Involvement of Ecdysone Signaling in the Expression of the *doublesex* Gene during Embryonic Development in the Silkworm, *Bombyx mori*

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Abstract

Steroid hormones, represented by estrogen and testosterone, act as sex hormones that play an essential role in the sexual differentiation of vertebrates. However, it remains unclear whether ecdysteroids, typical steroid hormones in insects, function as sex hormones. In this study, we investigated whether ecdysteroids or ecdysone signals are involved in the sexual differentiation of the silkworm (*Bombyx mori*) embryo. Quantitative analysis using LC-MS/MS demonstrated that there was no significant difference in the 20-hydroxyecdysone (20E) titer between sexes during embryonic development. Consistent with this result, expression levels of 2 genes encoding ecdysteroid-phosphate phosphatase (EPPase) and ecdysone 20-hydroxylase (E20Hase), which are essential for the biosynthesis of ecdysone and 20E in eggs, did not show a significant difference between male and female embryos. Expression levels of *ecdysone receptor* (*EcR*) and *E75*, which is one of a small set of genes induced directly by 20E, were also similar between the 2 sexes. However, knockdown of *EPPase* and one isoform of *EcR* (*EcR-A*)

resulted in decreased expression of *Bombyx doublesex* (*Bmdsx*), a master regulatory gene for sexual differentiation of the silkworm in both male and female embryos. In vitro analysis with cultured testes revealed that expression levels of *Bmdsx* were increased in a dose-dependent manner of the ecdysone analog, ponasterone A. These results suggest that ecdysone signaling may play a role in indirectly regulating the expression of some genes involved in sexual differentiation through inducing expression of *Bmdsx* in the silkworm.

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In mammals, sexual differentiation is induced by sex hormones produced in the gonad. The gender is determined by the action of the *SRY* (sex-determining region Y) gene located in the Y chromosome, and the gonad primordium begins to differentiate into the ovary or the testis [Berta et al., 1990; Jäger et al., 1990; Koopman et al., 1991]. The ovaries secrete estrogen, and the testes secrete testosterone, which act on follicle maturation and testicular development, respectively, resulting in the sexual development of the body. Estrogen and testosterone are both steroid hormones. In fish, testis-ova are formed when adult males are treated with estrogen [Egami, 1955]. In reptiles whose sex is determined by the temperature of

the environment, treatment with estrogen at male induction temperature induces gonadal feminization, and treatment with estrogen inhibitors at female induction temperature induces gonadal masculinization [Chardard et al., 1995; Richard-Mercier et al., 1995]. Similarly, in birds, it has been revealed that female-to-male sex reversal in gonads occurs when estrogen synthesis is inhibited [Wartenberg et al., 1992]. Thus, sexual differentiation is induced by sex hormones in many vertebrates including mammals.

Many “vertebrate-type” steroids, such as estradiol, testosterone, dihydrotestosterone, androsterone, and progesterone, have been chemically identified in insects [De Loof, 1987; Swevers et al., 1991; Darvas et al., 1997]. However, there is no evidence that they play a role in sexual differentiation or reproduction. Typical steroid hormones in insects are ecdysteroids, including ecdysone, ecdysterone, turkesterone, and 20-hydroxyecdysone (20E), which are essential for development, molting, and metamorphosis [Krishnakumaran and Schneiderman, 1970; De Loof, 2006; Margam et al., 2006]. In the silkworm, *Bombyx mori*, ecdysteroids are synthesized from cholesterol in the prothoracic gland by stimulation of prothoracic stimulating hormone secreted by the brain [Huang et al., 2008]. Ecdysone is converted into its active form, 20E, in the target tissue, and 20E binds to the ecdysone receptor (EcR), which forms a dimer with Ultraspiracle [Yao et al., 1993]. This complex then binds to the ecdysone response element (EcRE) to regulate the transcription of downstream target genes [Thummel et al., 1996].

In the silkworm, the continuous supply of 20E is required for embryonic development [Makka et al., 2002]. During the embryonic stage, 20E is provided not only by de novo synthesis, but also by dephosphorylation of maternally derived ecdysteroid conjugates (ecdysteroid-phosphates) [Horike and Sonobe, 1999; Sonobe et al., 1999; Makka and Sonobe, 2000]. In the de novo synthesis of 20E, hydroxylation at the C-20 position of ecdysone, which is catalyzed by ecdysone 20-hydroxylase (E20OHase), is a rate-limiting step [Makka and Sonobe, 2000]. The conversion of ecdysteroid-phosphates is catalyzed explicitly by ecdysteroid-phosphate phosphatase (EPPase) [Yamada and Sonobe, 2003].

A growing body of evidence supports that there is a close link between ecdysteroids and sexual differentiation in insects. The 20E titer in the hemolymph in several fly species (*Sarcophaga*, *Calliphora*, *Lucilia*, *Phormia*) is much higher in females than in males [Briers and De Loof, 1980; Briers et al., 1983a, b]. 20E is the only fac-

tor that can induce vitellogenin synthesis in adult *Sarcophaga* males [Huybrechts and De Loof, 1977]. Moreover, the feeding of 20E to males directs their behavior in a female direction [De Clerck and De Loof, 1983]. In *Drosophila melanogaster*, it is known that *EcR* and *E74*, a small set of genes induced directly by ecdysone, are required to maintain germline stem cells in the ovary [Morris and Spradling, 2012]. Similarly, *EcR* together with *E75*, a gene also induced directly by ecdysone [Segraves and Hogness, 1990], promotes stem cell maintenance in the *Drosophila* testis [Li et al., 2014]. Furthermore, ecdysone also affects sexual behavior in *Drosophila* [Ganter et al., 2007, 2011, 2012]. However, it remains obscure whether ecdysteroids or ecdysone signaling play a crucial role in sex determination, which usually occurs during embryonic development.

Sex determination in the silkworm occurs between 29 and 32 h after oviposition [Sakai et al., 2014]. The femaleness of the silkworm is predominantly determined by the presence of the W chromosome [Hashimoto, 1933]. During the sex determination stage, *Feminizer* (*Fem*), the master regulatory gene for femaleness, is transcribed from the W chromosome in females [Kiuchi et al., 2014]. *Fem* transcripts yield PIWI-interacting RNAs (piRNAs), causing degradation of transcripts from the *Masculinizer* (*Masc*) gene, which is an essential regulatory gene for maleness [Sakai et al., 2016]. In the absence of *Masc* expression, *Bombyx doublesex* (*Bmdsx*) pre-mRNA undergoes female-specific splicing to produce the female-type isoform of *Bmdsx* (*Bmdsx^F*), enhancing the female development of somatic cells [Suzuki et al., 2003; Kiuchi et al., 2014; Sakai et al., 2015]. The male stably expresses the *Masc* gene due to the lack of the W chromosome, inducing the expression of the male-specific isoform of the *Bombyx* homolog of the insulin-like growth factor II mRNA-binding protein gene (*Imp^M*) [Kiuchi et al., 2014; Sakai et al., 2015]. *Imp^M* is involved in the male-specific splicing of *Bmdsx* pre-mRNA, resulting in the production of the male-type isoform of *Bmdsx* (*Bmdsx^M*) [Suzuki et al., 2010]. *Bmdsx^M* promotes male development of the body [Suzuki et al., 2005]. *Bmdsx* starts sexual dimorphic expression within 48 h after oviposition [Sakai et al., 2014].

In the silkworm, by using the sex-limited strain, female embryos can be easily discriminated from male ones based on the pigmentation of the eggshell [Sakai et al., 2014]. Taking advantage of this useful feature, in this study, we investigated whether ecdysteroids or ecdysone signals are involved in sexual differentiation during the embryonic development of the silkworm. For this pur-

pose, ecdysone and 20E titers in male and female eggs were measured, and a comparison of the expression levels of ecdysone biosynthesis-related genes was made between the male and female embryos. This study provides several lines of evidence that there is a strong link between ecdysone signaling and *Bmdsx* expression by knockdown (KD) experiments of several ecdysone synthesis-related genes.

Materials and Methods

Silkworm Strains

To quickly and precisely discriminate female eggs from male eggs, the sex-limited black egg strain, S-2, was used as described previously [Sakai et al., 2015]. The developing eggs were incubated at 25°C. Larvae were reared on an artificial diet (Nihon Nosan) at approximately 25°C.

Extraction of Ecdysteroids from Eggs

Eggs of the S-2 strain were collected at determined points in time after oviposition and divided into males and females based on eggshell color. Twenty eggs of each sex were homogenized using a pestle in 350 µL sonic buffer (150 mM NaCl, 50 mM Tris pH 7.5, 2 mM EGTA pH 8.0). The resulting suspension was mixed with 350 µL of 1-butanol for 15 min at room temperature. The supernatant obtained by centrifugation (1,500 rpm, 4°C, 10 min) was collected, and the solvent was distilled off for 2 h at room temperature using an evaporator. The solvent-distilled sample was dissolved in 500 µL of 70% methanol and was shaken with 500 µL of hexane for 15 min to extract hydrophobic components such as cholesterol. The lower layer obtained by centrifugation (1,500 rpm, 4°C, 10 min) was collected, and the solvent was distilled off for 2 h at room temperature using an evaporator. The solvent-distilled sample was stored at -20°C until analysis.

Analysis by Liquid Chromatography-Mass Spectrometry

Prominence gradient HPLC (Shimadzu) and QTRAP 5000 (AB SCIEX) were used for LC-MS/MS analysis. The sample stored at -20°C as described above was redissolved in 100 µL of 100% methanol. The supernatant obtained by centrifugation (14,000 rpm, 4°C, 10 min) was used for the analysis. The conditions of LC-MS/MS were as follows. Column: PEGASIL ODS (2.0 × 100 mm), flow rate: 0.25 mL/min, gradient curve: 10–80% acetonitrile. The concentration of the component corresponding to 20E was calculated from the peak area value obtained from LC-MS/MS analysis.

RNA Extraction and Quantitative Real-Time RT-PCR

Total RNA was extracted from either single eggs or 10 pooled eggs using Isogen (Nippon Gene), as described previously [Suzuki et al., 2012]. cDNA synthesis and quantitative real-time RT-PCR (qRT-PCR) were performed according to the protocol of Suzuki et al. [2012]. The primer sequences used for qRT-PCR are shown in Table 1.

Preparation of Double-Stranded RNAs

DNA templates for double-stranded RNA (dsRNA) synthesis were amplified by RT-PCR using primers described in Table 2.

Table 1. Sequences of primers utilized for qRT-PCR

Target gene	Primers	Sequence (5'→3')
<i>EPPase</i>	EPPase F1 EPPase R1	GAAGGCAAAGAGCGTTGAAAG TCCAACCACACGTCGACTTAG
<i>E20OHase</i>	E20OHase F1 E20OHase R1	TGTCGAGAGCTTTTGGAGTAC ATCTGCATAAGCCTCGTGCAG
<i>EcR-A</i>	EcRA F3 EcRA R3	ACTGAAGAATCCAACGTCAAC ACCTCGTGTTCAGCTCCATG
<i>EcR-B1</i>	EcRB1 F1 EcRB R1	AGAGTAACGCAGACTTGGCAG CTGGACGAGGCTTTTAATAAG
<i>EcR-B2</i>	EcRB2 F1 EcRB R1	GATCTCAAAAGAGTAACGCAGT CTGGACGAGGCTTTTAATAAG
<i>E75A</i>	E75A F1 E75 R1	AGCTACGGGCGCTACGATGTG TGTATAGATCGCCGGAAGAAC
<i>E75B</i>	E75B F1 E75 R1	TGACGAGTGTAGTGAATGGTG TGTATAGATCGCCGGAAGAAC
<i>Bmdsx^F</i>	Bmdsx ^F F E2E5 R	AACTCGACACGCCAGAAAATG TGCATCATCCAATAACCTTCG
<i>Bmdsx^M</i>	Bmdsx ^M F E4E5 R	CCGCCAACCATGCCACCACTG GCATCATCCAATAACCCATAG
<i>Ago1</i>	Ago1 F1 Ago1 R1	CACTAGTATCTCCAGCAGCA ACATCATAGTGATGAACAAACC
<i>Bmvlg</i>	BmVLG F1 BmVLG R1	TCACAGTCTGTCCAGAGGAAG TCGCTGTGCTGTTCTCGTGAC
<i>EF-2</i>	EF2 F EF2 R	TCGTACCGTGAGACCGTAGCT ATGTCCTCTGGCAGACCATCA

Each primer contained a T7 promoter site. dsRNA synthesis and dsRNA injection into eggs were performed according to the protocol of Suzuki et al. [2012]. Briefly, fertilized eggs of *B. mori* S-2 strain were collected within 2 h after oviposition and were glued onto the surface of glass slides. dsRNAs were injected into the eggs at the preblastoderm stage (between 6–8 h after oviposition at 25°C) at a final concentration of 1 µg/µL.

Tissue Dissection and in vitro Culture

Testes were dissected out from day-0 fifth instar larvae of the w1pnd strain provided by Dr. Megumi Sumitani, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization (NARO). The dissected testes were washed 3 times with 1× PBS (TaKaRa). The testes were separately cultured using a 96-well cell culture plate (Iwaki). Each well contained 200 µL of TC-100 insect medium (Thermo Fisher). Ponasterone A (kindly provided by Dr. Shinji Nagata, University of Tokyo) was dissolved in 99.5% ethanol and added to the medium to give the desired concentrations prior to the culture. After 6 h of culture at 25°C, total RNA was extracted from each testis, and the expression level of *Bmdsx* was quantified by qRT-PCR according to the protocol described above.

Fig. 1. Quantification of the 20E titer in male and female eggs. **A** Quantitative analysis using mass spectrometer LC-MS/MS was performed to compare the 20E titer between male and female eggs. Eggs of the S-2 strain were collected at the indicated days post-oviposition (dpo) and divided into males and females according to eggshell color. Each sample was extracted from 20 pooled eggs of each sex and subjected to analysis. The concentration (ng/mL) of the component corresponding to 20E was calculated from the peak

area value obtained by LC-MS/MS analysis. Values represent the means $\pm$ SE of 3 independent experiments. **B** Changes of the expression level of *Bmdsx* quantified by qRT-PCR in male (blue line) and female (red line) eggs. The dots show the mean expression levels of the male-specific isoform of *Bmdsx* (*BmdsxM*) and of the female-specific isoform of *Bmdsx* (*BmdsxF*) in 20 pooled male and female eggs, respectively.

Table 2. Sequences of primers used for synthesis of dsRNA

Target gene	Primers	Sequence (5'→3')
<i>EPPase</i>	dsEPPase F	CCGGATCCTAATACGACTCACTATAGGGCGCAAGCACACGTGTACGCTTGCC
	dsEPPase R	CCGGATCCTAATACGACTCACTATAGGGCGAGTGTGTGATCGCGAGCCCAATG
<i>EcR-A</i>	dsEcRA F1	CCGGATCCTAATACGACTCACTATAGGGCGCAACTCACGCGCTTGTTC
	dsEcRA R1	CCGGATCCTAATACGACTCACTATAGGGCGACGACCCGCTCTCGCTCGTG
	dsEcRA F2	CCGGATCCTAATACGACTCACTATAGGGCGGTGACTGGGCCACAGTACG
	dsEcRA R2	CCGGATCCTAATACGACTCACTATAGGGCGCCGCGCGAGCTGACAAAGAG
<i>EcR-B</i>	dsEcRB F1	CCGGATCCTAATACGACTCACTATAGGGCGTCCGTTTTTACGGCTCAAAC
	dsEcRB R1	CCGGATCCTAATACGACTCACTATAGGGCGACATAACCATGGCCGGTG
	dsEcRB F2	CCGGATCCTAATACGACTCACTATAGGGCGCTTGCGACCACGATAAGACTAC
	dsEcRB R2	CCGGATCCTAATACGACTCACTATAGGGCGTCGACCGCACGCTTTTGTTG
<i>E75</i>	dsE75 F1	CCGGATCCTAATACGACTCACTATAGGGCGCTGTGCGATTTCGTCGTGTAC
	dsE75 R1	CCGGATCCTAATACGACTCACTATAGGGCGCCGGTTTGTAGTGGCAAG
<i>Ago1</i>	dsAgo1 F2	CCGGATCCTAATACGACTCACTATAGGGCGGGTTTGTTCATCACTATGATG
	dsAgo1 R2	CCGGATCCTAATACGACTCACTATAGGGCGTTGGTCTCACCATTGATG
<i>DsRed</i>	dsDsRed F	CCGGATCCTAATACGACTCACTATAGGGCGACATCCCCGACTACAAGAAG
	dsDsRed R	CCGGATCCTAATACGACTCACTATAGGGCGAGGTAGTGGCCGCCGTCCTTC

Results

Quantification of Ecdysone and 20E Titters in Male and Female Eggs

Quantitative analysis using mass spectrometer LC-MS/MS was attempted to compare ecdysone and 20E titters between sexes at the embryonic stage. For this pur-

pose, male and female eggs at 2, 3, 4, 5, 6, 7, 8, 9, and 10 days post-oviposition (dpo) were subjected to LC-MS/MS analysis. Although we could not quantitate ecdysone, the 20E titer in male and female eggs was quantified as shown in Figure 1A. The 20E titer in both sexes increased as embryonic development progressed, peaked at 6 dpo, and then gradually decreased (Fig. 1A). However, there

Fig. 2. Quantification analysis of mRNA levels of ecdysone-related genes in male and female eggs. Eggs of the S-2 strain were collected at the indicated days post-oviposition (dpo) and divided into males and females according to eggshell color. Total RNA was extracted from 20 pooled eggs of each sex and subjected to cDNA

synthesis. **A–G** Changes of the expression level of *EPPase* (**A**), *E20OHase* (**B**), *EcR-A* (**C**), *EcR-B1* (**D**), *EcR-B2* (**E**), *E75A* (**F**), and *E75B* (**G**) quantified by qRT-PCR in male (blue line) and female (red line) eggs. Values represent the means ± SE of 3 independent experiments.

was no significant difference in the 20E titer between sexes at all the stages examined in this study.

Quantification Analysis of mRNA Levels of Ecdysone-Related Genes in Male and Female Eggs

In order to verify the above results from another angle, we decided to investigate the expression level of 2 genes encoding *EPPase* and *E20OHase*, both of which are important for the biosynthesis of ecdysone and 20E in eggs. As described above, *EPPase* dephosphorylates ecdysone 22-phosphate accumulated in eggs, yielding free ecdysone [Yamada and Sonobe, 2003]. *E20OHase* catalyzes the hydroxylation at the C-20 position of ecdysone and is essential for the de novo synthesis of 20E in eggs [Makka and Sonobe, 2000]. Quantification of *EPPase* and *E20OHase* mRNAs by qRT-PCR demonstrated that

the expression levels of both genes reached the highest peak at 4 dpo, and then gradually decreased with embryo development (Fig. 2A, B). However, no significant differences were seen in the expression levels of the 2 genes between males and females. These results were consistent with the fact that *EPPase* and *E20OHase* are involved in the biosynthesis of 20E in the egg since the expression level of *EPPase* and *E20OHase* peaked before the 20E titer became highest as shown in Figure 1A.

The same analysis was performed to investigate expression levels of the 20E primary response genes *EcR* and *E75*. The expression level of *EcR-A*, one of the isoforms of *EcR*, peaked at 7 dpo (Fig. 2C) when the 20E titer still had a high level (Fig. 1A). *E75B*, which is one of the *E75* isoforms, showed an expression pattern similar to that of *EcR-A* (Fig. 2G). These results were consistent with the

Fig. 3. Effect of *EPPase* knockdown (KD) on *Bmdsx* expression. Expression levels of the genes in male and female eggs were quantified by qRT-PCR 4 days after injection of dsRNA targeting the respective mRNAs. dsRNA targeting *DsRed* served as a negative control dsRNA. **A** *EPPase*. **B** Ecdysone responsive genes in negative control eggs and *EPPase* KD eggs. **C** *BmdsxF* and *BmdsxM* in negative control eggs and *EPPase* KD eggs. * $p < 0.05$ (t test).

findings that expressions of *EcR* and *E75* are both induced directly by ecdysone [Segraves and Hogness, 1990; Morris and Spradling, 2012]. On the other hand, the expression levels of other isoforms of *EcR* (*EcR-B1* and *EcR-B2*) reached the highest peak already at 4 dpo (Fig. 2D, E). Differing from the expression patterns of the genes examined above, *E75A*, which is another isoform of *E75*, showed higher expression levels during 2 and 4 dpo (Fig. 2F). There was no significant difference in the expression levels of all analyzed genes between male and female eggs. Taken together, it seemed unlikely that ecdysone titer, ecdysone biosynthesis, and ecdysone signaling showed a difference between sexes in the embryonic stage.

In order to examine the possibility that sex differentiation of the silkworm embryo is under the control of ecdysone signaling, the expression level of *Bmdsx* at various embryogenic stages was quantified by qRT-PCR. As shown in Figure 1B, the expression level of *Bmdsx* in male and female embryos gradually increased with the progress of embryogenesis and reached the highest peak at 6 dpo. The expression pattern was almost similar to the developmental change in the 20E titer (Fig. 1A). These results suggest that *Bmdsx* expression in the embryo may be under the control of ecdysone signaling.

Effect of *EPPase* Knockdown on *Bmdsx* Expression in Embryos

Although the above results indicate that ecdysone signaling is not likely involved in sexual development, the expression level of the master regulatory gene for sexual differentiation, *Bmdsx*, seemed to correlate with the developmental changes in the 20E titer in embryos. To examine whether the expression level of *Bmdsx* is indeed under the control of 20E, we investigated the effect of the RNAi-mediated KD of *EPPase*, which dephosphorylates ecdysone 22-phosphate accumulated in eggs, supplying a lot of free ecdysones [Yamada and Sonobe, 2003]. The dsRNA injection targeting *EPPase* significantly repressed the expression of the target gene (Fig. 3A). However, efficiency for *EPPase* KD was slightly lower in male embryos. To evaluate whether *EPPase* KD caused a reduction in ecdysone and 20E titers, we quantified expression levels of several ecdysone responsive genes examined above. Expression levels of all the examined genes in the *EPPase* KD embryos significantly decreased compared with those in negative control embryos (Fig. 3B). Especially, the extent of reduction in the expression level of *EcR-A* and *E75B* in the *EPPase* KD eggs was greater than in other ecdysone responsive genes. Based on these results, it would be expected that *EPPase* KD might cause a reduction in ecdysone and 20E titers in eggs.

Fig. 4. Effect of knockdown (KD) of ecdysone responsive genes on *Bmdsx* expression. **A, C, E** Expression levels of the genes in male and female eggs were quantified by qRT-PCR 4 days after injection of dsRNA targeting the respective mRNAs. **A** *EcR-A*. **C** *EcR-B1* and *EcR-B2*. dsRNA targeted a region shared by both *EcR-B1* and *EcR-B2* mRNAs. **E** *E75A* and *E75B*. dsRNA targeted a region shared by both *E75A* and *E75B* mRNAs. **B, D, F** Expression levels of *Bmdsx* in the *EcR-A* KD eggs (**B**), *EcR-B* KD eggs (**D**), and *E75* KD eggs (**F**) 4 days after injection of the dsRNAs. *Bmdsx* was specifically quantified by qRT-PCR in female eggs, while in males, primers specifically annealing to *BmdsxM* were used for the qRT-PCR analysis. * $p < 0.05$ (t test).

The same analysis was performed to quantify the expression level of *Bmdsx*. A reduced expression level of *Bmdsx* was observed in both female and male *EPPase* KD embryos (Fig. 3C). These results suggest that the expression level of *Bmdsx* in male and female embryos is under the control of ecdysone signaling. Either *EcR-A* or *E75B* may be involved in the regulation of *Bmdsx* expression in embryos since a relatively greater extent of reduction in the expression levels of *EcR-A* and *E75B* was observed in the *EPPase* KD embryos (Fig. 3B).

Effect of *EcR* and *E75* Knockdowns on *Bmdsx* Expression in Embryos

To further verify whether the expression of *Bmdsx* in embryos is under the control of ecdysone signaling, we next investigated the effect of KD of *EcR* and *E75*, whose expression levels were decreased in the *EPPase* KD embryos. As shown in Figure 4A, dsRNA injection targeting *EcR-A* successfully reduced the expression level to less than 10% of that in the negative control embryos (Fig. 4A). KD of *EcR-A* caused a significantly reduced expression level of *Bmdsx* both in male and female embryos (Fig. 4B).

Fig. 5. Ecdysone analog-mediated induction of *Bmdsx* expression. **A** Comparison of the chemical structures of 20E and ponasterone A. **B** Ponasterone A (50, 100, and 150 pM) was injected into eggs at the preblastoderm stage. Expression levels of *EcR-A*, *EcR-B1*, and *EcR-B2* in eggs were quantified by qRT-PCR 4 days after injection. **C, D** Testes of fifth instar larvae were excised and cultured in me-

dium without or with ponasterone A at the indicated concentrations. After 6 h of culture, total RNA was extracted from the testis and expression levels of *Bmdsx* (**C**) and a *Bombyx* ortholog of the *vasa* gene (*Bombyx mori vasa-like protein gene*, *Bmvlg*) (**D**) were quantified by qRT-PCR. * $p < 0.05$; ** $p < 0.01$ (t test).

Although we successfully knocked down 2 other *EcR* isoforms, *EcR-B1* and *EcR-B2* (Fig. 4C), the expression level of *Bmdsx* was not affected by the KD of *EcR-B* isoforms (Fig. 4D). Similarly, KDs of *E75A* and *E75B* caused no significant change in the *Bmdsx* expression level (Fig. 4E, F). These results demonstrated that ecdysone signaling mediated by *EcR-A* directly or indirectly regulates the proper expression of *Bmdsx* during the embryonic stage.

Ecdysone Analog-Mediated Induction of *Bmdsx* Expression

Next, to examine whether an increased 20E titer promotes the expression of *Bmdsx* in embryos, we injected ponasterone A (Fig. 5A), which was shown to induce molting in the silkworm and to act efficiently as a ligand for *EcR* [Kobayashi et al., 1967; Saez et al., 2000], into fertilized eggs at the preblastoderm stage. However, most injected eggs died within 4 days after injection either due to technical problems in the injection technique or the presumably toxic effect of ponasterone A. The expression level of *EcR* quantified by qRT-PCR using cDNAs pre-

pared from barely survived eggs showed no significant change between control eggs and ponasterone A-injected eggs (Fig. 5B). We used an in vitro system to overcome this problem. Kambyssellis and Williams [1972] succeeded in the in vitro production of sperms in cultured testis from silkworm pupae by adding ecdysone to the medium. According to their method, testes of fifth instar larvae were excised and cultured in medium with or without ponasterone A. After 6 h of culture at 25°C, total RNA was extracted from the testis, and the expression level of *Bmdsx* was quantified by qRT-PCR. The expression level of *Bmdsx* in the cultured testis increased in a dose-dependent manner of ponasterone A (Fig. 5C). However, if ponasterone A treatment causes some changes in the cell type composition in the testis (i.e., more germ cells/less somatic cells), then such changes may lead to an apparent increase in *Bmdsx* expression levels. To investigate whether the ponasterone A treatment affects the number of somatic cells versus germ cells in the testis, we quantified the expression level of a *Bombyx* ortholog of the *vasa* gene (*Bombyx mori vasa like protein gene*, *Bmvlg*), a well-

Fig. 6. Effect of *Ago1* knockdown on *Bmdsx* expression. Gene expression levels were determined by qRT-PCR 4 days after injection of dsRNA. **A** Expression level of *Ago1* in male and female eggs. **B** Expression level of *Fem* in negative control female eggs and *Ago1* KD female eggs. **C** Expression level of *Imp^M* in negative con-

trol male eggs and *Ago1* KD male eggs. **D** Expression level of *Bmdsx* in negative control eggs and the *Ago1* KD eggs. *Bmdsx^F* was specifically quantified by qRT-PCR in female eggs, while in males, primers specifically annealing to *Bmdsx^M* were used for the qRT-PCR analysis. * $p < 0.05$ (t test).

known germ cell marker gene in various animals including the silkworm [Raz, 2000; Nakao et al., 2006; Cao et al., 2012]. In contrast to the *Bmdsx* expression, no apparent changes were observed in the expression level of *Bmvlg* (Fig. 5D), suggesting that ponasterone A treatment did not affect the number of somatic cells versus germ cells under our experimental conditions. These results strongly suggest that stimulation by 20E increases the expression level of *Bmdsx*.

Effect of *Ago1* Knockdown on *Bmdsx* Expression in Embryos

In order to examine the possibility that EcR-A directly regulates *Bmdsx* expression, we searched for a sequence identical to EcRE or EcRE-like sequences in the region near the transcription start site of *Bmdsx* or within the *Bmdsx* gene body, but could not find any (data not shown). Therefore, it is conceivable that EcR indirectly regulates *Bmdsx* expression. In *Drosophila*, transcription of *let-7* microRNA is known to be directly regulated by EcR [Chawla and Sokol, 2012]. Interestingly, ecdysone stimulates *let-7* miRNA expression through EcR, resulting in changes of expression levels of sex-determining genes such as *Sex-lethal*, *transformer*, and *dsx* [Fagegaltier et al., 2014]. To test whether the miRNA pathway is also involved in the regulation of *Bmdsx* expression, we investigated the effect of KD of Argonaute-1 (*Ago1*), an essential component for the formation of miRNA-induced silencing complexes [Baumberger and Baulcombe 2005; Mallory and Vaucheret, 2010], on *Bmdsx* expression in embryos. As shown in Figure 6A and D, KD of

Ago1 caused significant decrement in the expression level of *Bmdsx* in male and female embryos. The same KD experiments did not affect expression levels of *Fem* and *Imp^M* (Fig. 6B, C). As is the case in *Drosophila*, it is highly probable that EcR may also regulate the expression of *dsx* in the silkworm via miRNAs such as *let-7* miRNA.

Discussion

In order to investigate whether ecdysteroids or ecdysone signals are involved in sexual differentiation at the sex determination stage of the silkworm, in this study, we first measured ecdysone and 20E titers in male and female eggs by LC-MS/MS. However, we could not detect a compound whose retention time of the LC precisely matched the ecdysone standard. It is known that most ecdysone in the silkworm egg is accumulated in the phosphorylated state [Yamada and Sonobe, 2003] and that ecdysone is rapidly epimerized in the egg [Sonobe et al., 1999; Makka and Sonobe, 2000]. Therefore, the amount of free ecdysone extracted from eggs in this study (20 eggs per sample) may be insufficient for the analysis.

The 20E titer in both sexes increased as embryonic development progressed, peaked at 6 dpo, and then gradually decreased (Fig. 1A). This result was almost similar to the changes in the level of 20E during embryonic development reported previously [Yamada and Sonobe, 2003], supporting the high reliability of our quantification of 20E titer in the egg. Our results demonstrated that there was no significant difference in the 20E titer between the

sexes during embryonic development. However, we still cannot completely rule out the possibility that the ecdysone titer, which we failed to quantify in this study, may differ between male and female eggs. Further research is needed to clarify whether a sexual difference in ecdysone titer is observed during the embryonic stage.

Not only the 20E titer, but also the expression levels of genes related to the biosynthesis of ecdysone and 20E in the egg did not show a significant difference between males and females during the embryonic stage (Fig. 2A, B). Moreover, no significant difference in the expression levels of 20E primary response genes was observed between male and female embryos (Fig. 2C–G). These results suggest that ecdysteroids such as ecdysone and 20E are unlikely to act as a sex hormone that is important for sexual differentiation during embryogenesis in the silkworm. Nevertheless, KD of *EPPase* and *EcR-A* resulted in decreased expression of *Bmdsx* in male and female embryos (Fig. 3A, C, 4A, B). From these findings, it could be possible that ecdysone signaling plays a role in indirectly controlling the expression of some genes involved in sexual differentiation through inducing expression of *Bmdsx*, which is a master regulator of sexual differentiation in the silkworm.

Since EcRE or EcRE-like sequences were not found in the region near the transcription start site of *Bmdsx* or within the *Bmdsx* gene body, it is conceivable that EcR indirectly regulates *Bmdsx* expression. In *Drosophila*, ecdysone stimulates *let-7* miRNA expression through EcR, resulting in changes in the expression level of *dsx* [Fagegaltier et al., 2014]. Our results, where *Ago1* KD decreased the expression level of *Bmdsx*, support that there is a close link between the miRNA pathway and *Bmdsx* expression. In *B. mori*, the expression level of *let-7* miRNA is higher at the end of each instar and the beginning of each molt than at other periods, coinciding with the pulse of ecdysone as a whole [Liu et al., 2007]. Consistent with this finding, analysis using transgenic silkworms carrying a miRNA sponge targeting the *let-7* seed region demonstrates that *let-7* miRNA regulates molting and metamorphosis in *B. mori* [Ling et al., 2014]. As is the case in *Drosophila*, it is highly probable that EcR may also regulate the expression of *dsx* in the silkworm via miRNAs such as *let-7* miRNA.

In *Drosophila*, the expression of *dsx* is not maintained at a constant level throughout life and also varies in each tissue. For example, expression of *dsx* in the genital primordium is kept at a low level until the third instar larval stage but increases rapidly from the late third instar larval stage to the pupal stage when differentiation of genital

primordia starts [Chatterjee et al., 2011]. On the other hand, *dsx* is scarcely expressed in the fat bodies from the larval stage to the early pupal stage, but its expression level increases explosively from the late pupal stage to adults [Chatterjee et al., 2011]. Gonads and genital primordia in the silkworm show the most intensive growing during the pupal stage [Tazima, 1978]. It would be reasonable to consider that the expression of *Bmdsx* also changes dramatically depending on the tissue and developmental stage and that some of such changes are controlled via ecdysone signaling. There is still room to investigate whether ecdysone signaling also affects *Bmdsx* expression in developmental stages other than the embryonic stage.

Injection of ponasterone A, a well-known 20E analog, into fertilized eggs did not increase the expression level of EcR (Fig. 5B). The expression levels of all the EcR isoforms tended to decrease rather than increase depending on the concentration of ponasterone A. This may be due to the deleterious effect of higher concentrations of ponasterone A. Another plausible explanation is that ponasterone A might be inactivated immediately after injection because of the rapid epimerization of ecdysone in eggs as reported previously [Sonobe et al., 1999; Makka and Sonobe, 2000]. In a previous study, Makka et al. [2002] injected 20E into diapause eggs (18–21 h after oviposition) in order to examine the effect of 20E on embryonic development. Only approximately 7% of eggs were able to develop beyond the gastrula stage without entering diapause. The authors speculated that the low efficiency of injected 20E to induce embryonic development might be due to the immediate inactivation of 20E, since exogenous ecdysone and 20E are indeed promptly inactivated due to epimerization and phosphorylation [Makka and Sonobe, 1998, 2000; Sonobe et al., 1999]. Similarly, it is highly plausible that injected ponasterone A was also promptly inactivated and, therefore, had no effect on EcR and *E75* expressions as observed in this study.

On the other hand, an increased expression level of *Bmdsx* in the cultured testis was observed in a dose-dependent manner of ponasterone A (Fig. 5). Spermatogenesis occurred in the cultured testis of Cynthia silkworms only when ecdysone was added to the medium [Kambyzellis and Williams, 1972]. In *Drosophila*, spermatocyte meiosis and spermatogenesis can be induced in cultured spermatocytes under the presence of 20E [Ogata et al., 2012]. It is also known that EcR is required for promoting germline stem cell maintenance in the *Drosophila* testis [Li et al., 2014]. *dsx* controls the sexually dimorphic development of somatic gonadal cells via nonautonomous cell-cell signaling [DeFalco et al., 2008] and is responsible

for how the somatic gonad influences sex determination in the germline [Steinmann-Zwicky, 1994]. If we consider these findings together, it could be speculated that ecdysone signaling may promote spermatogenesis by inducing the expression of *dsx* at the proper level with proper timing. Among the EcR isoforms, EcR-A shows the highest expression in spermatocytes and is thought to play an important role in germline cells in *Drosophila* [Ogata et al., 2012]. This is reminiscent of our results where KD of *EcR-A* severely reduced the expression level of *Bmdsx* (Fig. 4B). EcR-A may have a conserved function to transmit ecdysone signals to *dsx*.

Another example where ecdysone triggers the differentiation of sexually dimorphic traits is reported in the tussock moth *Orgyia recens*. Females of this species have vestigial wings, whereas males have normal wings [Yamada, 1982]. During the early pupal stage, female wing discs degenerate drastically, while male wing discs develop normally. In vitro culture experiments demonstrate that only female wing discs start degradation after 20E stimulation [Lobbia et al., 2003]. This finding suggests that the same 20E stimulus is converted to a signal that results in a different sexual development. Such signaling conversion may be achieved by sex-specifically expressed factors such as *dsx*. Further studies are needed to uncover more detailed molecular mechanisms underlying the close link between ecdysteroids and sexual differentiation.

In this study, we focused only on ecdysone signaling via EcR. However, several lines of evidence suggest that the activity of some isoforms of the Ca^{2+} -ATPases in the different membrane systems such as plasma membrane, rough endoplasmic reticulum, and smooth endoplasmic reticulum are subject to regulation by lipophilic signaling substances such as endogenous sesquiterpenoids (e.g.,

farnesol [De Loof et al., 2014]) and hormones (e.g., steroid hormones [Bhargava et al., 2000; Strehler and Zacharias, 2001]). Based on these findings, De Loof [2015] proposed a novel paradigm named “Calcigender,” where differential Ca^{2+} homeostasis enabled by the interplay between farnesol-like endogenous sesquiterpenoids (juvenile hormones in insects) and steroid hormones (ecdysteroids in insects) governs the female-male physiological dimorphism. Depending on the species, the plasma membrane Ca^{2+} pumps can occur in more than 1 allele (4 alleles in human [Strehler and Zacharias, 2001], only 1 coding gene in *Drosophila* but 11 isoforms [De Loof et al., 2014]).

If sex-specific allele or splice variants of Ca^{2+} -ATPases exist, it can be easily imagined that they will produce the sexual difference in Ca^{2+} homeostasis in response to ecdysone and 20E stimuli. Further studies are needed to verify the validity of this hypothesis.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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