-1 and SHEP. Future experiments, such as chromatin immunoprecipitation (ChIP) to assess HP1-1 occupancy and histone modifications at *dsx1*, as well as assays probing HP1-1–SHEP interactions, will be necessary to rigorously test this proposed mechanism.

CHAPTER 3

A depletion of Pc has no effect on *dsx1* expression

3.1 Introduction

Daphnia magna has been widely adopted in ecological, toxicological, and developmental studies due to its ability to switch between parthenogenetic and sexual reproduction. In favorable environments, *D. magna* reproduces asexually to produce genetically identical female offspring. In contrast, under stress conditions or following exposure to juvenile hormone analogs, genetically identical male offspring are produced. This reproductive strategy enables the use of clonal individuals to investigate environmentally induced biological processes, including sex determination, while eliminating genetic variation as a confounding factor.

Sex determination in *D. magna* is driven by the DM-domain transcription factor *doublesex1* (*dsx1*) gene, which is essential for male development. Precise regulation of *dsx1* ensures appropriate sexual differentiation: it is transcriptionally activated in males by the transcription factor Vril1, while post-transcriptional repression by the RNA-binding protein SHEP and the lncRNA *DAPALR* prevents its translation in females. Epigenetic mechanisms may also contribute to sex-specific *dsx1* expression. Previous studies in *D. pulex* have shown enrichment of the repressive histone modification H3K27me3 at the *dsx1* locus in females, consistent with a silenced chromatin state (Kvist et al., 2020). Moreover, targeted enhancer mutagenesis at the *dsx1* locus was successful in male but not female embryos, further supporting the presence of chromatin-level repression in females (Mohamad Ishak et al., 2017).

While the previous chapter demonstrated that HP1-1 plays a key role in transcriptional repression of *dsx1* in females, the involvement of other chromatin regulators such as Polycomb (Pc) proteins remains unexplored in this context. Pc proteins are known readers of H3K27me3 and are central components of Polycomb Repressive Complexes (PRCs) that mediate gene

silencing in various organisms. In mammals, Pc homologs are implicated in sex differentiation through repression of alternative sexual pathways (Garcia-Moreno et al., 2019). However, whether Pc contributes to sex-specific gene repression in environmentally sex-determined species such as *Daphnia* is still unclear.

This chapter investigates the role of Pc in the transcriptional regulation of *dsx1* in *D. magna*. Specifically, it examines the genomic structure and evolutionary conservation of the Pc ortholog, its temporal expression pattern during embryogenesis, and the effect of *Pc* knockdown on *dsx1* expression using a *dsx1*-reporter strain.

3.2 Materials and methods

3.2.1 *Daphnia* strains and culturing conditions

The *D. magna* (NIES clone), obtained from the National Institute for Environmental Studies (NIES; Tsukuba, Japan), and the transgenic *dsx1*-reporter strain were cultured under the same laboratory condition mentioned in Chapter 2 (see 2.2.1 *Daphnia* strains and culturing conditions). The male daphniids were obtained with the same method described in Chapter 2 (see 2.2.2 Artificial male production by Fenoxycarb exposure).

3.2.2 Identification of Pc in *D. magna*

The genomic location of the Polycomb (Pc) ortholog in *D. magna* was identified using a tblastn search against the *Daphnia magna* genome assembly (ASM2063170v1.1) available in the NCBI database. The amino acid sequence of the *Drosophila melanogaster* Pc protein (accession number: NP_524199.1) was used as a query.

To verify orthology, multiple sequence alignment and phylogenetic analyses were performed using Pc homologs from a range of invertebrate and vertebrate species. Accession numbers and species information are listed in Table 11. Sequence alignments were performed using ClustalW with standard parameters (gap opening penalty: 6.00 for pairwise; 10.00 for multiple alignment; gap extension penalty: 0.21 and 0.24, respectively). The phylogenetic tree

was constructed using the neighbor-joining method and *p*-distance model, as implemented in MEGA X (Kumar et al., 2018), and rooted using the *Arabidopsis* LHP1 protein as an outgroup.

Table 11 Accession number of proteins used for Pc multiple sequence alignment

Common name	Scientific name	Gene name (Definition in NCBI)	Accession number
Water flees	<i>D. magna</i>	chromo protein homolog 2	XP_045033348.1
	<i>D. pulex</i>	chromobox protein homolog 8-like	XP_046449614.1
Silk moth	<i>B. mori</i>	chromodomain protein polycomb	NP_001188363.1
Fruit fly	<i>D. melanogaster</i>	polycomb	NP_524199.1
Red flour beetle	<i>T. castaneum</i>	polycomb	EFA10731.1
Zebrafish	<i>D. rerio</i>	Cbx2 protein	AAI64860.1
Chicken	<i>G. gallus</i>	chromobox protein (CHCB3)	BAA33402.1
Mouse	<i>M. musculus</i>	chromobox protein homolog 2	NP_031649.2
Human	<i>H. sapiens</i>	chromobox protein homolog 2 isoform 1	NP_005180.1
Thale cress	<i>A. thaliana</i>	like heterochromatin protein (LHP1)	ANM68248.1

3.2.3 Total RNA extraction, cDNA synthesis and RT-qPCR

Total RNA was extracted from male and female embryos at different developmental time points (0, 12, 24, 48, and 72 hours post-ovulation) following the same procedure as described in Chapter 2 (Section 2.2.4. Reverse transcription was conducted using PrimeScript III Reverse Transcriptase with random primers, as outlined in Section 2.2.5. RNA quality was confirmed via spectrophotometry, and cDNA was synthesized for subsequent RT-qPCR analysis of *Pc* expression levels. The RT-qPCR was performed using the primers in Table 12, the same reagents, conditions, and normalization strategy (*L32* as reference gene) as described in Chapter 2, Section 2.2.6.

Table 12 Primer sequences for RT-qPCR using in this study

Gene name	Primer sequence (5'- 3')
<i>L32</i>	Forward: GACCAAAGGGTATTGACAACAGA
	Reverse: CCAACTTTTGGCATAAGGTACTG
<i>Pc</i>	Forward: CAGCCAACCAACAATACACCA
	Reverse: CTCCAGGTAAAGCAGGTGAAAGA
<i>dsx1</i>	Forward: CCATTCATCATTACCAAATCCCTTC
	Reverse: AAGTTTGGTGTAGGGGAGGATGAG

3.2.4 Gene knockdown by RNAi

To examine the function of *Pc* in *D. magna*, RNA interference (RNAi) was used to knock down *Pc* expression, following the same overall strategy as described for *HPI-1* in Chapter 2, Section 2.2.8. Target siRNA sequences were designed using the Block-iT RNAi Designer tool (Thermo Fisher Scientific) based on the identified *Pc* coding region. The specific sequences are listed in Table 13. Two dTdT overhangs were added at the 3' ends of each strand to enhance stability.

The siRNAs were dissolved in DNase/RNase-free water (Life Technology Inc., Grand Island, NY, USA) and injected into embryos of the *dsx1*-reporter strain, following the same microinjection protocol as described in Chapter 2, Section 2.2.7. Each injection solution contained 2 mM Lucifer Yellow as a fluorescent tracer. Injected embryos were kept at 23 °C and monitored for survival and developmental progression. Embryos were collected at 18 hours post-injection for downstream analyses, including fluorescence observation and RNA quantification.

Table 13 siRNA sequences used in this study

Name of siRNA	Sequence (5'-3')
siCtrl	CGGUCUCGCUGAAGUCGGUAAGAAA
siPc1	GGGCGUCUUCUAGAAGCCUUCGAAA
siPc2	GCUAAGUACCUCAUCGUCUUCUGAA

3.2.5 Fluorescence photography and quantification

Samples were analyzed and photographed with a Leica DC500 CCD Digital Camera attached to a Leica M165FC fluorescence microscope (Leica Microsystems, Mannheim, Germany) as described in Chapter 2 (see 2.2.10 Fluorescence photography and quantification).

3.3 Results

3.3.1 Characterization of Pc in *D. magna*

To examine the potential presence of *Pc* ortholog in *D. magna*, the *D. magna* assembly ASM2063170v1.1, available on the NCBI database, was searched. Through this search, one *Pc* exhibiting high similarity to *D. melanogaster* was identified. Putative *Pc* in *D. magna* is encoded by gene containing 4 exons into 507 amino acids located on linkage group (LG) 7 (Figure 14). Predicted *Pc* was aligned with *Pc* proteins from several species to identify conserved domains. *Pc* in *D. magna* consists of two conserved domains: the CHROMatin Organization MOdifier Domain (CHROMOdomain; CD) at the N-terminus and the Polycomb Repressive box (PcR-box) at the C-terminus (Figure 14 and 15). To analyze the evolutionary relationship of *D. magna* *Pc*, a phylogenetic tree with other proteins was constructed through the neighbor-joining method. The phylogenetic tree revealed that *Daphnia* *Pc* ortholog is most closely related to the insects' *Pc* ortholog (Figure 16). These results confirmed that the proteins found in *D. magna* genome are *Pc* with high similarity to other organisms *Pc*, specifically in their conserved domains.

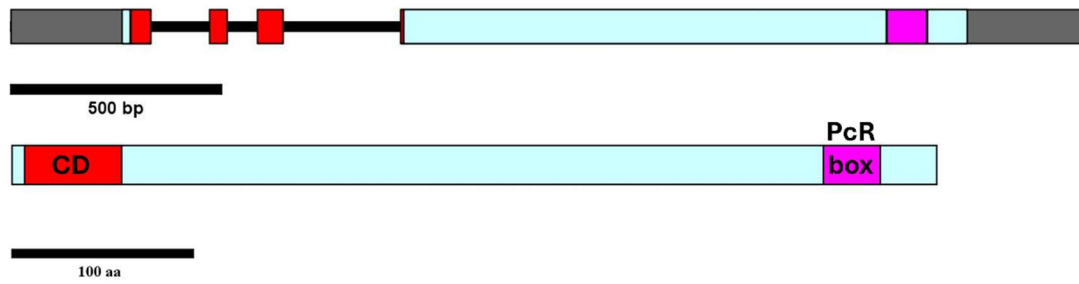


Figure 14 Genomic annotation of Pc gene in *D. magna*

The exons are shown in grey boxes and Pc ORFs are shown in blue boxes. The chromodomain (CD) and Polycomb repressive box (PcR-box) are shown in red and magenta boxes within ORF.

(A) Chromodomain

<i>D. magna</i> Pc	8	D	R	V	F	A	A	E	R	I	L	K	K	R	I	R	G	H	P	E	Y	L	V	K	W	K	G	W	S	T	K	H	N	T	W	E	P	E	E	N	I	L	D	G	R	L	L	E	A	F	E	R	R	E	R	E	-	-	A	A	T	66	
<i>D. pulex</i> Pc	8	D	R	V	F	A	A	E	R	I	L	K	K	R	S	R	R	G	H	P	E	Y	L	V	K	W	K	G	W	S	N	K	H	N	T	W	E	P	E	E	N	I	L	D	G	R	L	L	E	A	F	E	R	R	E	R	E	-	-	A	A	T	66
<i>T. castaneum</i> Pc	5	D	H	V	Y	A	A	E	R	I	I	K	K	R	V	R	R	G	V	V	E	Y	Y	V	K	W	K	G	W	S	Q	K	H	N	T	W	E	P	E	E	N	I	L	S	R	L	I	D	L	F	E	R	S	Q	K	-	-	L	D	P	62		
<i>B. mori</i> Pc	8	D	S	V	F	A	A	E	R	I	M	K	R	I	R	K	N	K	V	E	Y	Y	V	K	W	K	G	W	K	P	K	H	N	T	W	E	P	E	E	N	I	L	D	P	R	L	I	Q	S	F	M	K	G	E	-	-	-	E	V	63			
<i>D. melanogaster</i> Pc	23	D	L	V	Y	A	A	E	K	I	I	Q	K	R	V	K	K	G	V	E	Y	R	V	K	W	K	G	W	N	Q	R	Y	N	T	W	E	P	E	V	N	I	L	D	R	R	L	I	D	I	E	Q	T	N	K	S	-	G	T	P	82			
<i>G. gallus</i> CHCB3	9	E	Q	V	F	D	A	E	C	I	L	N	K	R	T	R	K	G	K	L	E	Y	L	V	K	W	R	G	W	S	S	K	H	N	S	W	E	P	Q	E	N	L	L	D	P	R	L	L	V	A	F	N	K	R	E	Q	E	K	E	L	I	69	
<i>D. rerio</i> Cbx2	8	E	H	V	F	A	V	E	S	I	E	K	K	R	I	R	K	G	R	V	E	Y	L	V	K	W	R	G	W	S	P	K	Y	N	T	W	E	P	E	E	N	I	L	D	P	R	L	L	I	A	F	Q	N	R	E	R	Q	E	Q	L	M	G	68
<i>M. musculus</i> Cbx2	9	E	Q	V	F	A	A	E	C	I	L	S	K	R	L	R	K	G	K	L	E	Y	L	V	K	W	R	G	W	S	S	K	H	N	S	W	E	P	E	E	N	I	L	D	P	R	L	L	A	F	Q	K	K	E	H	E	K	E	V	Q	N	69	
<i>H. sapiens</i> CBX2	9	E	Q	V	F	A	A	E	C	I	L	S	K	R	L	R	K	G	K	L	E	Y	L	V	K	W	R	G	W	S	S	K	H	N	S	W	E	P	E	E	N	I	L	D	P	R	L	L	A	F	Q	K	K	E	H	E	K	E	V	Q	N	69	

(B) PcR-box

<i>D. magna</i> Pc	453	H	R	K	N	P	-	L	V	D	Q	I	F	I	T	D	V	T	V	N	M	M	T	V	T	I	R	E	C	K	T	V	S	G	F	F	R	H	R	P	N	486
<i>D. pulex</i> Pc	478	H	R	K	N	P	-	L	V	D	Q	I	F	I	T	D	V	T	V	N	M	M	T	V	T	I	R	E	C	K	T	V	S	G	F	F	R	H	R	P	N	511
<i>T. castaneum</i> Pc	350	L	A	R	N	P	-	V	A	D	Q	V	F	I	T	D	V	T	V	N	L	K	T	V	T	I	R	E	C	K	T	E	K	G	F	F	K	D	R	D	383	
<i>B. mori</i> Pc	242	M	A	R	S	P	-	V	A	D	Q	I	F	I	T	D	V	T	V	N	L	Q	T	V	T	I	T	E	C	R	T	E	K	G	F	F	R	S	R	E	P	275
<i>D. melanogaster</i> Pc	349	L	P	A	K	C	N	I	S	N	R	V	V	I	T	D	V	T	V	N	L	E	T	V	T	I	R	E	C	K	T	E	R	G	F	F	R	E	R	D	M	382
<i>G. gallus</i> CHCB3	477	R	P	T	R	S	-	L	I	E	H	V	F	V	T	D	V	T	A	N	L	V	T	V	T	V	K	E	S	P	T	S	V	G	F	F	S	I	R	N	Y	510
<i>D. rerio</i> Cbx2	499	L	R	E	F	K	P	F	F	G	N	I	I	I	T	D	V	T	A	N	C	L	T	V	T	F	K	E	Y	V	T	-	-	-	-	-	-	-	-	-	523	
<i>M. musculus</i> Cbx2	486	K	P	T	R	S	-	L	I	E	H	V	F	V	T	D	V	T	A	N	L	I	T	V	T	V	K	E	S	P	T	S	V	G	F	F	N	L	R	H	Y	519
<i>H. sapiens</i> CBX2	499	K	P	T	R	S	-	L	I	E	H	V	F	V	T	D	V	T	A	N	L	I	T	V	T	V	K	E	S	P	T	S	V	G	F	F	N	L	R	H	Y	532

Figure 15 Multiple sequences alignment of Pc

Alignment of the (A) Chromodomain and (B) PcR-box of the different Pc orthologs from different organisms. The color is based on the physicochemical property of the amino acid-based on ClustalW.

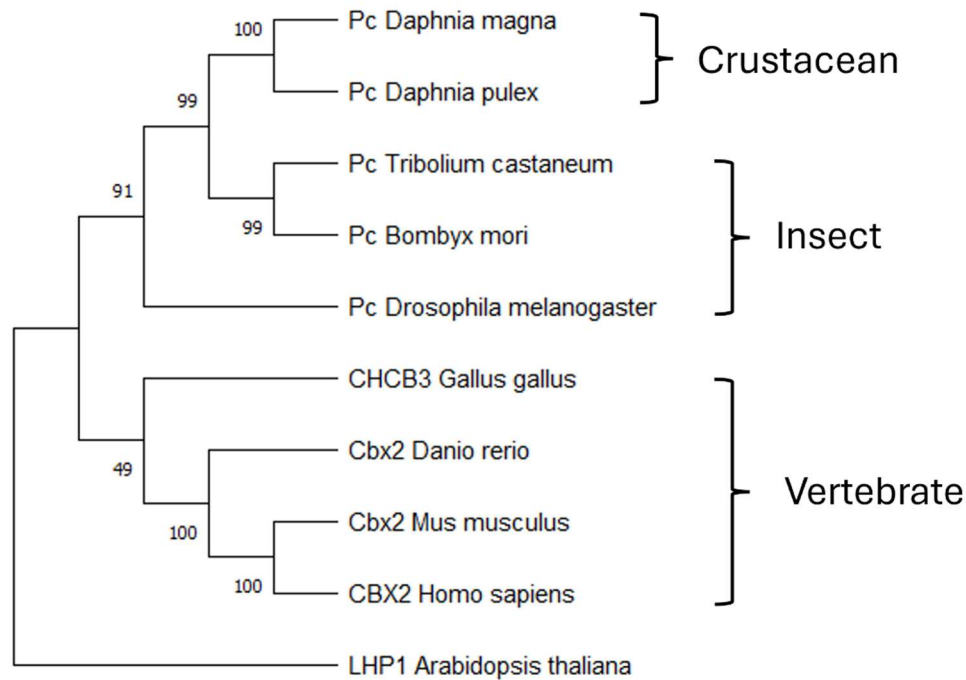


Figure 16 Phylogenetic tree of *Pc*

The percentages of the replicate tree in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary distances were computed using the *p*-distance method.

3.3.2 Asexual dimorphic expression of *Pc* during embryogenesis

To examine whether the expression of *Pc* shows sexually dimorphic expression like *dsx1*, the temporal expression of *Pc* during embryonic developmental stages was conducted by RT-qPCR. *Pc* was expressed in both male and female embryos, and no evidence of sexual dimorphism in their expression was observed (Figure 17). *Pc* exhibited the highest expression after ovulation (0 hours post-ovulation, 0 hpo), and a decline to the lowest expression at 12 hpo followed by slightly elevated at 24 hpo and maintained throughout embryonic developmental stages. This result suggests that *Pc* mRNA is maternally loaded into the embryo and zygotic expression takes place after 12 hpo.

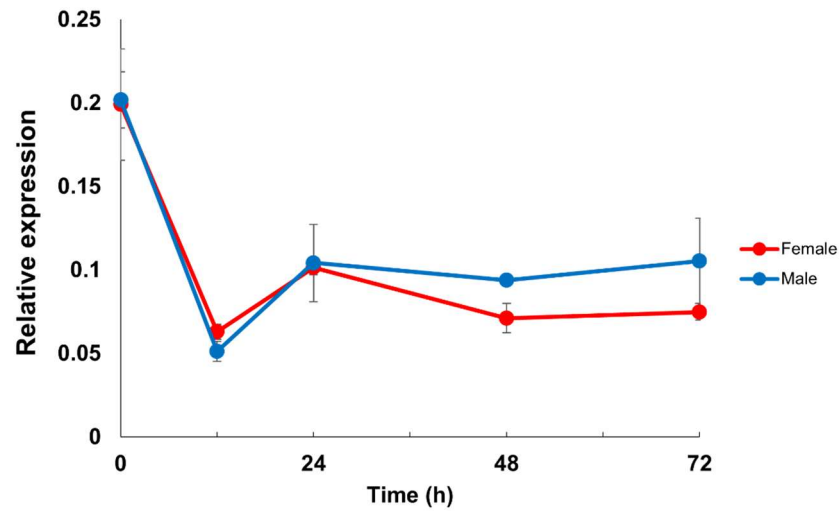


Figure 17 Temporal expression profile of *Pc* in embryonic developmental stages of *D. magna*.

The expression levels of *Pc* in female (red) and male (blue) embryos were determined at 0, 12, 24, 48, and 72 hours post-ovulation (hpo). Results are shown as relative expression normalized with the ribosomal protein *L32*. Error bars indicate the standard error of the mean ($n = 3$).

3.3.3 Pc silencing has no effect on *dsx1* expression

Loss-of-function analyses were performed to investigate the functions of the *D. magna* *Pc* gene, particularly in the context of sex determination. The RNAi method (Kato, Shiga, et al., 2011) was used to deplete *Pc* expression in of the *dsx1*-reporter strain during embryogenesis (Nong et al., 2017). The mCherry fluorescence in this strain represents the endogenous expression of *dsx1*, and H2B-GFP is expressed ubiquitously. This allows us to investigate which tissues or cells express *dsx1*.

Standard protocol in previous studies for knockdown target gene in *D. magna* by using siRNA is at the final concentration of 100 μ M (Kato, Shiga, et al., 2011). However, injected with a standard concentration of siRNA caused arrested development at early stage resulted in undifferentiated mCherry fluorescence, *Pc* siRNA (siPc) at the final concentration of 25 μ M was used for *Pc* knockdown in female or destined to be male embryos obtained from *dsx1*-reporter strain. Gene encoding *Maltose-binding protein (MalE)* in *E. coli* was used to designed negative control siRNA (siCtrl). At stage 6 (18 h after injection), a significant reduce of *Pc* transcript levels (Figure 18A) caused arrested development and led to lethality in siPC injected both female and male embryos (Table 14, Figure 19); however, *dsx1* expression (Figure 18B) and the relative fluorescence intensity did not significantly change compared to siCtrl injected one in both female and male embryos (Figure 20). The specificity of *Pc* knockdown using siRNA was confirmed by using an independent siRNA, siPc2 (Table 14 and Figure 18). This finding suggests that *Pc* may not have direct function on *dsx1* expression.

Table 14 Summary of *Pc* RNAi in *dsx1*-reporter strain

Sex	siRNA	Injected	Hatched	Swimming juvenile	Alteration of mCherry fluorescence at 18 h
Female	siCtrl	18	18	18	0% (0/18)
	siPc1	8	2	0	0% (0/8)
	siPc2	16	6	0	0% (0/16)
Male	siCtrl	8	8	8	0% (0/8)
	siPc1	4	4	0	0% (0/4)
	siPc2	3	3	0	0% (0/3)

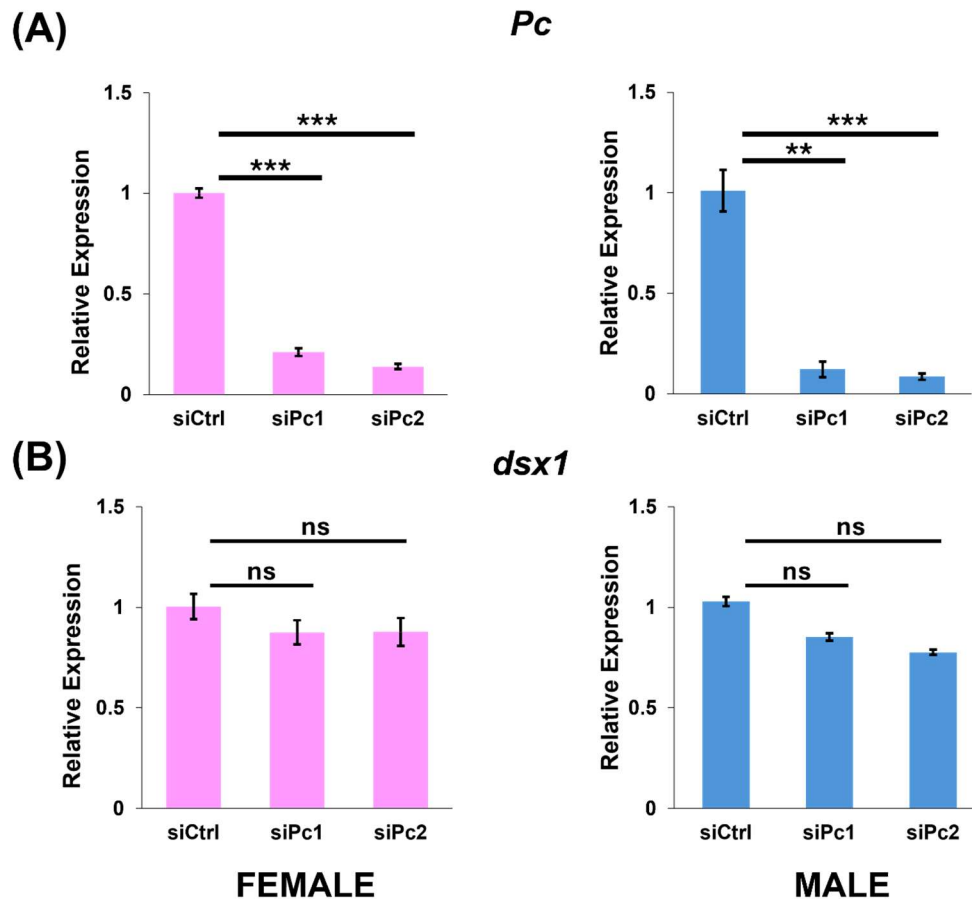


Figure 18 Gene expression profile in *Pc* knockdown in *dsx1*-reporter embryos.

(A) Quantitative RT-PCR analysis of *Pc* and (B) *dsx1* transcript levels in female (pink) and male (blue) embryos injected with control siRNA (siCtrl), *Pc*-siRNA1 (siPc1), or *Pc*-siRNA2 (siPc2). RT-qPCR results are shown as expression levels relative to *L32* gene. Error bars indicate the standard error of the mean (n = 3). *** $p < 0.001$, ** $p < 0.01$, ns: not significant (Student's *t*-test)

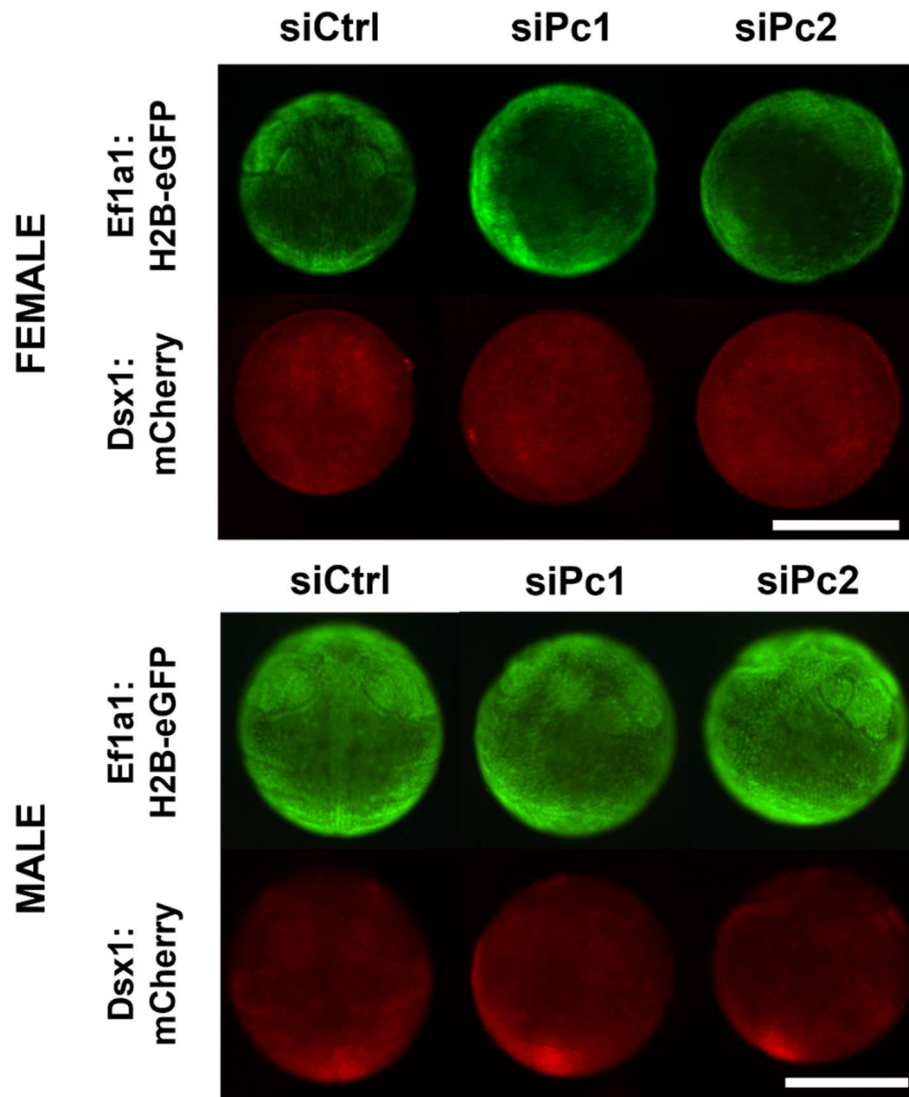


Figure 19 Phenotypes of *Pc* RNAi females and males

Ventral view of *dsx1*-reporter embryos injected with siCtrl, siPc1, or siPc2 at the final concentration of 25 μ M observing at stage 6 (18 h after injection). mCherry fluorescence allowed visualization of *dsx1* expression while GFP fluorescence in the nucleus enabled observation of body structures.

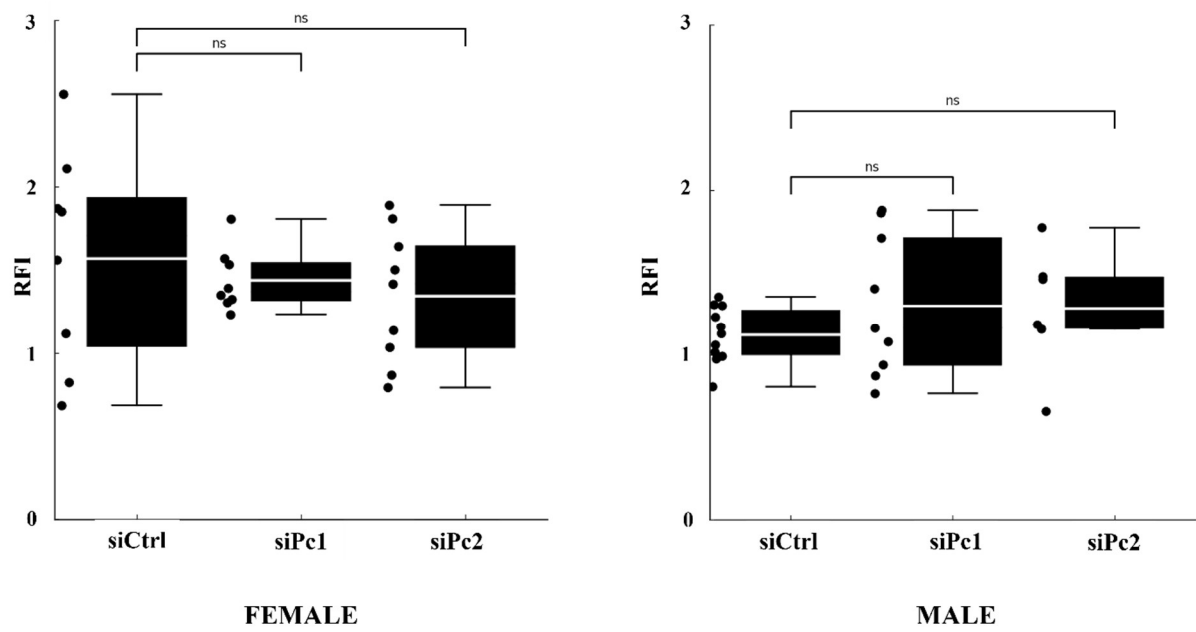


Figure 20 Quantitative analysis of mCherry fluorescence following *Pc* knockdown

Relative mCherry fluorescence intensity (RFI) of mCherry in *dsx1*-reporter female (left) and male (right) embryos injected with control (siCtrl), *Pc* siRNA-1 (siPc1), or *Pc* siRNA-2 (siPc2). Each dot represents one embryo. ns: not significant (Student's *t*-test).

3.4 Discussion

Polycomb (Pc) is well-established chromatin-associated protein involved in gene silencing and has been implicated in the regulation of sex-specific gene expression across in mouse and human (Biaison-Lauber et al., 2009; Eid et al., 2015; Garcia-Moreno et al., 2019; Katoh-Fukui et al., 2012; Sproll et al., 2018). In the closely related species *Daphnia pulex*, a significantly higher enrichment of H3K27me3 was observed at the *dsx1* locus in females, suggesting that the locus resides in a closed chromatin state to prevent its expression and thereby promote female development (Kvist et al., 2020). Given that H3K27me3 is a canonical repressive histone modification recognized by Pc protein, the present study aimed to investigate the functional role of Pc in the sex determination pathway of *D. magna*, with particular emphasis on its potential involvement in the transcriptional regulation of *dsx1*.

Polycomb (Pc) is an evolutionarily conserved protein found across a wide range of eukaryotic organisms. Homologs of Pc have been identified in species ranging from *D. melanogaster* (Pc) to humans (CBX2, CBX4, CBX6, CBX7 and CBX8) (Kaustov et al., 2010; Lewis, 1978). Members of the Pc family are characterized by two conserved domains: the chromodomain (CD) at the N-terminus and the Polycomb Repressive (PcR) box at the C-terminus (Senthilkumar & Mishra, 2009). The CD mediates the recognition and binding to H3K27me3, a well-established epigenetic mark associated with gene silencing (Cai et al., 2021; Fischle et al., 2003; Fukuda et al., 2025; Igoikina et al., 2019; Pailles et al., 2022). The PcR box is responsible for PRC1 complexes assembling via protein-protein interaction, enabling interactions with other chromatin-associated proteins (Bezsonova et al., 2009). In *D. magna*, one Pc was identified in this study containing the two conserved domains and are closely related to Pc in *D. melanogaster*, suggesting a conserved molecular function on gene silencing.

The expression of *Pc* in *D. magna* did not exhibit sexual dimorphism during embryonic development, indicating that it might have a role in both female and male. Moreover, the

highest transcript levels of *Pc* were detected at 0 hours post-ovulation (hpo), followed by a decline and subsequent stabilization throughout embryogenesis. This expression pattern suggests that *Pc* mRNA is maternally deposited, supporting its early developmental function.

Pc knockdown in *D. magna* had no observable effect on *dsx1* expression or induced sex reversal, in contrast to mammals such as mice and humans, where *Pc* homologs contribute to sex-specific gene regulation (Garcia-Moreno et al., 2019; Katoh-Fukui et al., 1998, 2012). These results indicate that *Pc* may not be required for *dsx1* repression in females. However, *Pc* depletion caused embryonic arrest and lethality in both sexes even at reduced siRNA concentration (25 μ M), consistent with findings in other model organisms and underscoring its essential role in early embryonic development (Cheutin & Cavalli, 2018; Chou et al., 2019; Katoh-Fukui et al., 1998; Lewis, 1978). Collectively, these findings suggest that while *Pc* may not be the primary effector maintaining *dsx1* repression in *D. magna*, it seems essential for general developmental processes. Interestingly, *D. pulex* females exhibit H3K27me3 enrichment at the *dsx1* locus, suggesting that this repressive histone mark could contribute to transcriptional silencing (Kvist et al., 2020). Because *Pc* is canonical reader of H3K27me3, it was hypothesized that *Pc* might mediated *dsx1* repression in *D. magna* females. Yet the knockdown results show that repression occurs through a *Pc*-independent mechanism, despite the presence of repressive H3K27me3 marks. One possibility is that *dsx1* silencing in females is stabilized predominantly through HP1-dependent heterochromatinization, with H3K27me3 functioning redundantly or in a context not captured under the tested conditions. Another possibility is that H3K27me3 contributes to a bivalent chromatin state at the *dsx1* locus, balancing repression with activation marks such as H3K4me3. In this scenario, *Pc* may help maintain *dsx1* in a poised state rather than enforcing strict silencing, consistent with models of bivalent regulation described in other organisms (Akmammedov et al., 2019; Bernstein et al., 2006; Kvist et al., 2020; Voigt et al., 2013).

To clarify this regulatory architecture, future experiments should profile histone modifications in a cell-type-specific manner, comparing *dsx1*-expressing cells from males with *dsx1*-non-expressing cells from both females and males. Analyzing the relative enrichment of H3K27me3 and H3K4me3 at the *dsx1* locus in these contexts would reveal whether bivalent or alternative chromatin configurations drive *dsx1* regulation in a sex- and cell-specific manner, and whether Pc-independent repression pathways operate in females.

CHAPTER 4

General Discussion and Conclusion

4.1 General discussion

This study investigated the roles of two conserved chromatin regulators, Heterochromatin Protein 1 (HP1-1) and Polycomb (Pc), in the regulation of *dsx1*, a key sex-determining gene in *D. magna*. While both proteins are associated with transcriptional repression in other systems, their functional contributions in an environmental sex determination context have been unclear (Baumann & De La Fuente, 2011; Biason-Lauber et al., 2009; Eid et al., 2015; Garcia-Moreno et al., 2019; Katoh-Fukui et al., 1998, 2012; Liu et al., 2005; Smolko et al., 2018; Sproll et al., 2018). The findings presented here suggest that HP1-1 and Pc act through distinct pathways, with different implications for sex determination and development.

Both HP1-1 and Pc possess conserved domains characteristic of their respective families, and neither displayed sex-biased expression during embryogenesis. Functional experiments revealed that depletion of *HP1-1* affected embryonic development in both sexes and led to derepression of *dsx1* and male-like elongated first antenna in juvenile stage in females. These results are consistent with the idea that HP1-1 contributes to the repression of *dsx1* in females. In contrast, the knockdown of *Pc* did not alter *dsx1* expression but caused embryonic lethality and morphological defects, suggesting that Pc is required for general developmental processes but may not directly regulate *dsx1* under the tested conditions. Taken together, these findings indicate that HP1-1 plays a role in maintaining *dsx1* silencing in females, whereas Pc does not appear to be directly involved in this process. This distinction highlights potentially different strategies employed by heterochromatin protein- versus Polycomb-associated pathways in *D. magna*. Importantly, these results should be interpreted with caution: while HP1-1 depletion clearly affected *dsx1* expression, overexpression did not

reduce *dsx1* levels in males, suggesting that additional cofactors or chromatin features are required for effective repression. Similarly, the absence of an effect from *Pc* knockdown does not rule out a role for other Polycomb group components, or functions at later developmental stages not captured in this study.

Based on the findings of this study, we propose that HP1-1 contributes to the establishment of a transcriptionally repressive chromatin environment at the *dsx1* locus in females (Figure 21). This heterochromatin-mediated silencing likely represents an important epigenetic barrier preventing male-specific gene expression in the female developmental context. Notably, although male and female *Daphnia* are genetically identical, *dsx1* expression is sexually dimorphic, supporting a role for epigenetic mechanisms in environmental sex determination. *HP1-1* gene itself does not display sex-biased expression during embryogenesis, suggesting that its contribution to *dsx1* repression depends on the chromatin context at the *dsx1* locus. The masculinization observed following *HP1-1* knockdown in female embryos is consistent with this interpretation.

Given the complexity of chromatin regulation, it is likely that multiple layers of control, including both transcriptional and post-transcriptional mechanisms, act in parallel to ensure robust repression of *dsx1* in females. In this model, chromatin-level repression mediated by HP1-1 operates alongside translational inhibition by the RNA-binding protein SHEP, providing complementary safeguards against inappropriate activation of the male developmental program. In males, these repressive mechanisms are alleviated through Vrille-mediated transcriptional activation and *DAPARL*-mediated translational derepression, collectively enabling the production of Dsx1 protein and commitment to the male developmental pathway (Kato et al., 2018; Mohamad Ishak et al., 2017; Perez et al., 2021). This integrated model underscores the importance of actively repressing the opposing sexual

fate to stabilize sex-specific development, consistent with the general principle that repression of alternative pathways is essential for robust cell fate decisions.

MALE



THIS STUDY FEMALE

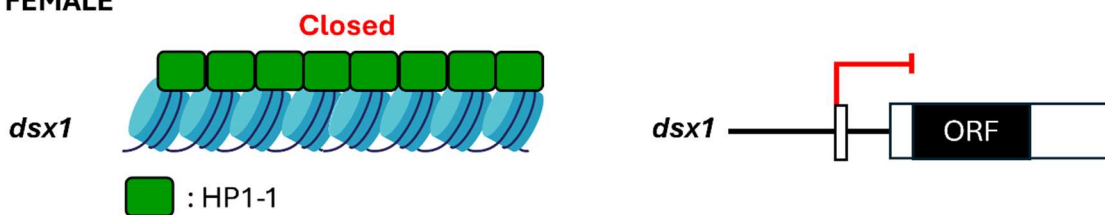


Figure 21 Proposed HP1-1 function in regulation of *dsx1* expression in *D. magna*

To deepen our understanding of the transcriptional regulation of *dsx1*, further investigation is required to elucidate the upstream mechanisms that establish and maintain its sex-specific expression. Current findings support a model in which chromatin-mediated repression silences *dsx1* in females, while activation occurs in males. However, the precise epigenetic landscape at the *dsx1* locus—including the distribution of histone modifications such as H3K4me3, H3K9me3, and H3K27me3—remains to be characterized in a sex- and stage-specific manner. Such analyses would clarify how these modifications contribute to either gene silencing or activation. In addition, assessing the developmental and sex-specific binding profile of HP1-1 would provide important evidence regarding its potential role in establishing repressive chromatin at the *dsx1* locus. Together, these future studies will offer deeper insights into the epigenetic regulation of sex determination and contribute broadly to our understanding of chromatin-based gene control in environmentally sex-determined invertebrates.

4.2 Conclusion

This study provides new insight into the epigenetic regulation of sex determination in *D. magna*, highlighting the role of chromatin-level mechanisms in controlling the expression of the key sex-determining gene *dsx1*. Recent studies in *D. pulex* have identified sex-specific histone modification patterns at the *dsx1* locus, suggesting an epigenetic basis for its regulation. To investigate this in *D. magna*, the present study uncovered the following findings:

1. Four HP1 and One Pc orthologs were identified in *D. magna*. Three HP1 homologs possess both CD and Chromoshadow Domain (CSD), while HP1CD contains three tandem CDs. Pc contains two conserved domains: the Chromodomain (CD) and Polycomb Repressive Box (PcR-box). The expression of all these genes showed no sexual dimorphism during embryogenesis; and
2. HP1-1 represses *dsx1* transcriptionally in females, contributing to the establishment of the female developmental pathway; lastly
3. Pc does not appear to regulate *dsx1* expression in either sex, though it is essential for embryonic development.

This study sheds light on the role of chromatin-level regulation in the environmental sex determination pathway of *D. magna*, offering new insights into the molecular diversity and evolutionary dynamics of sex determination systems.

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PUBLICATION

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