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Environmental Sex Determination in the Branchiopod Crustacean *Daphnia magna*: Deep Conservation of a *Doublesex* Gene in the Sex-Determining Pathway

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Abstract

Sex-determining mechanisms are diverse among animal lineages and can be broadly divided into two major categories: genetic and environmental. In contrast to genetic sex determination (GSD), little is known about the molecular mechanisms underlying environmental sex determination (ESD). The *Doublesex* (*Dsx*) genes play an important role in controlling sexual dimorphism in genetic sex-determining organisms such as nematodes, insects, and vertebrates. Here we report the identification of two *Dsx* genes from *Daphnia magna,* a freshwater branchiopod crustacean that parthenogenetically produces males in response to environmental cues. One of these genes, designated *DapmaDsx1*, is responsible for the male trait development when expressed during environmental sex determination. The domain organization of DapmaDsx1 was similar to that of Dsx from insects, which are thought to be the sister group of branchiopod crustaceans. Intriguingly, the molecular basis for sexually dimorphic expression of *DapmaDsx1* is different from that of insects. Rather than being regulated sex-specifically at the level of pre–mRNA splicing in the coding region, *DapmaDsx1* exhibits sexually dimorphic differences in the abundance of its transcripts. During embryogenesis, expression of *DapmaDsx1* was increased only in males and its transcripts were primarily detected in male-specific structures. Knock-down of *DapmaDsx1* in male embryos resulted in the production of female traits including ovarian maturation, whereas ectopic expression of *DapmaDsx1* in female embryos resulted in the development of male-like phenotypes. Expression patterns of another *D. magna Dsx* gene, *DapmaDsx2*, were similar to those of *DapmaDsx1*, but silencing and overexpression of this gene did not induce any clear phenotypic changes. These results establish *DapmaDsx1* as a key regulator of the male phenotype. Our findings reveal how ESD is implemented by selective expression of a fundamental genetic component that is functionally conserved in animals using GSD. We infer that there is an ancient, previously unidentified link between genetic and environmental sex determination.

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Introduction

Sex determination is a fundamental biological process. It affects not only the sexual differentiation of gonads, but also the development of most organs, and leads to sex-specific differences in behavior, physiology and morphology. Organisms have evolved a variety of different sex-determining systems [1,2] that can be broadly divided phenomenologically into two categories: genetic and environmental [3]. Genetic sex determination (GSD) is attributed to the genetic segregation of genes, often residing on sex chromosomes that initiate alternate sex-determining developmental pathways. Environmental sex determination (ESD) is initiated by environmental cues that presumably trigger alternative genetic signals, which regulate male or female sex-determining genes [4]. Although GSD is a more prevalent system in animals, ESD is also phylogenetically widespread, occurring in such diverse taxa as rotifers, nematodes, crustaceans, insects, fishes, and reptiles [5]. Environmental cues involved in ESD include temperature, photoperiod, nutrition, and population density [5]. Temperature is the most widely studied environmental cue, particularly in the case of reptiles where the temperature at which the egg is incubated determines sex [6]. ESD has arisen repeatedly during evolution [7], which may imply the adaptive significance of this system in environments [8].

It has long been suggested that selection forces drove the transition between GSD and ESD [9,10]. Previous experiments using a temperature-sensitive mutation created artificially in *Drosophila melanogaster* and *Caenorhabditis elegans* also demonstrated how GSD could rapidly evolve into ESD as a consequence of a mutation in a single control gene [11,12]. In addition, orthologs of some genes involved in GSD have been examined in species that use ESD, especially in temperature-dependent sex-determining reptiles [13]. Some of those are expressed in the gonads during the temperature-sensitive period [13]. These observations have led to the hypothesis that both ESD and GSD should have the same origin and share similar genetic components in their sex-determining

Author Summary

Sex determination is a fundamental biological process that can be broadly divided into two major categories. In genetic sex determination (GSD), sex-specific differentiation results from intrinsic genetic differences between males and females, whereas environmental sex determination (ESD) relies on environmental signals to induce male or female sex determination. In contrast to model organisms that utilize GSD system, environmental sex-determining organisms are poor genetic models. Therefore, although candidate genes involved in ESD have been found in vertebrates, their functions have remained largely unknown, impairing our understanding of ESD and making the comparison of sex-determining genes between both systems difficult. Here, we report the identification of a gene responsible for the production of males during environmental sex determination in the crustacean *Daphnia*. This gene is homologous to the *Doublesex* gene that is functionally conserved in animals that use GSD. Expression of *Doublesex* was increased primarily in male-specific structures. Gain- and loss-of-function analyses established that *Daphnia Doublesex* gene is a major effector that regulates the male phenotype in *Daphnia*. We infer that there is an ancient, previously unidentified link between genetic and environmental sex determination.

pathways [4]. However, a complete functional analysis of temperature dependent ESD has not yet been performed. Therefore, analyzing function of genes involved in ESD and unraveling the sex-determining pathways are crucial to understand the origin and evolution of sex-determining pathway.

The water flea *Daphnia magna* is a branchiopod crustacean, which is a common inhabitant in fresh water ponds in Europe and Asia. *D. magna* is known to switch between parthenogenetic and sexual reproduction when environmental quality declines [14]. Normal, healthy populations are entirely female. However, shortened photoperiod, a lack of food and/or increased population density, lead to the clonal production of males that are genetically identical to their sisters and mothers. First instar male juveniles are easily distinguished from the females by their elongated first antennae [15]. During maturation, daphnids undergo morphological sexual differentiation of various somatic tissues including the first thoracic leg that is armed with the copulatory hook in males, which becomes larger in the fifth instar [16]. Gonads develop and finally settle at both sides of the gut during embryogenesis in both males and females [17]. It has been reported that the gonads exhibit morphological sex differences in the first instar juveniles [18,19]. The appearance of males allows sexual reproduction to occur [20,21] when females begin producing haploid eggs requiring fertilization.

Recently, we and others have shown that juvenile hormone analogs (JHA) induce male production in cladoceran crustaceans without environmental cues [22,23]. Interestingly, exposure of *D. magna* to JHA at the stage corresponding to the environmentally-sensitive period for the other cladoceran *Moina* sex determination [24], reliably produces exclusively male broods, suggesting that juvenile hormone could be a key molecule for understanding environmental sex determination [22,25,26]. Together with the growing genome and transcriptome resources for *Daphnia* [27-30], this system is ideal to study genes responsible for ESD.

Mechanisms underlying genetic sex-determining pathways have been extensively studied in model organisms such as *D. melanogaster*, *C. elegans* and mouse and key genes have been identified [31-33]. A *Doublesex* (*Dsx*) gene was originally identified in *D. melanogaster* as a critical transcription factor considered to be at the end of sex determination cascades in GSD, that directly targets genes conferring sexually dimorphic traits [34]. Dsx contains two conserved domains: one is the Dsx/Mab-3 (DM) domain at the N-terminus that is evolutionarily conserved even in vertebrates [35] and another is the oligomerization domain at the C-terminus [36]. Genes encoding DM-domain (DM-domain genes) were discovered to play a related role not only in *C. elegans* [37,38], but even in vertebrates [39]. In contrast, results from numerous studies have shown that other genetic sex-determining genes are widely diverse among species [1,2,40].

To understand the molecular and evolutionary relationships between GSD and ESD, we analyzed the function of two *Dsx* genes from *D. magna* using gene manipulations that we have developed [41]. We provide evidence that one of the homologs, termed *DapmaDsx1*, plays an important role in directing the major sexually dimorphic development of *D. magna*. Intriguingly, the function of *Dsx* is significantly conserved between *Daphnia* and genetic sex-determining insect species, which are thought to be the sister group of branchiopod crustaceans [42]; however, the factors that regulate *Dsx* gene expression are independently co-opted in each lineage. Our functional demonstration that *Dsx* controls sexual dimorphism in an environmental sex-determining organism supports the hypothesis that genetic and environmental sex determination are similar at their most fundamental level.

Results

Molecular Cloning of Two *Doublesex* Genes from *Daphnia*

In an effort to understand environmental sex determination, we previously identified three DM-domain genes from *D. magna* and showed that two of the three DM-domain genes have sex dimorphic gene expression pattern in adult gonads [43]. However, none of these DM-domain genes exhibited sexually dimorphic expression patterns during embryonic development, suggesting that they are not involved in sex determination (Figure S1). This result prompted us to search for other DM-domain genes that might be involved in *Daphnia* sex determination. Two additional DM-domain genes were found in the *D. magna* EST database [27] and we cloned and sequenced cDNAs encoding each.

These newly identified DM-domain genes showed greater sequence similarity at the amino acid sequence level to known insect *Dsx* genes than to the previously identified *Dsx*-related genes. Therefore, these were designated *Daphnia magna Dsx* (*DapmaDsx*). One of the *DapmaDsx* genes (*DapmaDsx1*) encodes a protein of 330 amino acids from two mRNAs (*DapmaDsx1-α* and *DapmaDsx1-β*) that differ only in their 5'UTR (Figure 1A and Figure S2). The other (*DapmaDsx2*) encodes a protein of 314 amino acids (Figure S3). The predicted protein products of these two genes share 38% overall amino acid identity and both contain a DM-domain (Figure 1B). In addition, both genes also have an oligomerization domain that is characteristic for insect Dsx homologs (Figure 1C). Dimerization, which enhances specific DNA binding, is mediated by an extensive non-polar interface conserved within oligomerization domain. Importantly, in DapmaDsx2, two of three non-polar amino acids important in formation of the interface are substituted with the acidic amino acid, aspartic acid (Figure 1C). Phylogenetic analysis of these DM-domain genes confirmed that *Daphnia Dsx* genes are most closely related to the insect *Dsx* genes, but that the two *Daphnia* genes are paralogs that duplicated, forming a tandem gene cluster after the divergence of insects and crustaceans (Figure 1A and Figure S4).

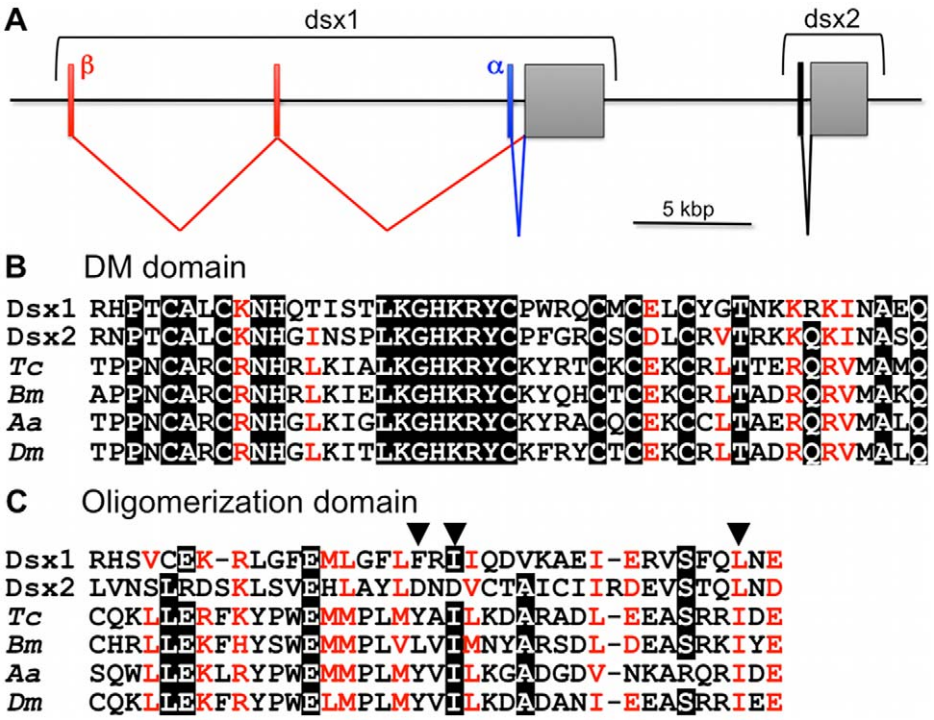

Figure 1. *Daphnia Dsx* genes. (A) Genomic organization of two dsx genes in *Daphnia magna*. Coding exons encoding Dsx1 and Dsx2 are indicated as grey boxes, respectively. Exons comprising DSX1-α, DSX1-β and DSX2 5′UTR are indicated as blue, red and black bars. (B, C) Alignment of deduced amino acid sequences of DM domains and oligomerization domains of dsx genes. Amino acid sequences were aligned using CLUSTAL-W. Identical amino acids are highlighted in black. Similar amino acids are shown in red. Positions of non-polar amino acids important in formation of the hydrophobic interface between oligomerization domains in *Drosophila* Dsx protein were indicated with solid triangles [36]. *Tc*, *Tribolium castaneum*: *Bm*, *Bombyx mori*; *Aa*, *Aedes aegypti*; *Dm*, *Drosophila melanogaster*.
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Sexually Dimorphic Expression of *Daphnia* Dsx

We next examined expression levels of *DapmaDsx* genes during development by quantitative real time PCR. Expression of both *DapmaDsx1* and *DapmaDsx2* genes increased over 72 h exclusively in male embryos (Figure 2A, dsx1, dsx2). This temporal expression correlates well with the development of sexually dimorphic organs (e.g., gonads and first antennae), in which morphological sex differences are observed at 72 h after ovulation when hatchlings begin to swim out from the brood chamber. This temporal expression pattern suggests that one or both of the *Daphnia Dsx* genes might play a critical role in sexual differentiation. To examine whether the two different *DapmaDsx1* promoters are used in a temporally independent manner, the expression levels of *DapmaDsx1-α* and *DapmaDsx1-β* mRNAs were evaluated. Both mRNAs increased only in males during early development, suggesting that both promoters function primarily during male development (Figure 2A, dsx1-α). *DapmaDsx1-β* mRNA could be detected immediately post-ovulation in males and females (Figure 2A, dsx1-β), suggesting that maternal *DapmaDsx1-β* mRNA is transferred to ovulated eggs.

We next examined whether *DapmaDsx* genes are expressed in male specific structures. Both *DapmaDsx1* and *DapmaDsx2* genes were highly expressed in the testis (Figure 2B). Whereas *DapmaDsx1-α* mRNA was expressed exclusively in the testis, *DapmaDsx1-β* mRNA was expressed in both testis and ovary. Whole mount *in situ* hybridization showed that *DapmaDsx1* and *DapmaDsx2* could both be detected in the first antennae and the first thoracic segments (Figure 2C and 2D), both of which are known to show sexually dimorphic characteristics. *DapmaDsx2* is also expressed in female first thoracic segments though apparently more weakly than in males. Male-specific expression was also observed in the compound eye, whose sex difference has not been reported in this species to date. Taken together, these male specific expression patterns are regulated temporally and spatially, supporting strongly the involvement of *DapmaDsx* genes in male differentiation in *Daphnia*.

Daphnia Dsx1 Is Necessary for Male Trait Development

The sexually dimorphic expression of *DapmaDsx* mRNAs led us to hypothesize that the expression level of the *DapmaDsx* transcripts could mediate sex determination. To test this hypothesis, we established a technique to introduce exogenous genes into ovulated eggs and developed a dsRNA-based gene knockdown technique for *D. magna* [41]. Eggs induced to become males by fenoxycarb exposure to the mother during a critical stage of oocyte development [22,26] were injected with *Dsx*-specific, or control dsRNAs, grown to the swimming juveniles and evaluated the phenotypes.

At the third instar stage, microinjection of the *DapmaDsx1*-specific dsRNAs resulted in development of the shortened first antenna whose length was the same as that of females in all of the *DapmaDsx1*-dsRNA-injected juveniles (Figure 3A). At the fifth instar stage, we dissected the feminized daphnids and found that the first thoracic appendage lacks a hook used in clasping the females and has a female-like long filament instead (Figure 3B). Correspondingly, repression of *DapmaDsx1* resulted in the development of ovaries during which oocytes accumulate yolk granules and lipid droplets as well as those of wild-type females (Figure 3C). In contrast, microinjection of *DapmaDsx2*-dsRNAs did not induce the formation of either female-like somatic or gonadal tissues (Figure 3A–3C, Table 1).

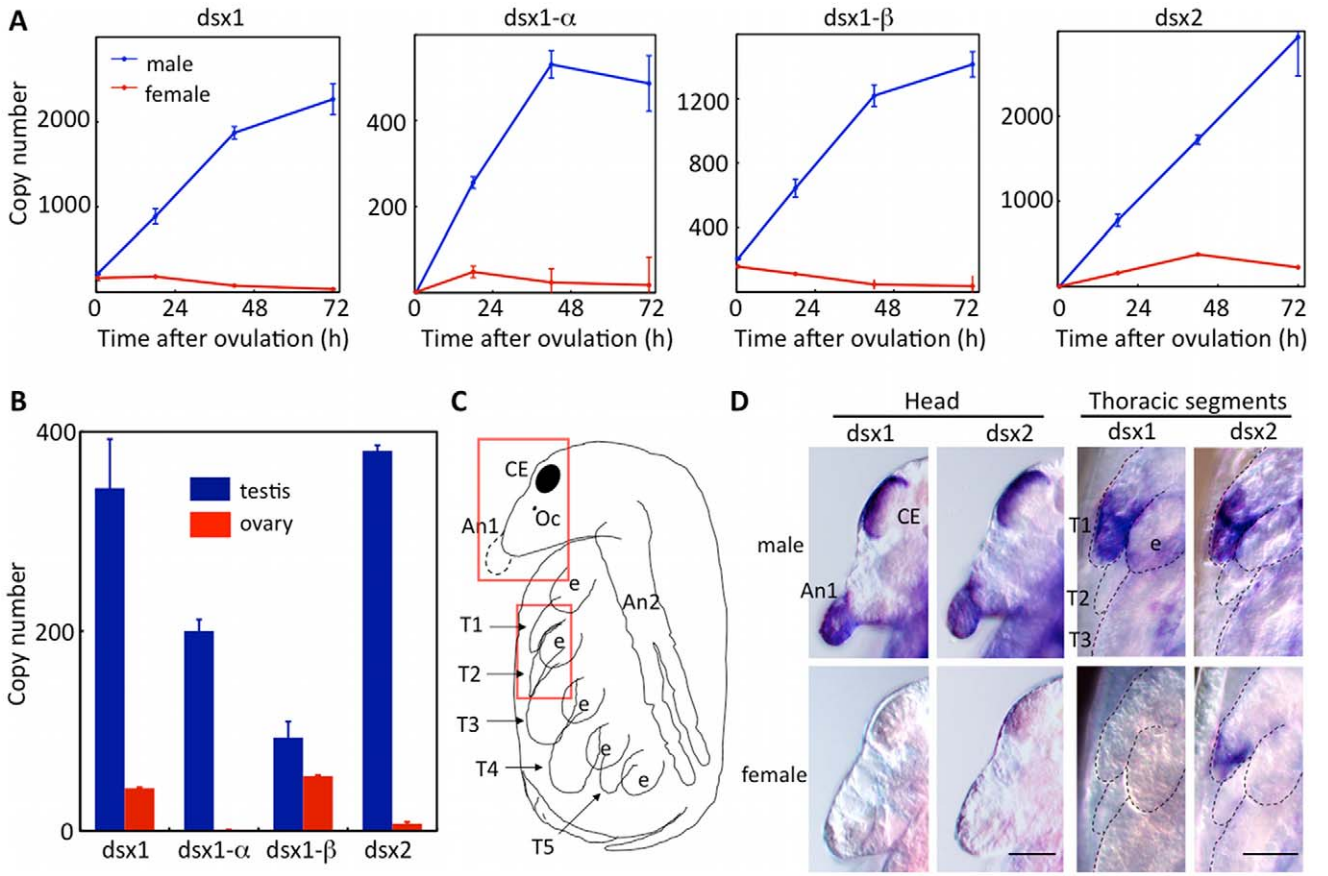

Figure 2. Temporal and spatial dimorphic gene expression of *Dsx* genes during development. (A) Male and female embryos were obtained and gene expression levels of *Dsx1* and *Dsx2* were determined at 18 h, 42 h and 72 h after ovulation by quantitative RT-PCR using primers corresponding to *Dsx1* and *Dsx2* coding sequence (CDS). Dsx1-α and DSX1-β transcripts were also quantified using primers specific to each 5′UTR. Copy numbers were estimated by quantification compared with an external standard and dividing by the number of embryos used. Bars indicate S.E.M. (B) Expression of dsx genes in adult gonads was quantified. PCR primers corresponding to *Dsx1* CDS, two types of 5′UTRs of *Dsx1* gene and *Dsx2* CDS were used for quantitative PCR. Bars indicate S.E.M. (C) Schematic illustration of *D. magna* late embryo. The red boxes indicate the areas shown in panel D. (D) Whole mount *in situ* hybridization in late embryos using DIG-labeled probes corresponding to *Dsx1* and *Dsx2*. The heads and the thoracic segments are magnified. Bars indicate 50 μm (heads) and 100 μm (thoracic segments). CE: Compound eye, Oc: Ocellus, An1: First antennae, An2: Second antennae, e: Epipod, T1-5: First to fifth thoracic segments.
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The quantity of *DapmaDsx1* and *DapmaDsx2* mRNA was decreased to 40% and 20%, respectively, relative to control-dsRNA-injected embryos. Reduction of transcripts from either of the two *DapmaDsx* genes did not change the mRNA level of the other (Figure 3D), suggesting that the *DapmaDsx* genes do not regulate each other's expression or stability. We confirmed the specificity of the knockdown by using non-overlapping ds-RNAs for each *DapmaDsx* gene (Figure S5). As expected, only *DapmaDsx1* RNAi induced the same phenotypic changes (Table 1). Since the size of testes appears to be somewhat reduced following the *DapmaDsx2*-dsRNA injection (Figure 3C), we cannot rule out some low-level requirement for DapmaDsx2 function. However, these results suggest that *DapmaDsx1* is necessary for sex determination.

To ask whether expression of *DapmaDsx1* or *Dsx2* might be sufficient to trigger male development in females, we developed a technique for transient transgenesis in *Daphnia* embryos by microinjection of capped, polyadenylated mRNAs into ovulated eggs. When the *DapmaDsx1* or *DapmaDsx2* mRNA was introduced into female eggs, only *DapmaDsx1* mRNA partially induced elongation of the first antenna in females at 72 h after microinjection (Figure 4 and Table 2). Unfortunately, due to the transient nature of this method, the masculinization of the first antennae was observed only in the first instar juveniles, whose gonads were too small to evaluate the sex-reversal. Taken together, these data show that although they are similar in amino acid sequence and expression pattern, *DapmaDsx1* and not *DapmaDsx2* plays a primary role for male trait development.

Daphnia Dsx1 Gene Lacks the Phylogenetically Conserved Splicing Variants

Recent phylogenetic analyses [44-46] and developmental genetics [47,48] suggest that insects may be a sister group to branchiopod crustaceans, a group that includes daphnids (water fleas) and brine shrimp *Artemia* [42]. As it is known that insect *Dsx* genes express sex-specific variants in their coding sequences, we next examined whether the sex-specific splicing of the *Daphnia Dsx1* gene also occurs. Only a single amplified cDNA could be detected from either male or female by RT-PCR with primers to amplify a coding sequence, although PCR products obtained from females were very faint (Figure 5A). We sequenced the cDNA fragments and confirmed no sex differences of the fragments.

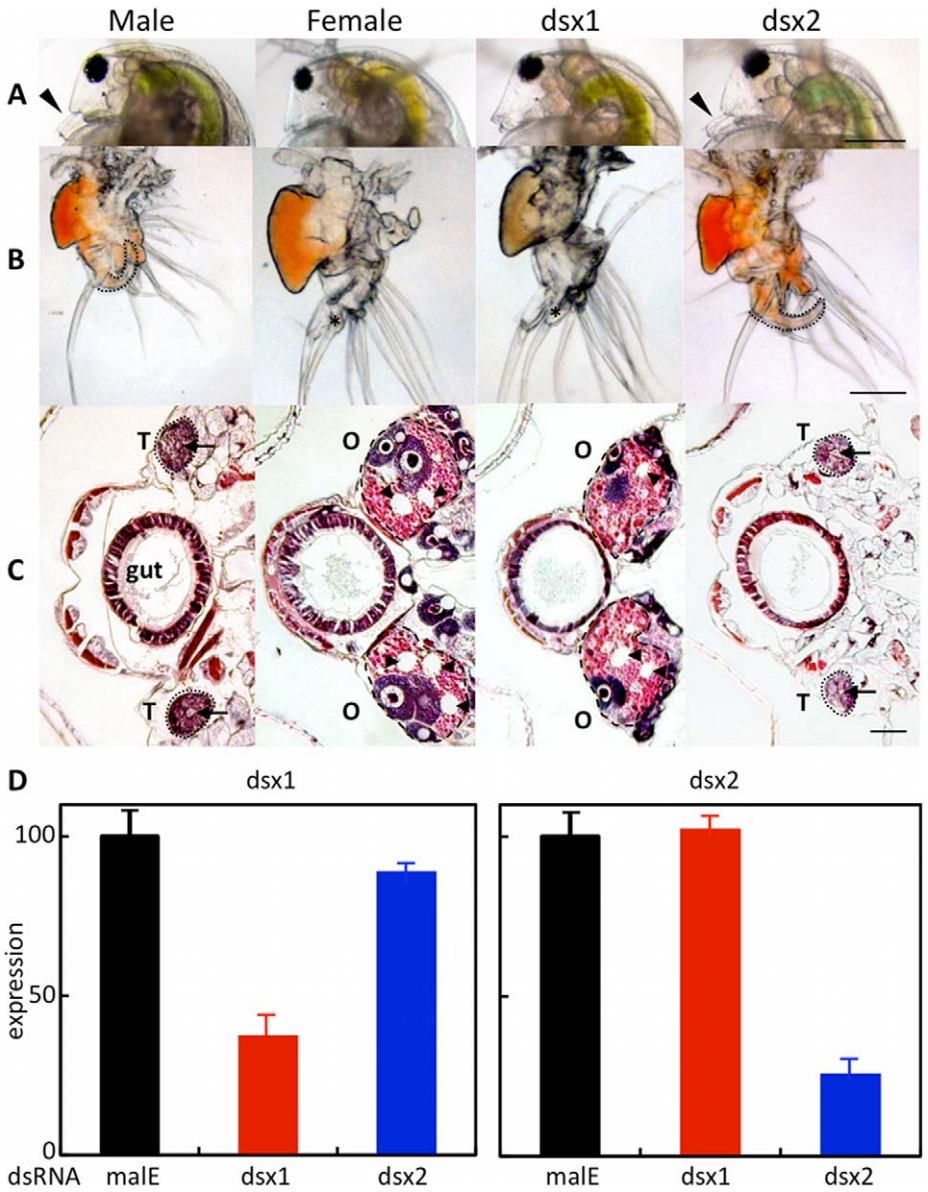

Figure 3. Dimorphic development of *Daphnia magna*. Eggs induced to become males were obtained from *D. magna*. After injection of the synthesized dsRNA, sexually dimorphic phenotypes were examined at the fifth or sixth instar except first antennae (third instar). The first two columns represent normal male and female phenotypes, respectively. The third and fourth columns represent phenotypes of individuals injected with #1-dsRNA of dsx1 and dsx2, respectively. (A): Lateral view of the head. Arrowheads indicate the first antennae. (B): First thoracic limb. Dotted line shows the outline of the stout chitinized hook. A female-type long filament corresponding to the hook is labeled with an asterisk. (C): Gonad. Daphnids were embedded in paraffin and sectioned, following by standard hematoxylin and eosin staining. Dorsal is left, ventral is right. Dotted circled lines show gonads at both sides of a gut. T and O indicate testis and ovary, respectively. Arrowheads indicate large lipid droplets lying among the eosinophilic yolk granules. Arrows indicate lumens into which the mature spermatozoa are released. (D): Gene expression profile of *Dsx1* and *Dsx2* in embryos injected with dsRNA of *Dsx1* (left panel) and in dsRNA of *Dsx2* (right panel). The *MalE* gene from *E. coli* was used as a control gene. Bars in (A), (B) and (C) indicate 200, 100, 50 μm, respectively.
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Further, to find altered *DapmaDsx1* mRNA length and abundance, we used a northern blot with an antisense probe hybridized to the coding region. In male adults, three transcripts of approximately 4 kb, 2.8 kb, and 2 kb were detected. Of the three, 2.8 kb transcripts were the most abundant. In contrast, only faint 2 kb transcripts were detected in female adults (Figure 5B). As length differences between 5'UTR-α and -β were only 112 bases, one explanation for large differences of the transcript lengths might be the 3'UTR lengths. To determine the 3'end of *DapmaDsx1* mRNAs, 3'RACE was performed. We identified four tandem polyadenylation sites located downstream of the stop codon and found that alternative usage of those sites was correlated well with production of the three *DapmaDsx1* transcripts. The canonical AAUAAA signal was used for 2.8 kb transcripts, the variant AAUAUA for the 4-kb, and for the shortest 2 kb transcripts the two variant signals were used (AAGAAA or AAUUGA, Figure 5D, Figure S2). Interestingly, RT-PCR analysis with primers to discriminate between 5'UTR-α and -β showed that female 2 kb transcripts were only *DapmaDsx1-β* mRNAs with the shortest 3'UTR that

Table 1. Summary of RNA interference using dsRNA.

dsRNA	Sex	Short first antennae	Ovarian development	First leg with a hook
malE*	♂	0% (0/40)	0% (0/31)	100% (13/13)
	♀	100% (45/45)	100% (28/28)	0% (17/17)
dsx1-#1	♂	100% (48/48)**	98% (47/48)**	0% (13/13)**
dsx1-#2	♂	100% (39/39)**	41% (7/17)**	0% (17/17)**
dsx2-#1	♂	0% (0/36)	0% (0/36)	100% (15/15)
dsx2-#2	♂	0% (0/45)	0% (0/24)	100% (18/18)

*malE gene from *E. coli* was used as control.
**$P<0.05$ versus malE (Fisher's exact probability test).
doi:10.1371/journal.pgen.1001345.t001

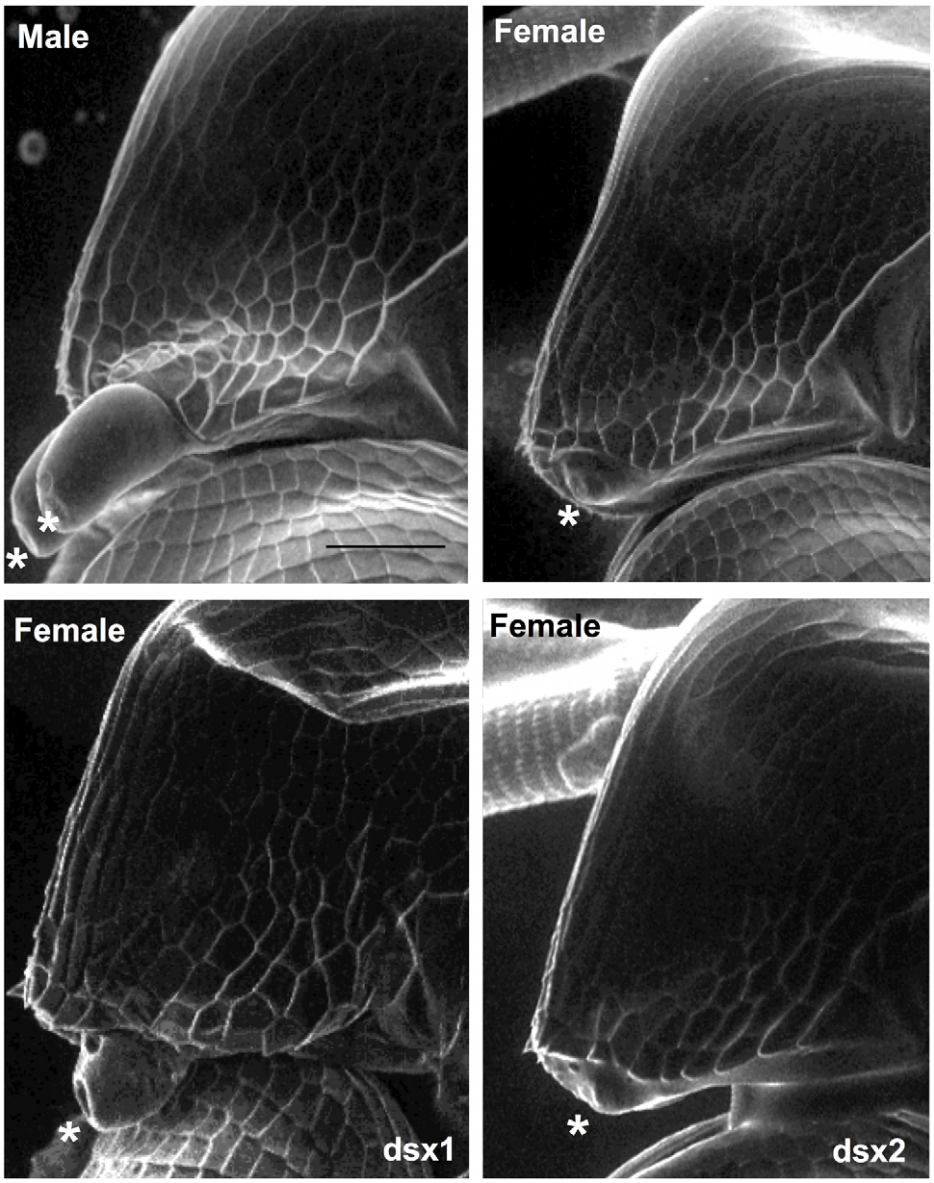

Figure 4. Elongation of 1st antenna by the expression of *Dsx1* gene. The mRNA was injected to embryos within one hour after ovulation and observed using electron microscope after 72 h. Male and female indicate normal phenotype of each sex. Dsx1 and Dsx2 indicate mRNA of *Dsx1* and *Dsx2*, which were injected to female eggs, respectively. Asterisk indicates first antennae. Bar indicates 100 μm.
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Table 2. Summary of effects caused by ectopic expression of *Dsx* genes in female embryos.

mRNA	Amounts (pg)	Eggs injected	Juveniles	Viability	Long first antennae
dsx1-α	1000	128	81	63%	21% (17/81)*
dsx1-β	1000	124	98	79%	8% (8/98)*
dsx2	500	94	54	58%	0% (0/54)
egfp	1000	60	49	82%	0% (0/49)

Equal molar amounts of dsx1-α, dsx1-β, and dsx2 mRNA were injected. egfp was used as control. * $P<0.05$ versus egfp (Fisher's exact probability test).
doi:10.1371/journal.pgen.1001345.t002

presumably were supplied to eggs as maternal mRNAs (Figure 5C).

Discussion

We report here that gain- and loss-of-function analyses in environmental sex-determining *Daphnia* have allowed us to characterize the function of *DapmaDsx1* in sexual dimorphism and provide insight into the molecular relationship between GSD and ESD. We and others previously showed that exposure to juvenile hormone analogs reliably produces male *Daphnia* [25,49]. This finding enabled us to examine genes in embryos directed to either male or female. Together with the growing genome and transcriptome resources and gene manipulation techniques for *Daphnia* [27-30,41,50], this species is a first crustacean model that provides novel insights into understanding evolution of the sex-determining pathway.

The molecular mechanisms of genetic sex determination have been well studied in a few model organisms, such as the mouse, fruit fly and nematode. DM-domain genes are highly conserved and involved in sexual differentiation of these species [38]. The DM-domain gene was also identified as a sex-determining gene in some populations of fish of the genus *Medaka* [51]. Moreover, recently, molecular analyses of GSD in the frog *Xenopus laevis* [52] and chicken *Gallus gallus* [53] demonstrate deep conservation of DM-domain genes in GSD. To our knowledge, this study is the first *in vivo* demonstration that the *Dsx* gene, a fundamental genetic component that is functionally conserved in animals using GSD, can also implement ESD. Interestingly, in reptiles with temperature-dependent sex determination, the *Dsx* ortholog, *Dmrt1* is

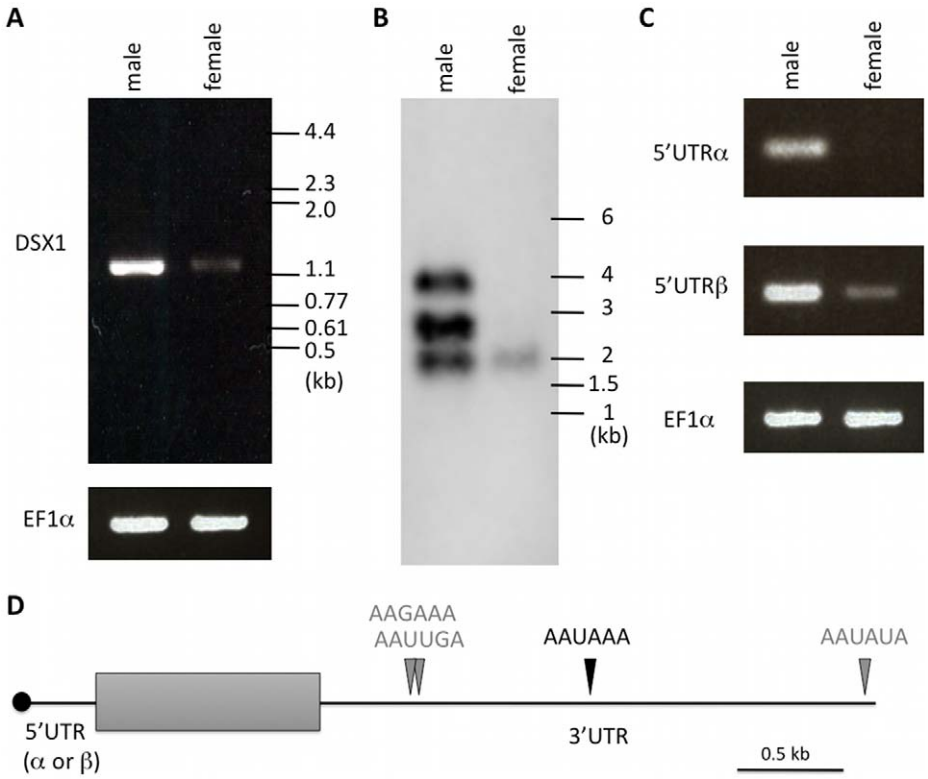

Figure 5. Structure of *Dsx1* mRNAs expressed in males and females. (A) RT-PCR using oligonucleotides corresponding to 5′- and 3′-ends of *Dsx1* CDS. The amplified cDNAs were resolved by agarose gel electrophoresis. (B) A northern blot probed for dsx1 mRNAs. Migration of markers with lengths indicated (kb) is shown at the right. (C) RT-PCR using oligonucleotides to amplify 5′UTR-α and -β of *Dsx1* gene. The amplified cDNAs were resolved by agarose gel electrophoresis. (D) Schematic illustration of *Dsx1* mRNAs with alternative isoforms due to usage of alternative promoters and polyadenylation signals. A grey box shows protein coding region; black line represents untranslated regions. Canonical and non-canonical functional polyadenylation signals identified by 3′RACE are indicated with black and grey arrowheads.
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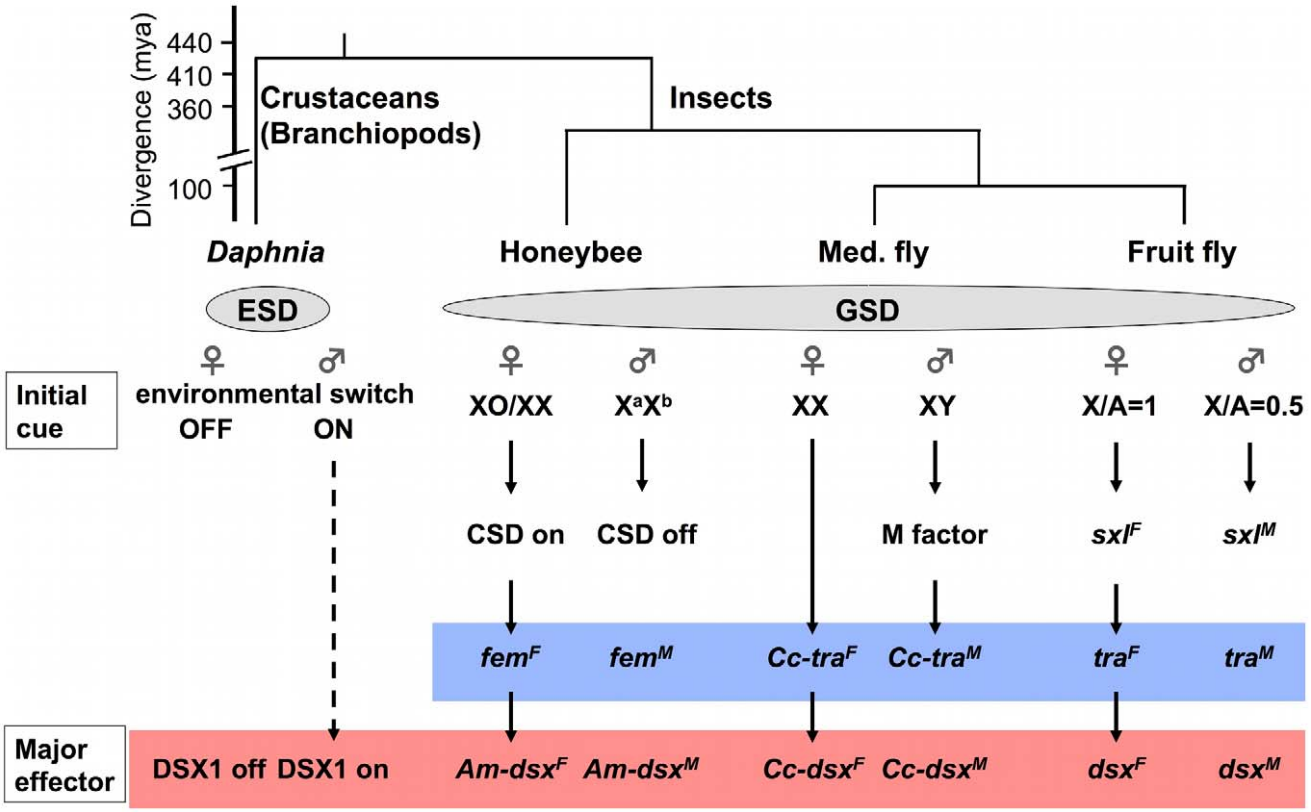

Figure 6. Simplified view of sex-determining pathways in the branchiopod crustacean *Daphnia* and insects. An ESD pathway in *Daphnia* is compared with GSD pathways in insect model species, honeybee (*Apis mellifera* [64]), Mediterranean fruit fly (Med. fly, *Ceratitis capitata*, [62]) and fruit fly (*Drosophila melanogaster* [78]). Conserved *Doublesex* and *Transformer* homologs are indicated with red and blue boxes, respectively. Phylogenetic relationships among the four species are shown above the pathways [42,64,71]. CSD, complementary sex determiner; *fem*, *feminizer*; *Am*, *Apis mellifera*: *Cc*, *Ceratits capitata*; *sxl*, *sex lethal*; mya, million years ago.
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regulated by temperature [54-56]. These indicate that DM-domain genes also play an important role in environmental sex determining organisms, supporting the hypothesis that both ESD and GSD have the same origin and share similar genetic components in their sex determining pathways.

Crustacean and Insect Doublesex Share Molecular Characteristics to Function as a Major Effector of Sexual Dimorphism

DapmaDsx1, one of two Dsx homologs from *Daphnia*, shares several important characteristic features of *Drosophila* Dsx protein to function as a major effector of sexual differentiation [40,57,58]. First, DapmaDsx1 protein is composed of two domains, the phylogenetically conserved DM-domain [35] and the insect-specific oligomerization domain. In contrast, the DapmaDsx2 protein appears to be unable to regulate sexual differentiation and contains mutations at important amino acids of oligomerization domain [36], suggesting that this domain might be necessary for establishing sexual dimorphic traits. Second, male-specific expression of *DapmaDsx1* is regulated temporally and spatially during development. Although it has long been believed that *Drosophila Dsx* gene was cell-autonomously expressed in all cells [59], Robinette *et al.* [60] recently reported that *Drosophila* also exclusively expressed *Dsx* in sexually dimorphic tissues and cells. Third, and perhaps most importantly, knock-down of *DapmaDsx1* in male embryos resulted in the production of female traits including ovarian maturation whereas ectopic expression of *DapmaDsx1* in female embryos resulted in partial masculinization of the first antennae. These results suggest that *Dsx* gene expression in sexually dimorphic tissues is a key process to induce sexual differentiation in crustacean *Daphnia* and insects.

Factors Regulating Expression of *Doublesex* Are Independently Co-Opted during Evolution in Each Lineage

In the fruit fly *D. melanogaster*, *Dsx* is spliced in the coding region by the Tra protein in a sex-dependent manner [34]. The female-specific RNA produced by alternative splicing is a functional mediator of Tra activity [61]. The female-specific splice variant of the *Tra* homologs encodes a functional protein not only in the Mediterranean fruit fly [62] and the house fly [63], but also in the honeybee *Apis mellifera* [64]. In all insect species studied to date (except the silkworm *Bombyx mori*) [65], Tra regulates sex-specific splicing of *Dsx*, which produces different mRNAs and proteins, resulting in sex-specific transcriptional activation and repression [66] (Figure 6). Sex-specific splicing of the *Dsx* gene by the Tra protein might be ancestral in insects. In contrast, *DapmaDsx* genes do not encode sex-specific Dsx proteins, but instead exhibit sexually dimorphic differences in the abundance of its transcripts. Interestingly, *Daphnia* has a homolog of the Tra protein but the *D. magna Tra* gene does not display any detectable sexually dimorphic differences in expression or splicing patterns [26]. We also performed knock-down of the *D. magna Tra* gene, but could not find any effect for development of sexually dimorphic traits (data

not shown). This is consistent with the apparent lack of a sex-specific splicing in the *Dsx1* gene. Although it is not yet clear if juvenile hormone directly activates the transcription of *DapmaDsx* genes, this remains an interesting possibility for future study. We found several motifs in promoter regions of the *Dsx1* and *Dsx2* genes, which resemble JH-responsive elements previously reported in *D. melanogaster* [67] and *D. magna* [68] (data not shown), suggesting that these motifs possibly function as elements to regulate the JH-dependent gene expression. The detection of unfavorable environmental conditions by *Daphnia* could be transmitted to the endocrine system, leading to the release of juvenile hormone to convey the environmental signals to sexually dimorphic cells. This would be a simple and elegant type of sex determination cascade. Understanding the molecular nature by which the transcription of the *DapmaDsx* genes is regulated remains an important future goal that will greatly enhance our understanding of not only sex determination, but also invertebrate hormonal systems.

Interestingly, expression of the *DapmaDsx1* gene utilizes alternative polyadenylation at tandem poly(A) sites, which can yield transcripts that have identical protein-coding sequences but different 3'UTR sequences. Alternative polyadenylation is often associated with tissue- or developmental stage-dependent gene expression [69,70]. We found that female *DapmaDsx1-β* mRNAs exclusively use the most promoter-proximal polyadenylation signals. The presence of alternative polyadenylation sites in *Dsx* genes has been reported in three insects, *D. melanogaster*, the phorid fly *Megaselia scalaris* and the mosquito *Anopheles gambiae*, indicating that regulation of the 3'UTR length might be a common mode to regulate expression of *Dsx* genes.

Despite having last shared a common ancestor with insects about 400 million years ago [42,71] and differences of the initial cue to determine sex, the *DapmaDsx1* maintained the domain structure essential for establishing sexual dimorphism, while regulation of its expression by other factors became complex and diverse. This is consistent with the prediction that new signals are co-opted upstream of a cascade during the course of evolution [72,73]. Thus, we have established that there were no boundaries between GSD and ESD in evolution of sex-determining genes at their most fundamental level.

Materials and Methods

Daphnia Strain and Culture Conditions

The *Daphnia magna* strain (NIES clone) was obtained from the National Institute for Environmental Studies (NIES; Tsukuba, Japan) and maintained as described previously [25]. In order to obtain male embryos, adult *D. magna* (about 2 weeks of age) were treated with a synthetic juvenile hormone mimic, fenoxycarb (1 μg/L), and eggs ovulated into the brood chamber were collected.

Cloning of DSX-Like Genes

The amino acid sequence of the *Drosophila melanogaster Dsx* gene was retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/) and used to search the *D. magna* EST database for related sequences. Two EST sequences were identified to have similarities with the *Drosophila Dsx* gene. The harvested daphnids were briefly washed and homogenized using the Physcotron NS-310E (Nichion, Tokyo, Japan). Total RNA was extracted with TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Poly (A)+ RNA was isolated from purified total RNA using Fast Track (Invitrogen) and converted to cDNA using Superscript III and random primers (both Invitrogen) according to the manufacturer's protocol. cDNAs corresponding to the EST sequences were obtained by PCR, and full length cDNAs were obtained by RACE (Cap Fishing, SeeGene, Seoul, South Korea) using the oligonucleotide sequences shown in Table S1. Sequence data from this article have been deposited with the DDBJ/EMBL/Genbank Data Libraries under Accession No. AB569296, AB569297, AB569298.

Phylogenetic Analysis of the DM Domain Genes

A phylogenetic tree of DM domain genes including newly cloned *D. magna Dsx* genes was constructed using amino acid sequences of DM-domain genes used in the previous study [43] and insect *Dsx* genes listed in Table S2. A multiple alignment was constructed using Clustal W [74] with the following settings (pairwise alignment parameters: gap opening penalty 6.00, gap extension penalty 0.21, identity protein weight matrix; multiple alignment parameters: gap opening penalty 10.00, gap extension penalty 0.24, delay divergent cutoff 30%, gap separation distance 4). Phylogenetic reconstruction was performed using the p-distance algorithm and the neighbor-joining method implemented in *MEGA* version 4 [75]. The phylogenetic tree was rooted to vertebrate DMRT7 outgroups (mouse; NP_082008, bovine; NP_0010332710, data not shown).

Quantitative PCR

Embryos were obtained from *D. magna* at two weeks of age. Ovulation occurred just after molting and was assigned to be 0 h. The embryos were collected 18 h, 42 h and 72 h after ovulation. Gonads were isolated and specific mRNAs were quantified as described previously [43]. The oligonucleotide sequences for PCR were indicated in Table S3.

Whole Mount *In Situ* Hybridization

Templates for the probe preparation were synthesized by PCR using gene-specific primers containing the T7 polymerase promoter sequence at their 5'-ends (Table S4). DIG-labeled probes were prepared as described by Butler *et al.* [76] and subjected to alkaline hydrolysis. Whole mount *in situ* hybridization was performed as described by Sagawa *et al.* [17]. Both antisense and sense probes were used to confirm the specificity of staining.

Double-Strand RNA (dsRNA) Preparation

Double-stranded RNA was synthesized using the MEGAscript high yield transcription kit (Ambion, Austin, TX, USA). Templates were prepared by PCR using gene-specific primers with the T7 polymerase promoter sequence at their 5'-ends (Table S5). The synthesized RNAs were purified using phenol/chloroform. Following ethanol precipitation, the RNA was resuspended in DNase/RNase-free distilled water (Invitrogen, Tokyo, Japan) and annealed [41]. Sequences corresponding to each dsRNAs were shown in Figure S2. dsRNA lengths were: Dsx1-#1, 778 bp: Dsx1-#2, 579 bp: Dsx2-#1, 703 bp: Dsx2-#2: 448 bp.

Synthesis of Capped and Polyadenylated mRNAs

DSX1-α, DSX1-β and DSX2 cDNAs were subcloned into pCS2 vector and used for RNA synthesis. To synthesize the control RNA, pEGFP-C1 vector was used. *In vitro* transcription with T7 RNA polymerase and poly-A tail addition were performed according to the manufacturers' protocol using commercial kits [mMESSAGE mMACHINE, and Poly(A) Tailing kit, respectively, Ambion]. Templates were prepared by PCR using primers corresponding to the 5'- and 3'-ends of the mRNA sequences. The T7 polymerase promoter sequence was attached to the 5' end of the forward primer.

Microinjection

Eggs were obtained from *D. magna* at two weeks of age just after the ovulation and placed in ice-cold M4 media [77] containing 40 mM sucrose (M4-sucrose). The synthesized dsRNA (1 mg/ml) containing 1 mM Chromeo 494 fluorescent dye (Active Motif Chromeon GmbH, Tegernheim, Germany) or mRNA was injected and incubated in a 96-well plate for appropriate times [41]. Equal molar amounts of DSX1-α, DSX1-β and Dsx2 mRNA were injected for the gain-of-function study. Injection volume was approximately 0.3 nL.

Microscopy

Embryos were dissected off the yolk, and photographed with a Zeiss Axioplan 2 Imaging microscope (Zeiss, Oberkochen, Germany). Adults and juveniles were observed and photographed using a Leica MZ APO dissecting microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). mRNA-injected juveniles were directly observed using an environmental scanning electron microscope (XL30 ESEM; Philips, Hillsboro, OR, USA).

Northern Blot Analysis

3 μg and 10 μg of male and female poly (A)+ RNA were used respectively. The RNAs were separated by electrophoresis on a 1.0% formaldehyde–agarose gel and then transferred to positively charged nylon membranes (Hybond-N+; GE Healthcare, Little Chalfont, England). RNA probes were prepared with a DIG RNA labeling kit (Roche Diagnostic GmbH, Manheim, Germany). Primers to amplify templates for the probe preparation were (5'-3'): forward: AAGAATTGTCCGTGGGGGCAC and reverse: TAATACGACTCACTATAGGGAAAGTTTGGTGTAGGGAG. The membranes were hybridized with DIG-labeled RNA probes for 11 hr at 68 °C with DIG easy hyb (Roche Diagnostic). DIG-labeled RNA was detected with an alkaline phosphatase-conjugated anti-DIG antibody using CDP star (Roche Diagnostic) according to the manufacture's protocol.

Supporting Information

Figure S1 Temporal embryonic gene expression profile of DM-domain genes. Embryonic gene expression levels of *dmrt11E*, *dmrt93B*, and *dmrt99B* in male (blue line) and female (red line) *Daphnia* at developmental points after ovulation. Copy numbers were estimated by quantification of plasmid DNA for each DM-domain gene and divided by the number of individuals examined. Bars indicate S.E.M.
Found at: doi:10.1371/journal.pgen.1001345.s001 (3.46 MB TIF)

Figure S2 Nucleotide and deduced amino acid sequences of dsx1 cDNA. Nucleotide sequences of UTR-α (A), UTR-β (B) are indicated. Nucleotide sequences of the common region including ORF and 3'UTR (C) are indicated with deduced amino acid sequence. Sequences corresponding to the #1-dsRNA and #2-dsRNA are highlighted in red and green. Squares indicate polyadenylation signals identified by 3'RACE.
Found at: doi:10.1371/journal.pgen.1001345.s002 (5.44 MB TIF)

Figure S3 Nucleotide and deduced amino acid sequences of dsx2 cDNA. The deduced amino acid sequence is shown below the nucleotide sequence. Sequences corresponding to the #1-dsRNA and #2-dsRNA are highlighted in red and green. The square indicates the putative polyadenylation signal.
Found at: doi:10.1371/journal.pgen.1001345.s003 (3.15 MB TIF)

Figure S4 Phylogenetic tree connecting the *dsx* genes. Branch lengths are proportional to the evolutionary distances. Numbers in each branch represent the bootstrap values obtained by Neighbor-Joining.
Found at: doi:10.1371/journal.pgen.1001345.s004 (8.92 MB TIF)

Figure S5 Shortened first antenna in males induced by RNAi using #2-dsRNAs of dsx1 and dsx2, respectively. The phenotype was examined at the third instar. Arrowheads indicate the first antenna. Bar indicates 200 μm.
Found at: doi:10.1371/journal.pgen.1001345.s005 (1.48 MB TIF)

Table S1 Primer sequences for 5'RACE and 3'RACE.
Found at: doi:10.1371/journal.pgen.1001345.s006 (0.03 MB DOC)

Table S2 Accession numbers of insect *Dsx* genes used for phylogenetic analysis.
Found at: doi:10.1371/journal.pgen.1001345.s007 (0.03 MB DOC)

Table S3 Primer sequences for quantitative real-time PCR.
Found at: doi:10.1371/journal.pgen.1001345.s008 (0.03 MB DOC)

Table S4 Primer sequences for probe preparation.
Found at: doi:10.1371/journal.pgen.1001345.s009 (0.03 MB DOC)

Table S5 Primer sequences for probe preparation.
Found at: doi:10.1371/journal.pgen.1001345.s010 (0.03 MB DOC)

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Author Contributions

Conceived and designed the experiments: YK HW TI. Performed the experiments: YK KK HW. Analyzed the data: YK KK HW TI. Contributed reagents/materials/analysis tools: YK HW. Wrote the paper: YK HW TI.

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