Doctoral Dissertation

Elucidating the role of ecdysteroid in embryogenesis of the branchiopod crustacean *Daphnia magna*

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Chapter 1 General introduction

1.1. Introduction

The endocrine hormone ecdysteroid, plays important roles in arthropod physiology, growth, and development (Lafont et al., 2012). Thus, they are believed to have contributed to the evolution of a diverse range of life strategies in arthropods, making them exceptionally abundant and ecologically diverse (Jockusch and Smith, 2015). The components of ecdysteroid such as its biosynthesis genes and signaling pathway is highly conserved among arthropod (Chelicerata, Myriapoda, Crustacea, and Insecta), which suggest its vital role in this clade (Qu et al., 2015; Schumann et al., 2018). Subsequently, this hormone has become focus of study in the field of arthropod endocrinology.

Elucidation of ecdysteroid has been benefitted from the advancement of biotechnology and molecular technique, combined with extensive use of insect model animals, such as fruit fly *Drosophila melanogaster* and silkworm *Bombyx mori*. Various knock out and knock down of ecdysteroid related genes in insect has demonstrated their function in various life stages (Gilbert, 2004; Rewitz et al., 2006). In contrast, our knowledge about ecdysteroid in other arthropod clades is scarce. This is partly not all arthropod is a convenient model animal, thus making functional analysis in the species difficult.

The freshwater crustacean *Daphnia magna* is an emerging model animal for genetic and endocrinology. *Daphnia* is the only crustacean to have both its genome sequenced and various genetic engineering tools developed. Therefore, it is a suitable model for elucidating ecdysteroid in non-insect arthropod. Moreover, being a crustacean, *Daphnia* share close relationship with commercially important species, such as shrimp and crabs, in which the knowledge of *D. magna* endocrinology can be

easily transferred to. However, the knowledge about role of ecdysteroid in *D. magna* is still limited.

1.2. The steroid hormone ecdysteroid

Ecdysteroid is a family of naturally occurring steroid hormone. It represents a large family of sterol derivatives compromising more than 300 members and their presence are varied among species (Lafont et al., 2012). Ecdysteroid can be found within animals (zooecdysteroid) and plants (phytoecdysteroid) (Baltaev and Shangaraeva, 2000; Tarkowská and Strnad, 2016). Among various ecdysteroid compounds, ecdysone (E) and its active form, 20-hydroxyecdysone (20E) are most studied and characterized because of their abundance (Lafont et al., 2012).

The study of ecdysteroid was initiated in 1954, when Butenandt and Karlson succeeded in isolating and purified this hormone from 500 kg of silkworm pupae (Butenandt and Karlson, 1954). Upon injecting the purified hormone to insect, molting was observed. Thus, this hormone is then referred as the molting hormone. Over the next 60 years, roles of ecdysteroid were re-defined, as this hormone was found to regulate not only molting, but also various physiological and developmental processes. For instance, in *Bombyx mori*, ecdysteroid was discovered (later known to be synthesized) in ovary, which then deposited into the embryos. In fruit fly *Drosophila melanogaster* larva, ecdysteroid controls deposition of new cuticle proteins, remodeling of the fat body, and prepupal development (Kozlova and Thummel, 2003). Both examples indicate that ecdysteroid also have roles for reproduction, embryogenesis, and metamorphosis.

1.2.1. Biosynthesis reaction

Ecdysteroid biosynthesis (ecdysteroidogenesis) occurs in specialized compartment named steroidogenic organs (prothoracic gland in insect, or Y-organ in crustacean). The activity of this organ is directly regulated by brain-secreted neuropeptide hormone called prothoracicotropic hormone (PTTH) (Smith and Rybczynski, 2012; Zitnan and Adams, 2012). Like all steroid hormones, precursor of ecdysteroid is sterol. Since arthropods lacking squalene synthase enzyme, they are unable to synthesize sterol *de novo* (Lafont and Koolman, 2009). Consequently, they need to rely sterol from dietary sources. Sterol derived from animal (zoosterol) are mostly cholesterol (C_{27}). Thus, arthropods feeding on animal-derived food utilized cholesterol directly. On the other hand, others feeding on plant and yeast obtain their sterol in the form phytosterol (C_{28} and C_{29}) (Tarkowská and Strnad, 2016) and need to first convert them into cholesterol by dealkylation reaction (Gilbert et al., 2002).

The early step of ecdysteroidogenesis begins with dehydrogenation of cholesterol into 7-dehydrocholesterol (7-dC). This well-understood reaction is catalyzed by 7,8-dehydrogenase enzyme encoded by *Neverland* (*Nvd*) gene (Yoshiyama et al., 2006). The next reaction to convert 7-dC into the first recognizable ecdysteroid product, 5β-ketodiol is less characterized, due to no intermediate product in this apparently multistep reactions ever observed (Lafont et al., 2012). This reaction is commonly termed as “Black box”, and considered to be the rate limiting reaction of ecdysteroidogenesis (Rewitz et al., 2009). However, two monooxygenase enzymes (*Spook*, *Spo* and *Cyp6t3*) and one dehydrogenase (*Shroud*, *Sro*) are thought to catalyze this conversion process (Enya et al., 2014; Niwa et al., 2010) (further explanation on Chapter 2).

The late hydroxylation step to convert 5β-ketodiol into ecdysone is well documented. The conversion process is catalyzed by series of four different monooxygenases belonging to cytochrome P450 (CYP450) superfamily. The genes encoding these enzymes are *Phantom* (*Phm*), *Disembodied* (*Dib*), and *Shadow* (*Sad*) (Chávez et al., 2000; Gilbert et al., 2002; Petryk et al., 2003). These genes commonly referred as Halloween genes, due to eerie phenotype observed in their mutant (Mykles, 2011). Ecdysone is then released into the hemolymph and modified further to its active form, 20-hydroxyecdysone (20E) by the last of Halloween gene, *Shade* (*Shd*) (Rewitz and Gilbert, 2008) in a target cells (Figs 1, 2). Cellular level of active ecdysteroid (20E) is kept in check by a degradation pathway catalyzed by CYP family of *Cyp18a1* (Guittard et al., 2011; Rewitz et al., 2010). This enzyme converts 20E into inactivated form of 20-hydroxyecdysonoic acid (Guittard et al., 2011).

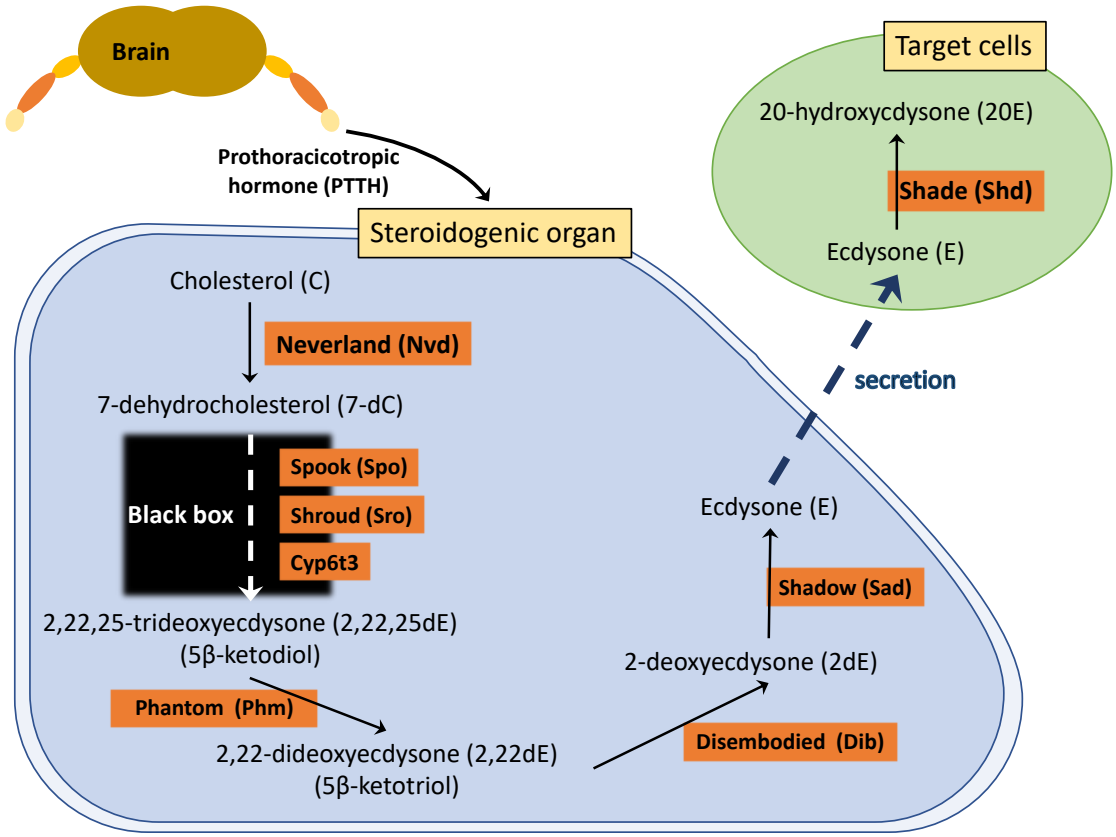


Figure 1 Putative biosynthetic pathway of ecdysteroid.

Enzymes are referred based on their nomenclature in insect. Nvd, neverland; Spo, spook; Spok, spookier; Spot, spookiest; Sro, shroud; Phm, phantom; Dib, disembodied; Sad, shadow; Sad, shade;

1.2.2. Ecdysteroid signaling mechanism

In target cells, a complex of intracellular receptors (Fig 2) mediates actions of ecdysteroid. These receptors are generally well conserved among arthropod species and consist of two proteins, the ecdysteroid receptor (EcR) and an ultraspiracle (USP) or retinoid X receptor (RXR) protein (Henrich, 2012). Both are member of nuclear receptor (NR) superfamily of which upon the binding of ligand (ecdysteroid) will act as transcription factor and initiate gene expression. The DNA binding domain (DBD) of these NRs recognizes specific sequence in the promoter region of ecdysteroid-responsive genes called ecdysteroid-responsive elements (EcREs) (Antoniewski et al., 1996; Henrich, 2012). The first set of genes activated by direct binding of EcR-USP complex are known as “primary” or “early” genes, which mostly consists of transcription factors (TFs) (Schumann et al., 2018). These TFs then in turn activate other sets genes (late genes), creating a hierarchical signaling cascade.

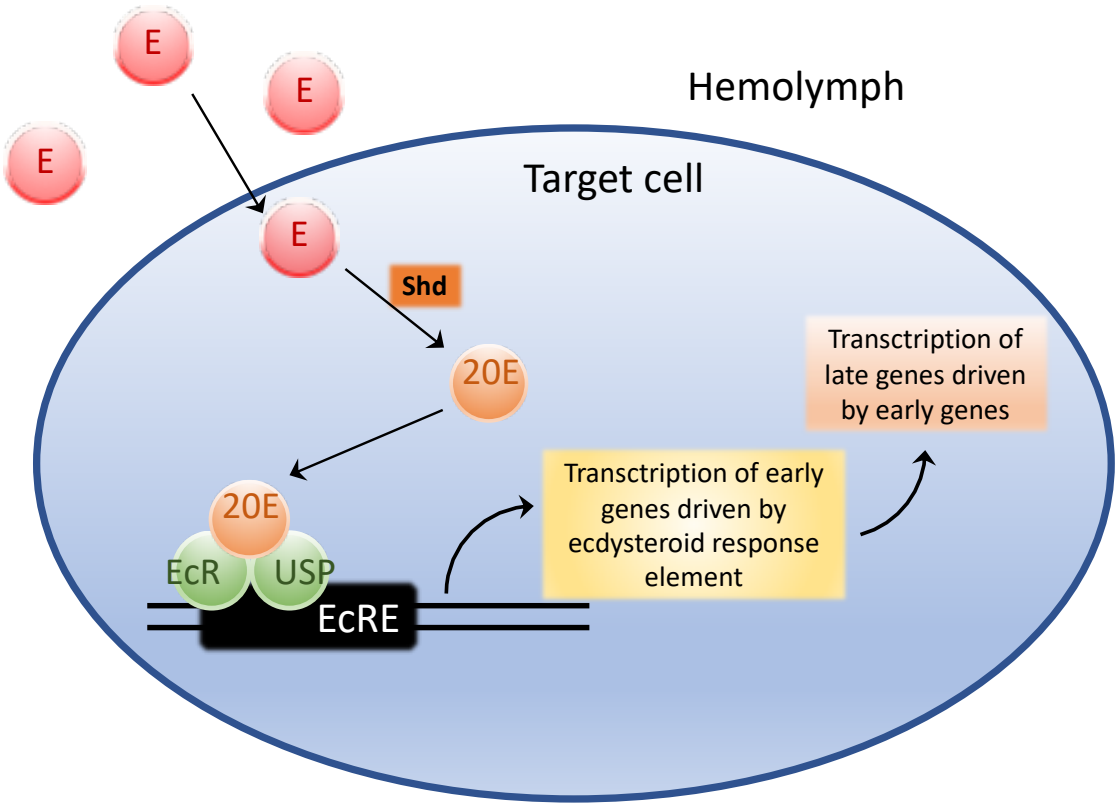


Figure 2 Simplified diagram of ecdysteroid signaling mechanism.
E from hemolymph was uptake by the target cell. Inside, an enzyme, Shd, catalyze the conversion of E into its active form, (20E). Binding of 20E with EcR and USP activates their transcriptional activity and start the expression of EcRE downstream genes which initiate a signaling cascade.
EcR, ecdysteroid receptor; USP, ultraspiracle protein; EcRE, Ecdysteroid response element; E, ecdysone; 20E, 20-hydroxyecdysone, Shd: shade.

1.3. The model organism *Daphnia magna*

The *Daphnia magna* is a branchiopod crustacean, which commonly inhabits ponds and lakes in Europe and Asia. This microcrustacean plays important roles in an ecosystem by becoming an essential component of fish foods and also contributing to water clarity by their ability to graze algae (Mu and Leblanc, 2002). Thus, *D. magna* serves as a crucial link from the primary producer and secondary consumer in the food pyramid. The *D. magna* exhibit a unique life cycle, where they can switch their reproductive strategy depending on the environmental condition they live in (Fig 3) (Hebert, 1978). In nutrient-rich environment, *D. magna* produce parthenogenetic eggs

and increases their population asexually. Alternatively, in response to deteriorating conditions such as shortening of photoperiod, lack of food or high population density, they produces clonal males, which allows them to mate and undergo sexual reproduction (Kleiven et al., 1992). This adaptive reproductive cycle is thought to be a survival strategy to overcome adverse conditions (Barton, 2009). In parthenogenetic cycle, *D. magna* reproduce approximately every three days and this reproduction cycle occurs synchronously with molting cycle (Figure 1.1) (Smirnov, 2017). Shortly after molting, the mother ovulates eggs from the ovaries into its brood chamber (oviposition). After 3 days, the mother releases juveniles into surrounding water. After this release, mother would molt again and repeat the cycle.

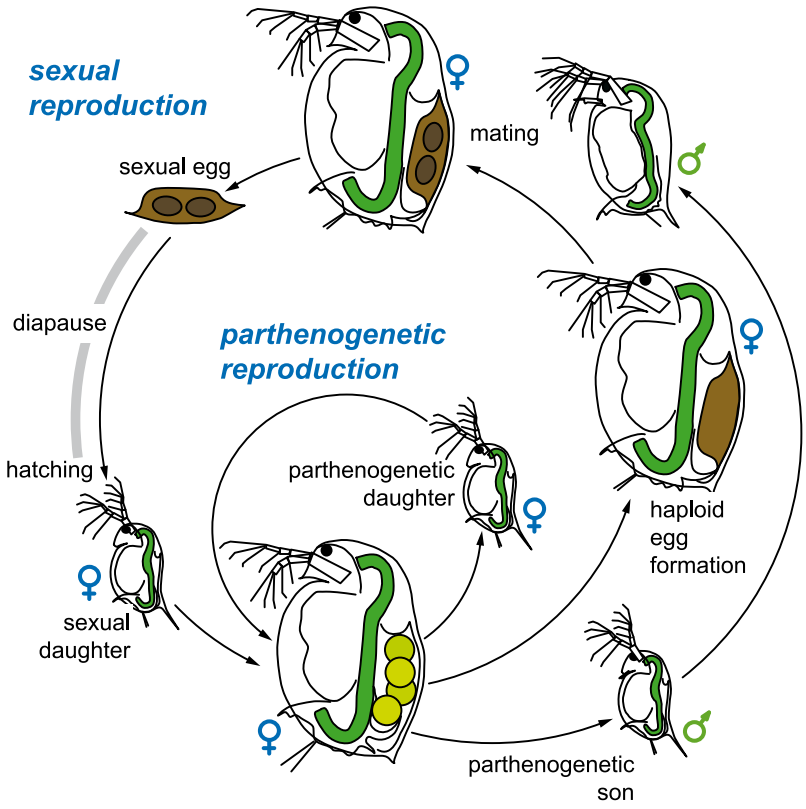


Figure 3 Life cycle of *Daphnia*
The diagram illustrates the parthenogenetic (asexual) and sexual cycle of *Daphnia*. In asexual cycle, female *Daphnia* produces diploid eggs that develop to be female offspring through parthenogenesis. When condition worsen due environmental stress females produces male offspring, and later haploid egg that requires male fertilization. The fertilized egg, (resting egg),

is enclosed in protective hard shell termed as ephippium and can endure harsh condition over a long time period. Picture source: https://commons.wikimedia.org/w/index.php?curid=47524211

The embryonic development of *D. magna* showed typical direct development (Olesen, 2004). Hatching of *D. magna* embryos occurs when appendage segmentation ends (Mittmann et al., 2014) in around 21-23 h at 22°C. In insects, this stage seems to be consistent with timing of blastokinesis (Panfilio, 2008). The hatched embryos remain inside the brood chamber and subsists on its own yolk supply (Smirnov, 2017). This stage is comparable to later stages of the hemimetabolan embryo, or pronymph (Truman and Riddiford, 1999). After 54-56 h, the hatched embryos molt to become juvenile and attain their complete morphology, with structures including setae of the second antennae and a tail spine. At this stage, they will be released from their mother's brood chamber. The juvenile will grow into several instar stages before reaching adult stage and attain reproductive maturation around 5-6 days later.

The *D. magna* has been long known as a classical model animal for ecotoxicology evaluation. Recently, with the advent of affordable next generation sequencing technology, genetic information of *D. magna* become publicly available, such as draft genome sequence and transcriptome data (Colbourne et al., 2011; Lee et al., 2019; Orsini et al., 2016). This information and the microinjection technique opened breakthrough in *D. magna* genome manipulation, such as RNAi technique (Kato et al., 2011), protein overexpression (Törner et al., 2014), transgenesis (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., 2016, 2015; Törner et al., 2018). This growing genomics and genes manipulation toolbox for *Daphnia*, plus their characteristic of fast life cycle and easy laboratory rearing have transformed *D. magna* as emerging genetic model animal. These advantages provide opportunities to extend

laboratory research to analyze gene function for elucidating various molecular mechanism.

1.4. Ecdysteroid study in *Daphnia magna* and its potential

Development of genetic manipulation in *D. magna* has opened possibility for thorough elucidation in its endocrinology, especially related to ecdysteroid. Previous study had been succeeded in characterizing the ecdysteroid receptors that actively bound by 20E in *D. magna* (Kato et al., 2007; Wang et al., 2007). Data mining from the recently published *Daphnia* genome database revealed the presence of ecdysteroidogenesis genes orthologs in *Daphnia* (Rewitz and Gilbert, 2008). Recently, with the use of microinjection technique, a reporter plasmid containing EcREs sequence followed by green fluorescence protein (GFP) gene was transiently introduced into *D. magna* embryo, thus allowing visualization of ecdysteroid during embryogenesis (Asada et al., 2014a).

However, despite advancement of ecdysteroid study conducted in *Daphnia*, there are large parts of the synthesis, regulation and roles of ecdysteroid that remain unknown. In *Daphnia*, as well as other non-insect arthropods, studies about their molecular functions are mostly drawn from comparative study from those reported in insect, rather than direct experiments (Lafont and Mathieu, 2007). Although this approach may be applicable for some fundamental concepts, further elucidation should be conducted to lay the foundation for in-depth analysis of their functions. Moreover, popular insect model animals such as *Drosophila* and *B. mori* are evolutionary distanced from crustacean, as the former diverged more than 500 million years ago (Glenner et al., 2006; Schwentner et al., 2017). Thus, some of their genes are more specialized due to gene gain/loss throughout evolution, and may serve different functions. For instance,

the dehydrogenation of cholesterol into 7-dC was catalyzed by two paralog of *Nvd* in *Daphnia* (Miyakawa et al., 2017), whereas in insect only have one. The appearance of second *Nvd* gene in *Daphnia* is unique and thought to be the result of gene duplication, which commonly occurred in this species. (Colbourne et al., 2011; Miyakawa et al., 2017; Sumiya et al., 2016).

Elucidation of ecdysteroid roles in *Daphnia* may have potential environment benefits. It has been demonstrated that some chemicals that are intentionally released into the environment interfere with ecdysteroid signaling (LeBlanc, 2007). These chemicals are known as ecdysteroid agonists and commonly used as pesticides. The ecdysteroid agonists have capability to mimic ecdysteroid mode of action by direct binding with its receptors. Thorough ecdysteroid study on insect may contribute to the development of selective ecdysteroid agonist which leaving non-target insect, e.g. beneficial in integrated pest management (IPM) programs, unaffected. For instance, tebufenozide is a non-steroidal ecdysone agonist commonly used as the insect growth regulator (IGR) (Smagghe and Degheele, 1998). It showed selective activity against non pest insect, but displayed non-target effect toward crustacean (Smagghe and Degheele, 1998; Song et al., 1997). Consequently, understanding the ecdysteroid in crustacean may contribute to development of pesticide chemicals that are safer to this taxonomic clade.

Lastly, recent advancement of genome editing in *D. magna* may also entertain the possibility to develop transgenic animal for detecting the presence of ecdysteroid agonist in the aquatic environment. This can be done by integrating a reporter construct containing hormone response element followed by reporter gene into *D. magna* genome by the use of targeted genome editing. The resulted transgenic *Daphnia* could be used

to study revealing spatio-temporal role of ecdysteroid, as well as detecting its presence in surrounding environment.

1.5. Objective of this study

Studies regarding ecdysteroid hormone, its biosynthetic genes, and signaling pathway has been benefitted with the use of insects as model animal. However, our knowledge is limited about this hormone in other arthropod species, Therefore, this study is aimed to elucidate the role of ecdysteroid hormone in *D. magna* and for furthering our understanding about ecdysteroid role in non-insect arthropods.

To begin with, I first elucidate the ecdysteroid role in embryogenesis by performing characterization and functional analysis of *Spo*, the rate-limiting gene for ecdysteroid biosynthesis (Chapter 2). In Chapter 3, I demonstrate the interaction between ecdysteroid biosynthetic regulations by sesquiterpenoid signaling. This is the first report of interaction between two major arthropod hormones in non-insect species. Lastly in Chapter 4, I develop an ecdysteroid reporter transgenic *Daphnia* to aid spatio-temporal study of ecdysteroid and development of ecdysteroid biosensor.

Chapter 2 Characterization and functional analysis of *Spook* for *de novo* ecdysteroidogenesis

2.1. Introduction

The ecdysteroid biosynthesis (ecdysteroidogenesis) is a multistep process involving various genes (Fig 1). Recently, advent of next generation sequencing technology revealed the high conservation of those ecdysteroidogenesis genes across other arthropod clades (Crustacea, Chelicerata, and Mryapoda) (Qu et al., 2015). In the last 20 years, characterization and functional analysis of ecdysteroidogenesis genes have been benefitted by the use of insect model organisms such as *Drosophila* and *Bombyx mori* (Gilbert et al., 2002; Niwa and Niwa, 2016). However, little is known about role and presence of those genes in other arthropod species.

In *Daphnia*, evidence for the presence of ecdysteroidogenesis genes in the genome has been reported (Rewitz and Gilbert, 2008). Subsequently, previous study has characterized *Nvd* and *Shd* (Sumiya et al., 2016), the genes which catalyze the first and final stage of ecdysteroidogenesis respectively. However, functional study of other ecdysteroidogenesis genes ortholog in *Daphnia*, remains lacking. Furthermore, the rate-limiting component of ecdysteroidogenesis in *Daphnia* is not characterized yet. Elucidating the rate-limiting factor in ecdysteroidogenesis would provide an insight for the overall ecdysteroid biosynthesis reaction in *D. magna*.

In previous chapter, I describe the biosynthesis pathway of ecdysteroid. Point of interest in this pathway is the conversion of 7-dC into 5ß-ketodiol. In this reaction, no intermediates between two products has ever observed. Therefore, it is termed as “Black box” reaction. Despite not clearly characterized, Black box reactions is thought to be catalyzed by 3 enzymes: CYP450 family monooxygenase of *Spook* (*Spo*) and CYP6T3, and dehydrogenase/reductase *Shroud* (*Sro*) (Enya et al., 2014; Niwa et al., 2010). The

lack of intermediate compounds could be resulted from the unstable nature of the intermediates, or the reactions itself is rate-limiting one, or both. Therefore, black box reaction is considered to be the rate limiting reaction of entire ecdysteroidogenesis. The concept of reactions within Black box as rate-limiting step in ecdysteroidogenesis is supported by the findings that *Spo*, one of the enzyme which catalyze the reaction inside Black box, is the only component which its phosphorylation and expression is directly induced by PTTH stimulation (Rewitz et al., 2009) (see section 1.2.1). Furthermore, bioinformatic analysis of *Spo* also indicate high sequence conservation with insects (Rewitz et al., 2007). This high conservation over vast evolutionary distance between insect and crustacean may indicate its evolutionary important role such as rate limiting function in the ecdysteroidogenesis (Rewitz and Gilbert, 2008).

In this chapter, I aimed to elucidate the ecdysteroidogenesis in *D. magna* embryo by characterizing and analyzing the function of *Spo*. Examining the *Spo* expression and function may provide information for understanding ecdysteroidogenesis in embryogenesis of *D. magna*.

2.2. Materials and methods

2.2.1. *Daphnia* strain and transgenic line

All daphnids were raised under the following conditions: 80 neonates (under 24 h) were transferred to 5 L medium and cultured at 22–24°C , under a constant light/dark photoperiod of 16 h/8 h. Artificial Daphnia Medium (ADaM) was used as the culture medium and prepared using reverse osmosis (RO) water, as reported previously (Klüttgen et al., 1994). *Daphnids* were fed daily with a 100 µL suspension of 8×10^9 cells/mL *Chlorella vulgaris* (Oitamedakabiyori, Oita, Japan) and 15 µL suspension of 0.15 g/mL baker's yeast (Marusan Pantry, Ehime, Japan) during the first week. Upon

reaching reproductive age, their offspring were removed once per day and fed daily with a 200 µL suspension of 8×10^9 cells/mL chlorella and 30 µL suspension of 0.15 g/mL baker's yeast.

The wildtype strain (NIES clone) was obtained from the National Institute of Environmental Studies (NIES, Tsukuba, Japan). In this study, I also utilized previously established transgenic *Daphnia* that express a H2B-GFP fusion protein under *Daphnia magna* elongation factor 1-α (Ef1α) promoter/enhancer (Kato et al., 2012). This transgenic daphnia would enable me to visualize and define embryo stages during the progression of embryogenesis.

2.2.2. RNA extraction and purification

Daphnia (adults or embryos) were collected in 2-mL tubes, immediately frozen in liquid nitrogen, and homogenized using a MicroSmash MS-100 machine (TOMY, Tokyo, Japan) in the presence of Sepasol-RNA I reagent (Nacalai Tesque, Kyoto, Japan), according to the manufacturer's instructions. Extracted total RNA was further purified using phenol-chloroform extraction and ethanol precipitation. Purified total RNA was dissolved in RNase free water (Invitrogen, Carlsbad, USA) and stored at –80°C until further use.

2.2.3. Cloning and sequencing

Adult *Daphnia* (115 inds) were collected and subjected to total RNA extraction and purification according to the above-mentioned procedure. Beforehand, eggs were removed from the brood chamber. Polyadenylated RNA was purified from 500 µg of total RNA using the PolyATtract mRNA Isolation System (Promega Corporation, Tokyo, Japan) and used for 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) using the GeneRacer (Invitrogen, Carlsbad, USA) and SMARTer RACE cDNA

Amplification (Clontech Laboratories, Mountain View, WI, USA) kits, respectively. The primers used for cDNA amplification are listed in Table 1. PCR was performed using KOD+ DNA Polymerase (Toyobo, Osaka, Japan). PCR products were verified by agarose gel electrophoresis, purified, cloned using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, USA), and sequenced.

Table 1 The primers used for *Spo* RACE experiments

Primer name	Primer sequence (5′−3′)
Spo_5RACE-GSP	Forward: - Reverse: TTCGTTCACCATCAAGCCGCTCTC
Spo_5RACE-GSP-nested	Forward: - Reverse: GAACGACGATCCCTCTGCAAGTAG
Spo_3RACE-GSP	Forward: GCCCTACACCGAAGCGACTATTCTC Reverse: -
Spo_3RACE-GSP-nested	Forward: TCCGGCTCGATTTCTTATCCAAGG Reverse: -

2.2.4. Phylogenic analysis

Amino acid sequences of *Spo* family genes were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/) as shown in Table 2 and the whole amino acids of each protein were used to construct the phylogenetic tree. Multiple sequence alignments of the amino acid sequences were constructed using the ClustalW (Thompson et al., 1994) in MEGA X program for MacOS (Stecher et al., 2020). The following settings were used for the analysis: pairwise alignment parameter: gap opening penalty = 6.00, gap extension penalty = 0.21, and identity protein weight; matrix multiple alignment parameter: gap opening penalty = 10.00, gap extension penalty = 0.24, delay divergent cut-off = 30%, and gap separation distance = 4. The phylogenetic reconstruction was performed using the p-distance algorithm and the neighbor-joining method was implemented in MEGA.

Table 2 Accession numbers of *Spo* ortholog genes used in this study.

Scientific name	Common name	Accession no.
Daphnia magna	Water flea	BCF86811 (this work)
Daphnia pulex	Water flea	EFX88041
Tribolium castaneum	Red flour beetle	EFA11558
Agrillus planipennis	Jewel beetle	XP_018330366
Anopheles gambiae	African malaria mosquito	XP_560266
Blattella germanica	German cockroach	PSN30774
Bombyx mori	Silkworm moth	BAH47267
Aedes aegypti	Yellow fever mosquito	XP_021694486
Drosophila melanogaster	Fruit fly	NP_001286943
Penaeus vannamei	Shrimp	XP_027213908
Portunus trituberculatus	Crab	MPC33641
Danio reiro	Zebrafish	NP_001099140
Mus musculus	Mouse	AAA37506

2.2.5. Quantitative RT-PCR (qRT-PCR)

Embryos were collected at 0, 6, 12, 18, 24, 36, 48, and 72 h after ovulation. These timepoints correspond to specific embryonic developmental landmarks described in Section 2.3.3. Samples were collected in three biological replicates and subjected to total RNA isolation as described in the section 2.2.2. Synthesis of cDNA was performed using a random primer from 1 μg of the purified total RNA with the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, USA). The absence of genomic DNA (gDNA) contamination was confirmed as described previously (Kato et al., 2018). PCR was performed in an Mx3005P (Stratagene, La Jolla, CA, USA) instrument using the Power SYBR Green PCR Master Mix (Invitrogen, Carlsbad, USA) with primers listed in Table 3. PCR amplification was performed in triplicate under the following conditions: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primer specificity was confirmed by analyzing dissociation curves. Expression levels of each gene were normalized against those of the ribosomal *L32* gene.

Table 3 The primer pairs used for qRT-PCR analysis of *Spo*

Gene name	Primer sequence (5′−3′)
Spo	Forward: GGGCTATGCTGTCGATTTCC Reverse: TTGTGCTGTTGTGCGTCTTC
L32	Forward: GACCAAAGGGTATTGACAACAGA Reverse: CCAACTTTTGGCATAAGGTACTG

2.2.6. RNA interference and microinjection

Small interfering RNAs for *Spo* were designed using the Block-iT RNAi designer (http://www.invitrogen.com/rnaidesigner.html). The siRNA sequences were: 5′ CCGUCUUCUUGCGAUCAAAA 3′. Two nucleotides dTdT were added to the 3′ end of the siRNA strand. Two additional siRNAs, one containing a random sequence (5′ GGUUAAGCCGCCUCACAU 3′) (siRNA-scrambled) (Asada et al., 2014b), and another one targeting the *Escherichia coli MalE* gene (siRNA-MalE) were used as controls. Microinjection was performed according to an established protocol (Kato et al., 2011). Briefly, freshly ovulated eggs from 2–3-week-old *Daphnia* were collected and placed in ice-cold M4 medium (Elendt and Bias, 1990) containing 80 mM sucrose (M4-Sucrose). Specific siRNA samples for each experiment were mixed with 5 μM AlexaFluor 568 fluorescent dye (Invitrogen, Carlsbad, USA) as an injection marker. After injection, intact eggs were transferred and cultured individually inside 96-well plates filled with 100 μL M4-Sucrose at 23°C. Injected embryos were collected in three biological replicates and subjected to total RNA isolation as described above. Ten micrograms of yeast tRNA (Ambion) were added to each sample as carrier RNA. Synthesis of cDNA was performed using a random primer with a PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa, Shiga, Japan). Gene expression levels were evaluated by qPCR using the primer pairs shown in Table 2.

2.2.7. Rescue experiment with 20- hydroxyecdysone (20E)

Shortly after siRNA injection, intact eggs were transferred into 100 μL M4-Sucrose containing 0.01% dimethylformamide (DMF) as control, or 1 μM (500 μg/L) 20-hydroxyecdsone (Tokyo Chemical Industry, Tokyo, Japan) in 0.01% DMF. Embryos were incubated at 23°C in the dark. At designated time points, development was observed microscopically

2.2.8. Fluorescence photography

Fluorescence micrographs of embryos were acquired with a Leica DC500 CCD digital camera mounted on a Leica M165FC fluorescence microscope (Leica Microsystem, Mannheim, Germany). GFP-expressing embryos were imaged using a GFP2 filter.

2.3.Results

2.3.1. Characterization of *Spo* gene in *D. magna*

To investigate the existence of *Spo* ortholog in *D. magna*, I performed a TBLASTN search using the amino-acid sequences of *Spo* obtained from several insect species against the *D. magna* genome database. One putative ortholog of *Spo* was found at scaffold 02011. In order to obtain the full sequence of the *Spo* cDNA, I performed 5′ and 3′ RACE from isolated polyadenylated mRNA of adult female *Daphnia*. Following cloning and sequencing, a single *Spo* cDNA was obtained and found to be 2,288 nucleotides in length. Aligning the *Spo* cDNA to *Daphnia* genome sequence revealed that it contains 3 exons and 2 introns, spanning 4,191 bp in the genome (Fig 4).

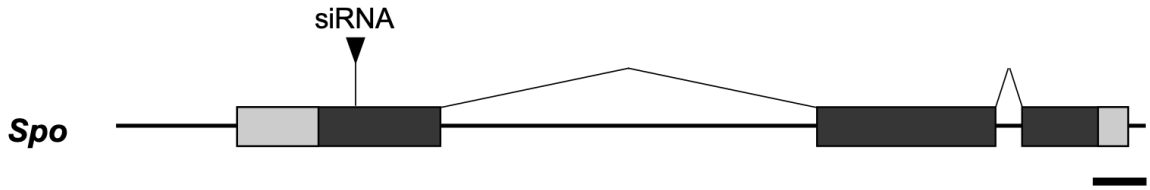
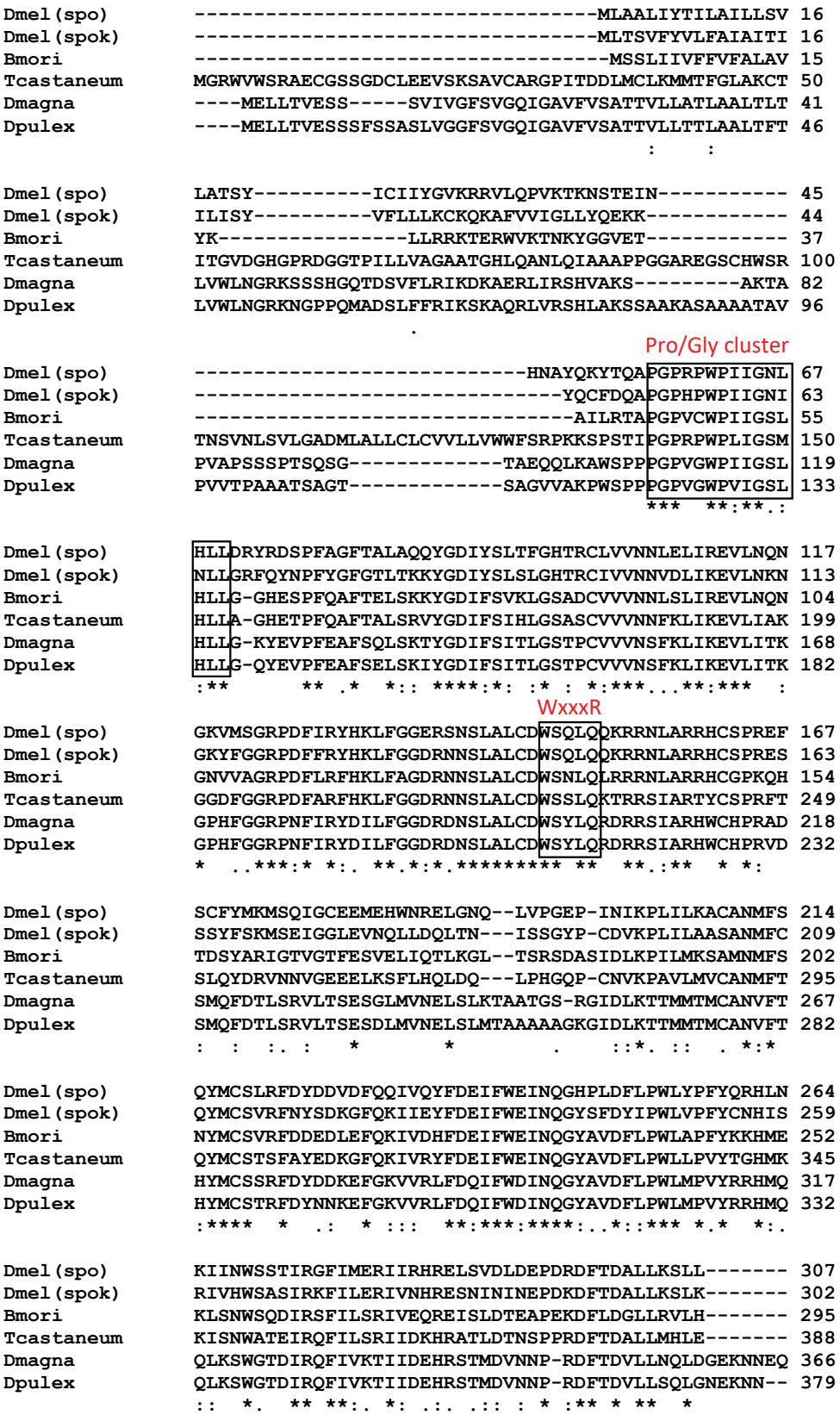


Figure 4 A schematic representation of *Spo* gene structure.
Coding sequences (CDSs) are showed as dark-colored boxes, untranslated regions (UTRs) are indicated as light-colored boxes. siRNA target sites are indicated by black triangles. Scale bar, 250 nt

The *Spo* coding sequence (CDS) was found to encode a 591-residue protein with a molecular weight of 66 kDa, which is typical for CYP450 protein family (Feyereisen, 1999). Multiple alignment with fruit fly (*D. melanogaster*), silkworm (*B. mori*), and red flour beetle (*Tribolium castaneum*) orthologs revealed that *D. magna Spo* contains typical CYP450 characteristic domains: Pro/Gly cluster, WxxxR, ExxR, PERF motif, and heme loop (Fig 5).


Pro/Gly cluster
WxxxR

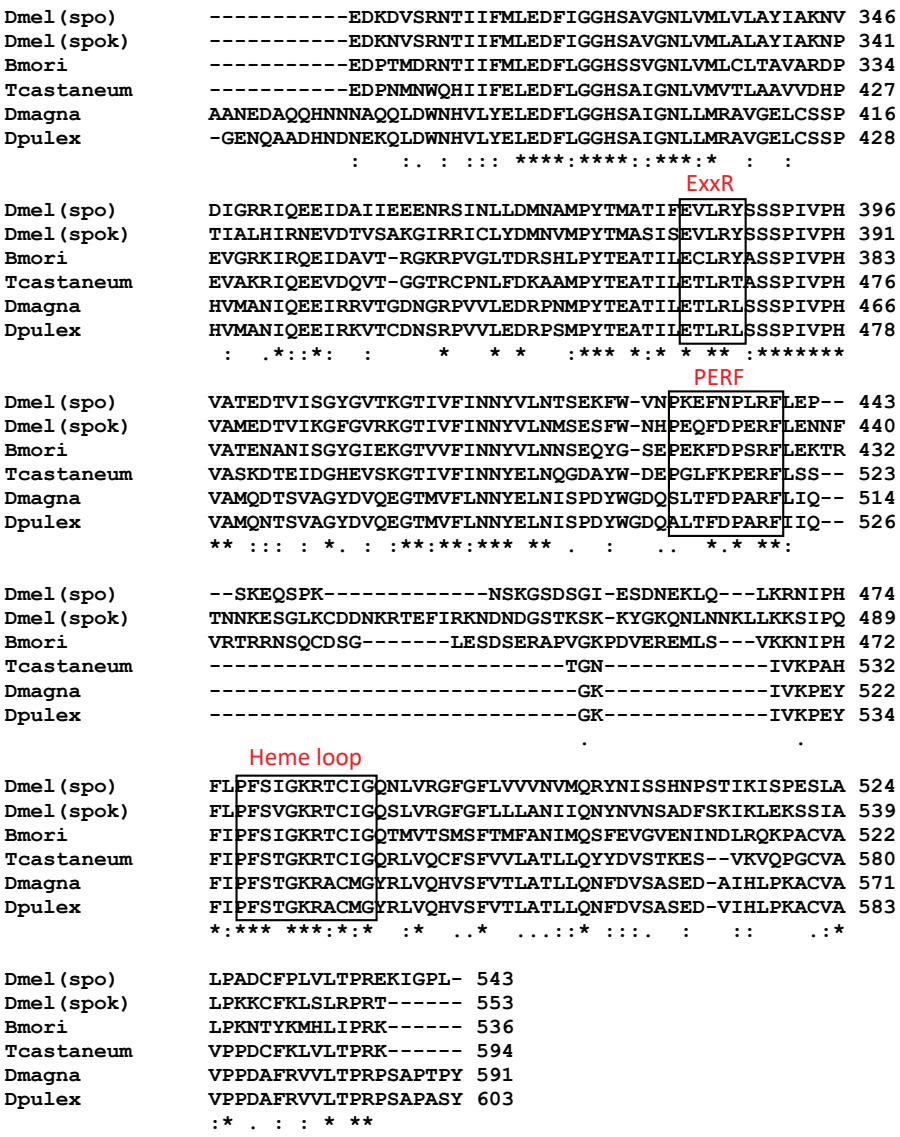


Figure 5 Multiple Alignment of Spo protein.
Dmagna, *Daphnia magna*; Dpulex, *Daphnia pulex* Dmel, *Drosophila melanogaster*, Tcastaneum, *Tribolium castaneum* and Bmori, *Bombyx mori*. Asterisks, semicolons, and dots indicate conserved, strongly similar, and weakly similar residues respectively. The position of characteristic P450 motifs are indicated by black boxes under the red characters. Red box indicates putative S-adenosyl-L-methionine (SAM) binding site

To analyze the evolutionary relationship of *D. magna Spo*, a phylogenetic tree with 12 other related proteins (Table 2) was constructed by neighbor-joining method, using the whole amino acid sequence (Figure 6). The result in Figure 6 exhibits that the topology of the phylogenetic relationship between *Spo* orthologs was in good agreement with taxonomic relationship between insects and crustaceans. Compared to

the *Spo* ortholog of shrimp and crab belonging to malacostraca crustacean, the branchiopod crustacean *Daphnia* is more closely related to insect *Spo* orthologs. This result supports the hypothesis that insects originated from branchiopod crustaceans (Glenner et al., 2006).

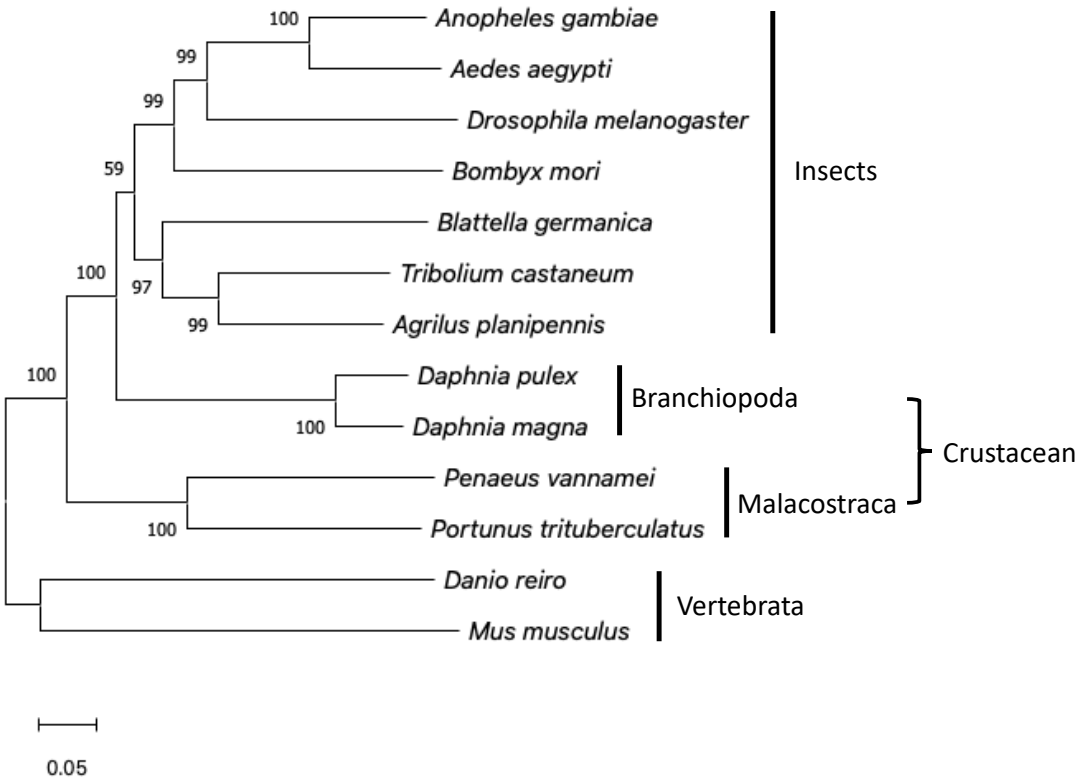


Figure 6 Phylogenetic tree using amino acid sequences of Spo.
The percentages of replicated tree in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The bar indicates branch length and corresponds to the mean number of the differences ($P<0.05$) per residue along each branch. Evolutionary distances were computed using the p-distance method.

2.3.2. *Spo* expression pattern during embryogenesis

I examined the expression pattern of *Spo* during embryogenesis progression by using qRT-PCR. Embryogenesis progression in *D. magna* can be classified into two stages based on the timing of molting. The first (embryo) stage is where cell division, gastrulation, and body segmentation occurs and it lasts for the first 50 h after oviposition.

Shortly after, the embryo experiencing pseudomolt where the embryonic membrane is shed. This mark the end of embryo and beginning of the second (juvenile) stage.

Spo expression was detected at high level during early embryo and juvenile stage (Fig 7). The expression showed peak at 6 h after ovulation which coincide with onset of gastrulation (Kato et al., 2012; Mittmann et al., 2014). Expression level then abruptly dropped at 12 h and steadily decreasing until the lowest level shortly after hatching at 24 h. Upon reaching the end of embryo stage, *Spo* expression then steadily increasing (Fig 7).

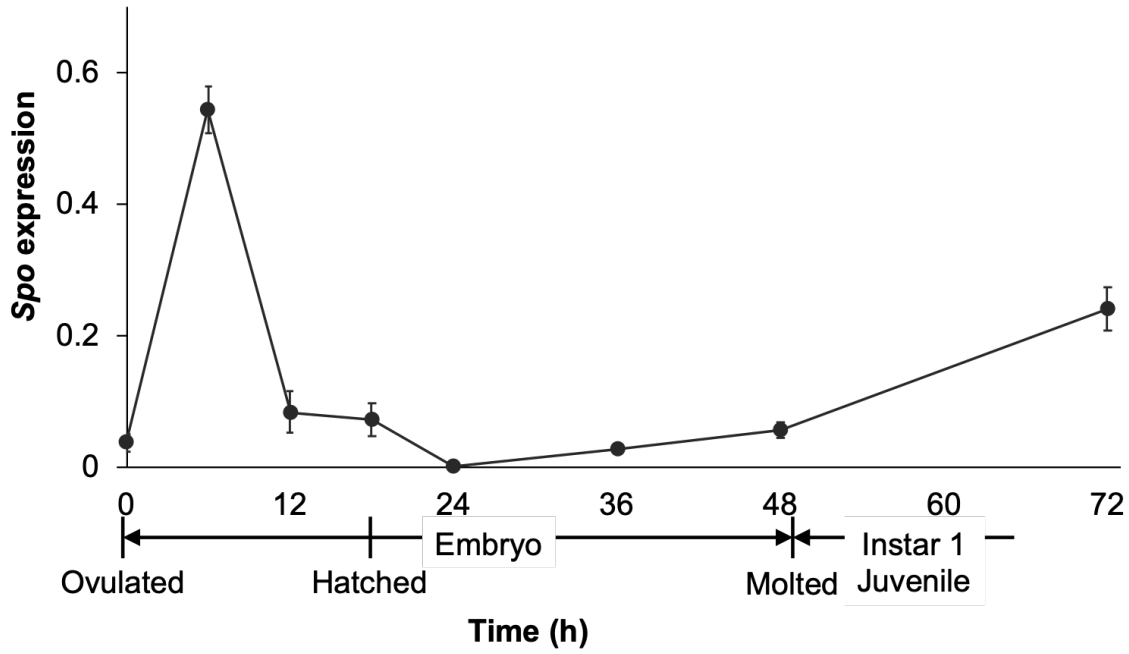


Figure 7 Expression of *Spo* during the progression of embryogenesis
Timing of hatching and molt to become instar 1 juvenile was shown at the bottom of the graph. Time is in hours after ovulation. Expression levels were normalized using reference gene expression levels (ribosomal L32). All values are the mean. Error bars represent SD (N=3).

2.3.3. *Spo* is essential for the progression of early embryogenesis

To investigate the role of *Spo* during embryogenesis, I performed loss of function analysis using RNA interference (RNAi). I designed a RNAi which targets the first exon at 5′ end of *Spo* transcript (hereby named siRNA-*Spo*) as shown in Fig 4.

To easily track its developmental progression in the embryo stage, I utilized a previously established transgenic *Daphnia* that ubiquitously expresses an H2B-eGFP fusion protein (Kato et al., 2012). Staging parameter was determined based on developmental landmarks as previously described (Kato et al., 2012; Mittmann et al., 2014). As shown in Fig 8, cleavage and blastula formation occurred during the first 6 h (Stage 1). Shortly after, the appearance of an invagination pit was indicative of gastrulation (Stage 2). Following gastrulation, cell mass migration proceeded, with the formation of three germ layers (Stage 3). At around 15 h, the formation of thoracic appendages and a pair of secondary antennae was observed (Stage 4). Hatching occurred around 22 h (Stage 5).

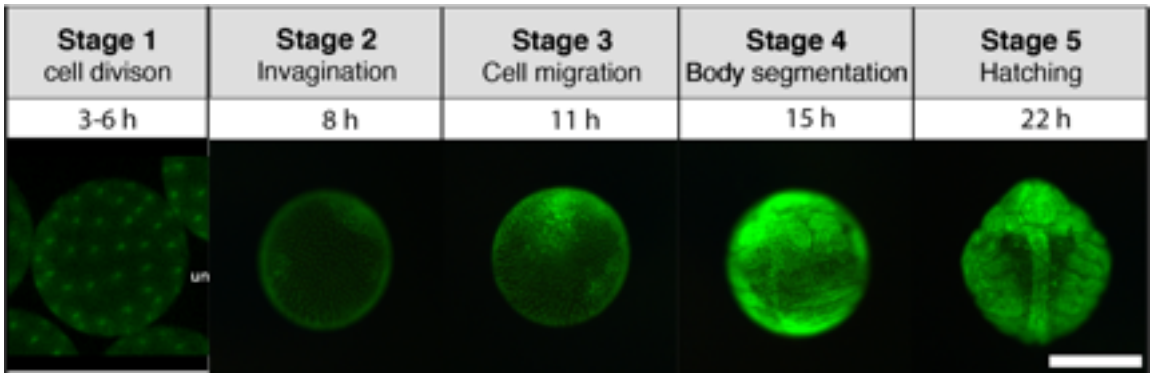


Figure 8 Developmental staging of *Daphnia magna* early embryogenesis
Developmental timing and corresponding stage in H2B-eGFP expressing *D. magna* cultured at 23°C. Time is in hours after ovulation (hao). Scale bar 200 µm.

Based on the eGFP expression pattern as presented in Fig 9, embryos injected with siRNA-*Spo* initially showed no developmental differences relative to siRNA-ctrl-injected embryos until stage 2, during cleavage and cell mass migration (Fig 9B). Subsequently, siRNA-Spo injected embryos showed a retardation in development and failed to proceed beyond stage 2 (Fig 9A, B; siSpo), in contrast to control embryos (Fig 9A, B; siControl). Eventually, around 75% of siRNA-Spo injected embryo burst and

died shortly after hatching at 22 h. These results may indicate that *Spo* is essential for the progression of early embryogenesis.

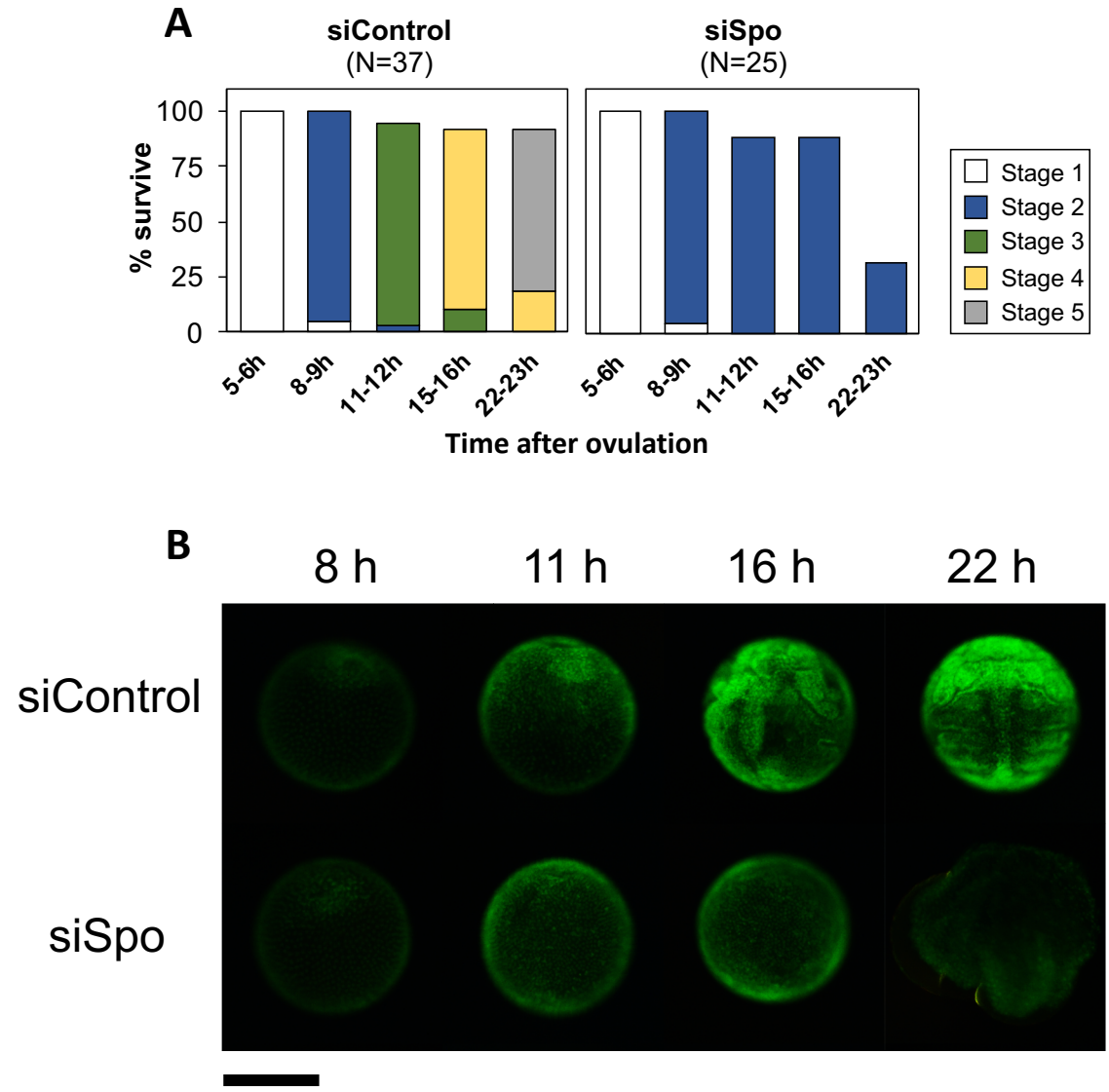


Figure 9 *Spo* knock down resulted in early developmental arrest
(A) Developmental progression and survival rate of siControl and siSpo Color bars represent each developmental stage described in Fig 7. Stage 1 (white), stage 2 (blue), stage 3 (green), stage 4 (yellow), stage 5 (gray).
(B) Representative images of embryos injected with siControl and siSpo, Transgenic embryos expressing H2B-GFP are used in these photographs, and are taken under GFP filter. Representative photographs from different stages are combined into a single image. Scale bar, 200 μm.

Time is indicated in hour after ovulation

To confirm that the *Spo* expression has been successfully knocked down during RNAi treatment, total RNA from 6 h siRNA injected embryos was isolated, and then the *Spo* expression level was measured by qRT-PCR. I confirmed the reduced expression of *Spo* upon injection of siRNA compared with control as shown in Fig 10, indicating that *Spo* was effectively silenced in the siRNA-injected embryos.

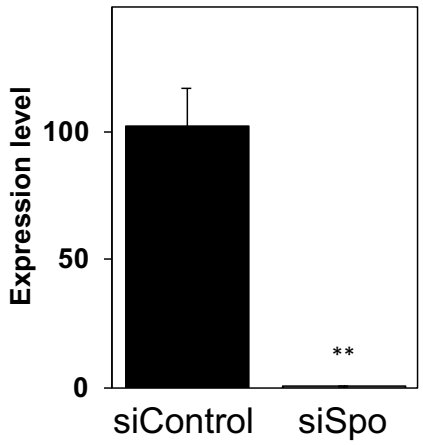


Figure 10 *Spo* expression in siRNA-injected embryos
Gene expression level of *Spo* in siControl and siSpo embryos at 6 h, as assessed by qRT-PCR. All values are the means. Error bars represent SD (N=3). **p<0.01 (Student's t-test).

2.3.4. *Spo* is required for *de novo* ecdysteroid biosynthesis

In order to demonstrate the role of *Spo* in ecdysteroid biosynthesis in *D. magna* embryogenesis, I attempted to rescue the phenotype of *Spo* RNAi embryos by supplementing ecdysteroid hormone analog 20-hydroxyecdysone (20E) in the culturing medium. To determine optimum 20E concentration for rescue, I initially performed exposure experiment using several concentrations of 20E to *D. magna* embryos. Freshly ovulated eggs were exposed by gradient concentration of 20E from 0 to 5000 μg/L. After 22–24 h, embryos were evaluated based on their hatching time and hatchability. As presented in Fig 11, exposure to 100 and 500 μg/L showed no significant effect on hatchability, although number of delayed hatching embryo increased in 500 μg/L

concentration. Embryos exposed more than 500 µg/L 20E started to show toxicity, where hatching rate was significantly reduced. This result suggest that 500 µg/L is the maximum exposure concentration before toxicity effect appears. Therefore, I decided to use 500 µg/L 20E concentration for subsequent rescue experiment.

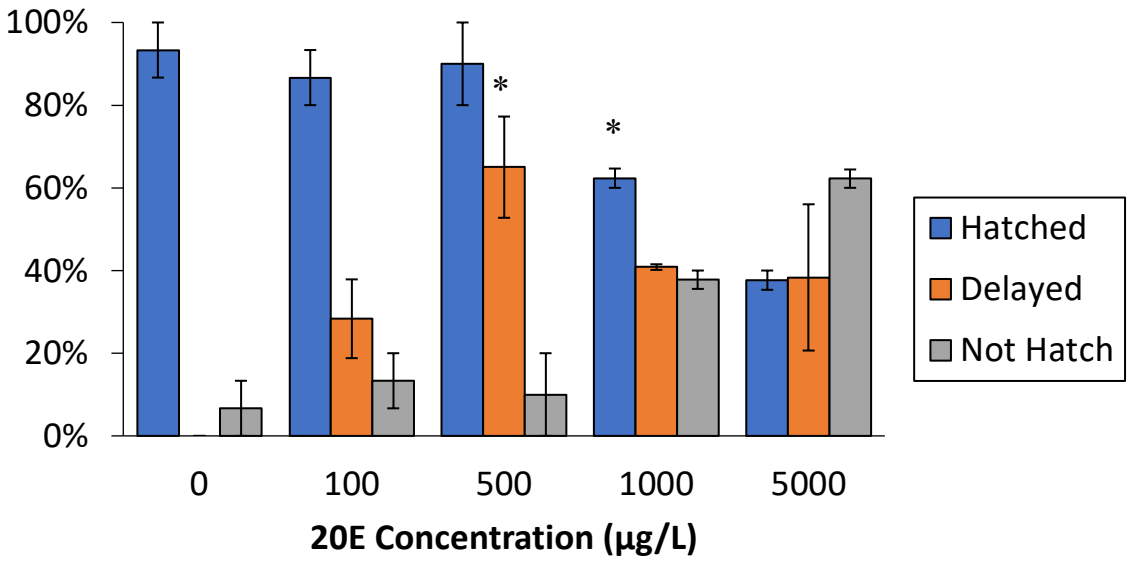


Figure 11 20-hydroxyecdysone exposure experiment to *Daphnia magna* embryo
Freshly ovulated embryos were collected and exposed with series of 20E concentration. At 24 h, embryos' survival rate was evaluated. Error bar represent SD (N=3). *p<0.05 (Student's t-test)

Rescue experiment was performed to H2B-eGFP expressing *Daphnia* injected with siRNA-Spo. As expected, 20E-exposed Spo RNAi embryos progressed beyond stage 2 (Fig 12A, B). Around 70% of rescued embryos progressed to stage 4 (Fig 12A, B). Of the rescued embryos, 10% developed further and formed naupliar and compound eyes after 46 h (Fig 12C) but did not become juveniles. These results demonstrate that *Spo* is essential for early embryogenesis, likely via *de novo* ecdysteroid biosynthesis.

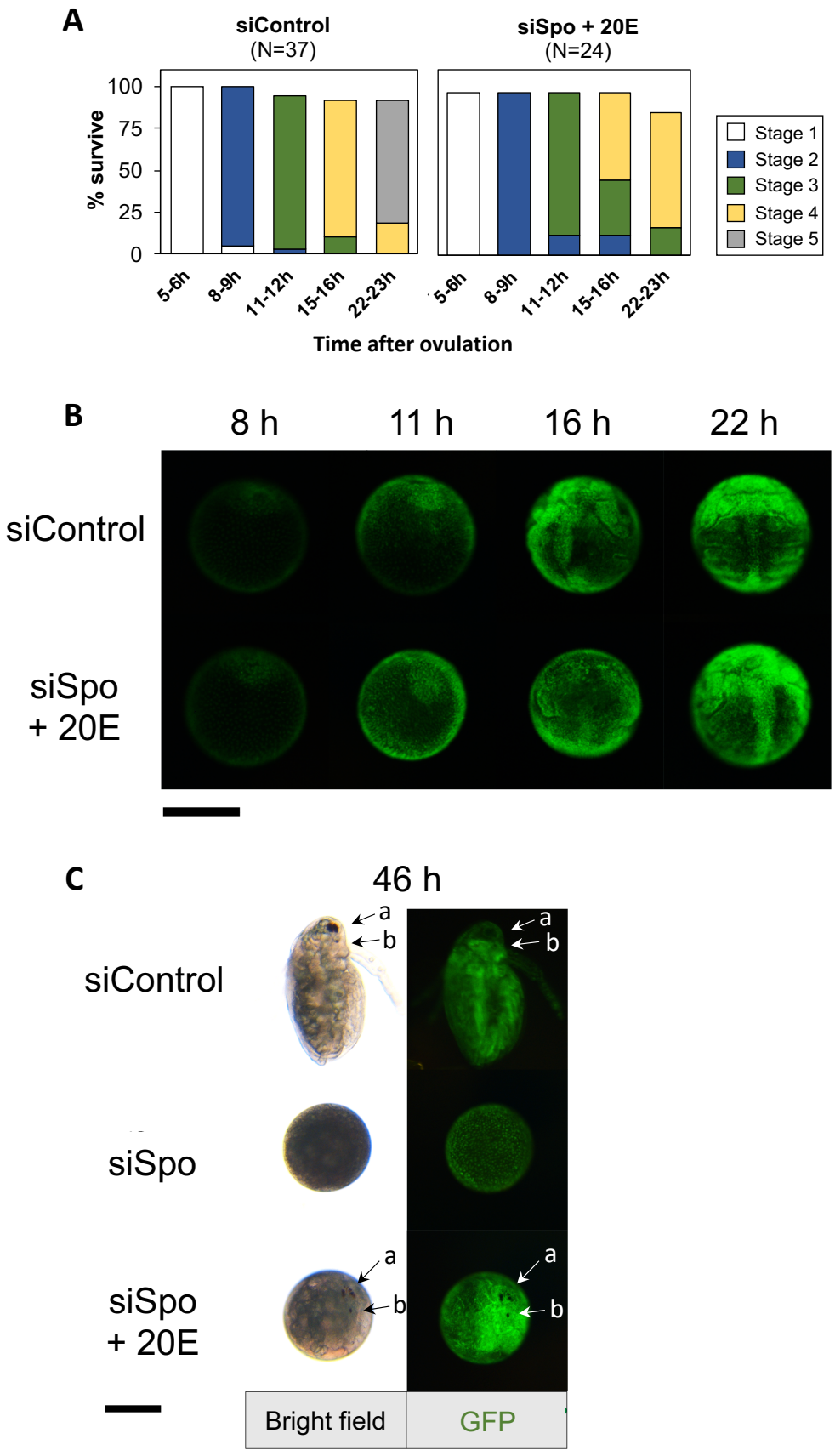


Figure 12 *Spo* knock down phenotype can be rescued by supplementing 20E

(A) Developmental progression and survival rate of siControl and siSpo supplemented with 20E. Color bars represent each developmental stage described in Fig 8. Stage 1 (white), stage 2 (blue), stage 3 (green), stage 4 (yellow), stage 5 (gray).
(B) Representative images of embryos injected with siControl and siSpo supplemented with 20E, Transgenic embryos expressing H2B-GFP are used in these photographs, and are taken under GFP filter. Representative photographs from different stages are combined into a single image. Scale bar, 200 μm.
(C) Retardation of embryo developmental progression. Compound eye (a) and naupliar eye (b) development observed at 46 h after ovulation. Scale bar, 200 μm.

Time is indicated in hour after ovulation

2.4. Discussion

In this chapter, I characterized and elucidated the role of *Spo* ortholog for *de novo* ecdysteroidogenesis in *D. magna*. *Spo* gene structure consists of two introns and three exons. The conservation of intron/exon organization of Halloween genes is common in arthropod, which support their vital role and common evolutionary origin (Betts et al., 2001; Rewitz and Gilbert, 2008). In *D. magna* however, number of intron/exon structure in *Spo* is unique compared to that of insects, as the latter only have one intron (Rewitz and Gilbert, 2008). This may be possible that insect lost the intron after their divergent from crustacean.

The expression pattern and functional analysis of *Spo* confirmed its indispensable role in ecdysteroidogenesis during *D. magna* embryogenesis. The *Spo* transcript was highly expressed during early embryonic stages and peaked at 6 hpo, which coincides with the onset of gastrulation (Kato et al., 2012), indicating that ecdysteroid is required for embryonic cell differentiation. *Spo* expression in the *D. magna* embryo showed a similar expression pattern to that in the early embryo of *D. melanogaster*, where *Spo* has been found to be expressed during pre-cellular blastoderm stage, prior to gastrulation (Ono et al., 2006). In *D. magna*, the RNAi-mediated knockdown of *Spo* resulted in embryonic arrest during gastrulation. This phenotype is consistent with

Halloween gene mutants previously reported in insects (Chávez et al., 2000; Niwa et al., 2004; Ono et al., 2006; Petryk et al., 2003), suggesting its conserved role in embryonic ecdysteroid biosynthesis.

Interestingly, Spo RNAi embryos showed no developmental differences before 8 hpo, which includes the processes cleavage and early gastrula formation. This may be due to embryos utilized maternally supplied ecdysteroid conjugates instead of performing *de novo* ecdysteroidogenesis. In a previous study, it is found that the expression of *EPPase* which convert inactive ecdysteroid conjugates into active ecdysteroid dropped sharply after 6 hpo, which was consistent with the activation of *Spo* (Asada et al., 2014b) (Fig 7). This pattern suggests that *Spo* begins to take over ecdysteroidogenesis after the depletion of maternally supplied ecdysteroid or EPPase. In the other words, my results suggest that *de novo* ecdysteroidogenesis in *D. magna* start as early as 6-8 h in embryogenesis.

Spo RNAi embryos supplemented with 20E partially resumed development, and hatching was never achieved (Figs 12A, B, C). This is partly because it is impossible to completely mimic the hormonal level changes of ecdysteroid during embryogenesis by artificial supplementation of ecdysteroid. Therefore, the fluctuating level of ecdysteroid is essential in determining developmental progression. This is consistent with the *Spo* expression pattern, which fluctuates during the course of embryogenesis progression (Fig 7). Therefore, further investigation is necessary to clarify the interacting factor which responsible to control ecdysteroid biosynthesis.

Chapter 3 Interaction between ecdysteroid biosynthesis and sesquiterpenoid

3.1. Introduction

Regulation of hormonal level fluctuation is key factor for developmental progression. In arthropods, two hormones, ecdysteroid and sesquiterpenoid are known to coexist in controlling growth and development. Recently study reported the cross-talk between these two hormones in the regulation of each other's biosynthesis in *D. melanogaster* larvae (Liu et al., 2017). In this study, sesquiterpenoid through its response genes reduced the size of prothoracic gland (PG), the portion of ring glands that synthesize ecdysteroid. Meanwhile, the ecdysteroid signaling cascade inhibits the expression of sesquiterpenoid biosynthesis genes in the corpora allata (CA) (Liu et al., 2017). The antagonism between these two hormones is likely a key element in the progression of metamorphosis (Liu et al., 2017).

The sesquiterpenoid hormone is synthesized from acetyl-CoA through the mevalonate pathway. The early steps of the mevalonate pathway to produce farnesyl pyrophosphate (FPP) are conserved among arthropods. The conversion of FPP into its final form of sesquiterpenoid, however, varies among taxa (Bellés et al., 2005; Noriega, 2014). In chelicerates and some crustaceans, methyl farnesoate (MF) has been reported as the final product of the sesquiterpenoid pathway, while in the majority of insect species, the final product of the sesquiterpenoid pathway is Juvenile Hormone III (JH III) (Miyakawa et al., 2014; Sin et al., 2015). The rate-limiting reaction of sesquiterpenoid hormone biosynthesis has been thought to be the final conversion into JH III or MF via S-adenosyl-methyltransferase (SAM)-dependent methylation by juvenile hormone acid methyltransferase (Jhamt) (Bellés et al., 2005; Shinoda and Itoyama, 2003).

In *Daphnia*, ortholog of *Jhamt* and other sesquiterpenoid biosynthesis genes as well as its signaling pathway has been reported (Miyakawa et al., 2013b; Toyota et al., 2015). Expression analysis related to the sesquiterpenoid biosynthesis genes and signaling in *D. pulex* confirmed its role in in male offspring production in adults (Toyota et al., 2015). However, no studies reported the functional analysis of *Jhamt* or sesquiterpenoid biosynthesis, and let alone, the cross-talk between sesquiterpenoid and ecdysteroid in *Daphnia*.

Therefore, in this chapter, I aimed to investigate the possible interaction between sesquiterpenoid in regulating ecdysteroid biosynthesis in *D. magna*. To achieve this, I first focused on elucidation of the sesquiterpenoid biosynthesis, by performing characterization and functional analysis of *Jhamt*, a rate-limiting gene for sesquiterpenoid biosynthesis. After that, I investigated the interaction or cross-talk between sesquiterpenoid and ecdysteroid biosynthesis.

3.2.Materials and method

3.2.1. *Daphnia* strains and transgenic line

Daphnia magna NIES clone was obtained from the National Institute of Environmental Studies (NIES, Tsukuba, Japan) and cultured under the same laboratory condition mentioned in section 2.2.1).

3.2.2. RNA isolation and purification

Daphnia (adults or embryos) were collected in 2-mL tubes, immediately frozen in liquid nitrogen, and homogenized using a MicroSmash MS-100 machine (TOMY) in the presence of Sepasol-RNA I reagent (Nacalai Tesque), according to the manufacturer's instructions. Extracted total RNA was further purified using phenol-

chloroform extraction and ethanol precipitation. Purified total RNA was dissolved in RNase free water (Invitrogen) and stored at –80°C until further use.

3.2.3. Cloning and sequencing of *Jhamt* transcripts

Adult *Daphnia* (115 inds) were collected and subjected to total RNA extraction according to the above-mentioned procedure. Beforehand, eggs were removed from the brood chamber. Polyadenylated RNA was purified from 500 µg of total RNA using the PolyATtract mRNA Isolation System (Promega) and used for 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) using the GeneRacer (Invitrogen) and SMARTer RACE cDNA Amplification (Clontech) kits, respectively. The primers used for cDNA amplification are listed in Table 4 below. PCR was performed using KOD+ DNA Polymerase (Toyobo). PCR products were verified by agarose gel electrophoresis, purified, cloned using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen), and sequenced.

Table 4 The primers used for *Jhamt* RACE experiments

Primer name	Primer sequence (5′−3′)
Jhamt_5RACE-GSP	Forward: - Reverse: TCCGTTCTGCCATGCGTTCGTAC
Jhamt_5RACE-GSP-nested	Forward: - Reverse: CCGTTGATGGTCTTTGATCCAG
Jhamt_3RACE-GSP	Forward: CCACTGGATCAAAGACCATCAACG Reverse: -
Jhamt_3RACE-GSP-nested	Forward: CATGTACGAACGCATGGCAGAAC Reverse: -

3.2.4. Phylogenic analysis

Amino acid sequences of Jhamt genes were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/) as shown in Table 5, and the whole amino acid sequences of each protein were used to construct the phylogenetic tree. Multiple sequence alignments of the amino acid sequences were constructed using ClustalW

(Thompson et al., 1994) in MEGA X for MacOS (Stecher et al., 2020). The same settings mentioned in section 2.2.4 were used for the analysis. The phylogenetic reconstruction was performed using the p-distance algorithm and the neighbor-joining method implemented in MEGA software.

Table 5 Accession numbers of *Jhamt* ortholog used in this study.

Scientific name	Common name	Accession no.
Daphnia magna	Water flea	BCF86812 (this work)
Daphnia pulex	Water flea	EFX90188
Tribolium castaneum	Red flour beetle	NP_00112078
Anopheles sinensis	Malaria mosquito	KFB41593
Bombyx mori	Silkworm moth	NP_001036901
Aedes aegypti	Yellow fever mosquito	XP_001651876
Drosophila melanogaster	Fruit fly	NP_001285980
Penaeus vannamei	Shrimp	XP_027227056
Portunus trituberculatus	Crab	ALT10380
Branchiostoma floridae	Lancelet fish	XP_019635073
Apis mellifera	Honey bee	NP_001314896
Dendroctonus ponderosae	Pine beetle	ERL88145

3.2.5. Quantitative RT-PCR

Embryo samples were collected and their total RNA extracted following description in section 2.2.2 and 2.2.4. qRT-PCR was performed in an Mx3005P (Stratagene) instrument using the Power SYBR Green PCR Master Mix (Invitrogen) with primers listed in Table 6 below. PCR amplification was performed in triplicate under the following conditions: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primer specificity was confirmed by analyzing dissociation curves. Expression levels of each gene were normalized against those of the ribosomal *L32* gene.

Table 6 The primer pairs used for qRT-PCR analysis

Gene name	Primer sequence (5′−3′)
Jhamt	Forward: GTGGGCCGAATACATGAAGG Reverse: ACGAAGGAACGGGTTGACAG
Met	Forward: CGGGTCGTTTGATTTTCCTTC Reverse: TCCTTCATTCCTTGCTCTTCG
Nvd2	Forward: CGTCGGTGACTGCATCGA Reverse: TGCCGTCGTTCCCATTG
Spo	Forward: GGGCTATGCTGTCGATTTCC Reverse: TTGTGCTGTTGTGCGTCTTC
Dib	Forward: CCAACGAACGGACCTGAATG Reverse: TCGAACGTCACCAAACCAAG Reverse: CGGCTGCCACTAGGTCGATA
L32	Forward: GACCAAAGGGTATTGACAACAGA Reverse: CCAACTTTTGGCATAAGGTACTG

3.2.6. RNAi and microinjection

Small interfering RNAs for *Jhamt* were designed using the Block-iT RNAi designer (http://www.invitrogen.com/rnaidesigner.html). Two siRNAs, one targeting 5′ region of Jhamt (siRNA-Jhamt#1) and the one targets 3′ (siRNA-Jhamt#2) (Fig). siRNA sequences were as follows: siRNA-*Jhamt*#1 (5′ GGACUUCGGUUGU GGUGAU 3′); siRNA-*Jhamt*#2 (5′ GGCACCAUCUGCAGAUGAA 3′). Two nucleotides dTdT were added to the 3′ end of the siRNA strand. As control, siRNA-control was used (see section 2.2.6). Microinjection was performed according to an established protocol (Kato et al., 2011). Briefly, freshly ovulated eggs from 2–3-week-old *Daphnia* were collected and placed in ice-cold M4 medium (Elendt and Bias, 1990) containing 80 mM sucrose (M4-Sucrose). Specific siRNA samples for each experiment were mixed with 5 µM AlexaFluor 568 fluorescent dye (Invitrogen) or Lucifer Yellow dye (Invitrogen) as an injection marker. After injection, intact eggs were transferred and cultured individually inside 96-well plates filled with 100 µL M4-Sucrose at 23°C. Injected embryos were collected in three biological replicates and subjected to total RNA isolation as described above. Ten micrograms of yeast tRNA (Ambion) were

added to each sample as carrier RNA. Synthesis of cDNA was performed using a random primer with a PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa). Gene expression levels were evaluated by qPCR using the primer pairs shown in Table 4.

3.2.7. Fenoxycarb exposure

Shortly after siRNA injection, intact eggs were transferred into 100 μL M4-Sucrose containing 0.01% dimethylformamide (DMF) as control, and 33 pM (0.01 μg/L) Fenoxycarb (Wako Pure Chemical, Osaka, Japan) in 0.01% DMF. Embryos were incubated at 23°C in the dark.

3.2.8. Statistical analysis

Differences between datasets were calculated using Student's *t*-test or Welch's *t*-test. For multiple comparisons, differences were assessed using Kruskal-Wallis analysis followed by the Dunn post-hoc test. P-values were further adjusted using the Benjamini-Hochberg procedure. All calculations were performed using R 3.6.2 for MacOS software (R Foundation for Statistical Computing).

3.3.Results

3.3.1. Characterization *of Jhamt* gene in *D. magna*

To investigate the presence of *Jhamt* ortholog in *D. magna*, I performed a BLAST search by using amino acid sequence of *D. melanogaster Jhamt* against *D. magna* genome database. I found one ortholog, which showed strong homology which located in scaffold 00915. Subsequently, I determined a full-length *Jhamt* cDNA by 5′ and 3′ RACE reactions and obtained a single 976 bp nucleotide sequence that encodes for 270 amino acids weighting 37 kDA. Mapping the cDNA into genome revealed that this gene spans 1,767 bp and consists of 5 exons and 4 introns (Fig 13). The deduced

amino acid residue obtained from the cDNA sequence was compared to the protein sequence of *Jhamt* ortholog of other animals. The multiple alignments revealed that *D. magna Jhamt* protein contains the conserved S-adenosyl-L-methionine (SAM) domain located in the N-terminus (Fig 14). This domain is the signature of the methyltransferase protein family (Niwa et al., 2008; Shinoda and Itoyama, 2003).

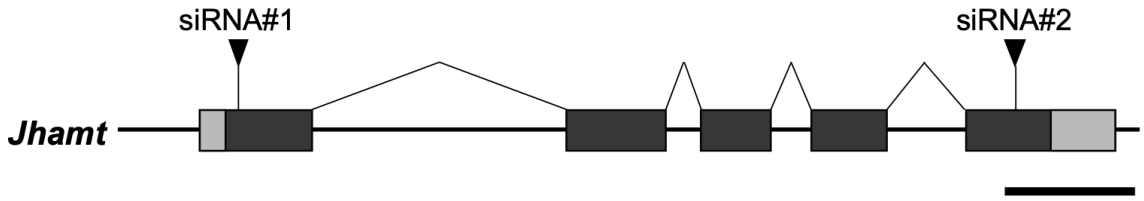


Figure 13 Schematic representation of Jhamt gene structure
Coding sequences (CDSs) are showed as dark-colored boxes, untranslated regions (UTRs) are indicated as light-colored boxes. siRNA target sites are indicated by black triangles. Scale bar, 250 nt

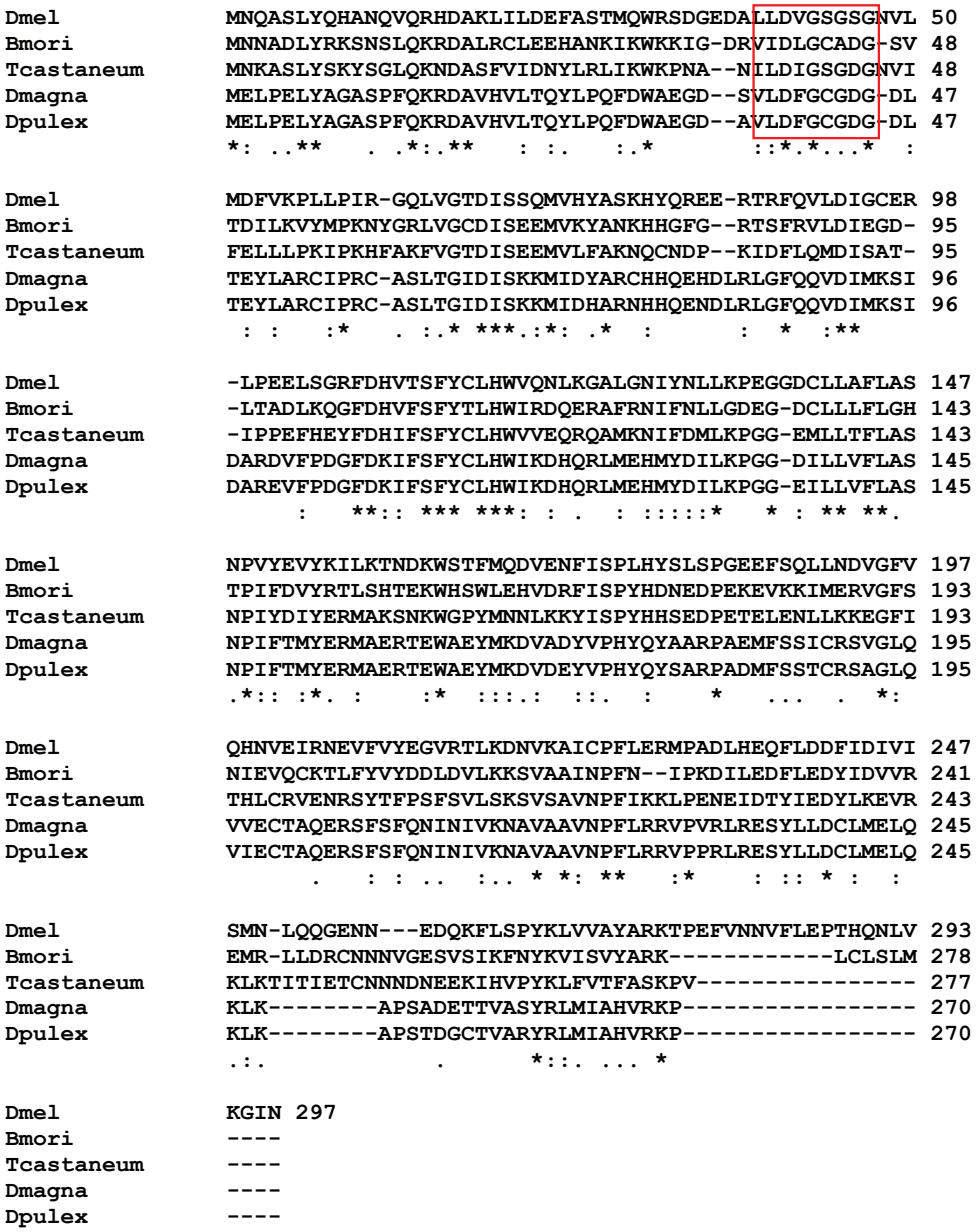


Figure 14 multiple alignment of Jhamt protein
Dmagna, *Daphnia magna*; Dpulex, *Daphnia pulex* Dmel, *Drosophila melanogaster*, Tcastaneum, *Tribolium castaneum* and Bmori, *Bombyx mori*. Asterisks, semicolons, and dots indicate conserved, strongly similar, and weakly similar residues respectively. The red box indicate conserved SAM motifs.

To analyze the evolutionary relationship of *D. magna* Jhamt protein to other animals, a phylogenetic tree from other 11 Jhamt related proteins listed in Table 5 was constructed (Figure 15). The phylogenetic tree was built through neighbor-joining method, using the whole amino acid sequences.

The phylogenetic tree revealed that *Daphnia Jhamt* ortholog is more closely related with malacostraca (shrimp, crab) crustacean rather than those of insects. (Figure 6).

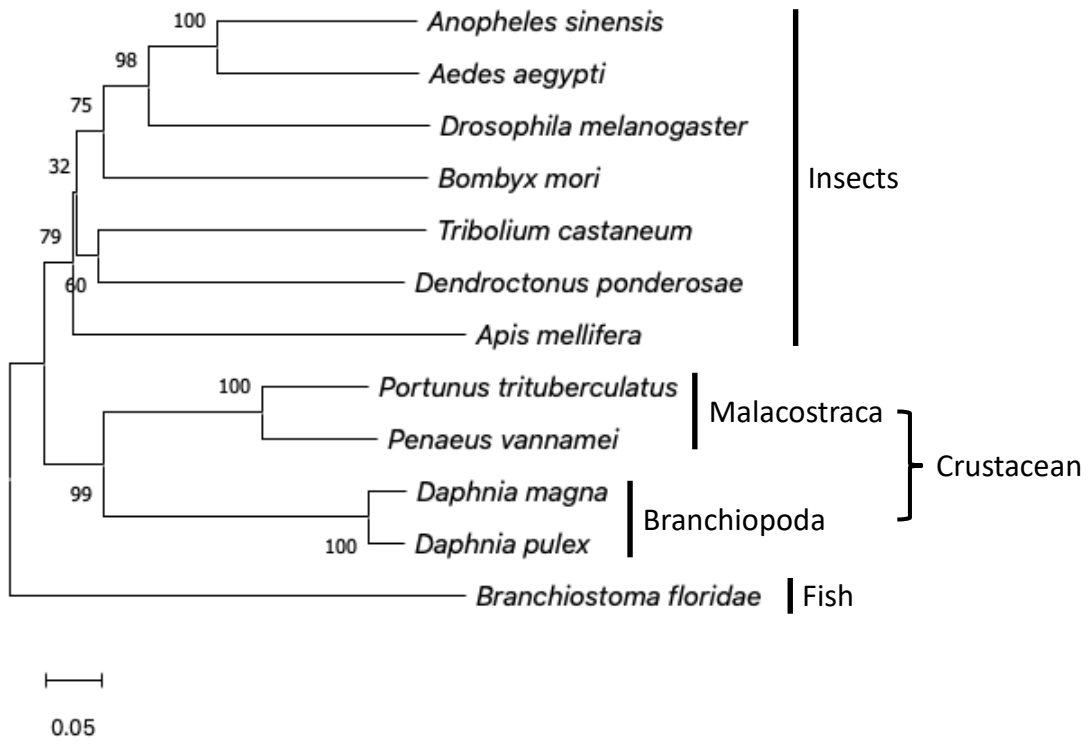


Figure 15 Phylogenetic tree of the amino acid sequences of the Jhamt.
The percentages of the replicate tree in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The bar indicates branch length and corresponds to the mean number of the differences (P<0.05) per residue along each branch. Evolutionary distances were computed using the p-distance method.

3.3.2. Expression of *Jhamt* during embryogenesis

I measured the temporal expression pattern of *Jhamt* by qRT-PCR during predetermined time points during *D. magna* embryogenesis (Fig 16). *Jhamt* mRNA was rarely detected during entire embryogenesis, with the exception of an expression surge at 24 h.

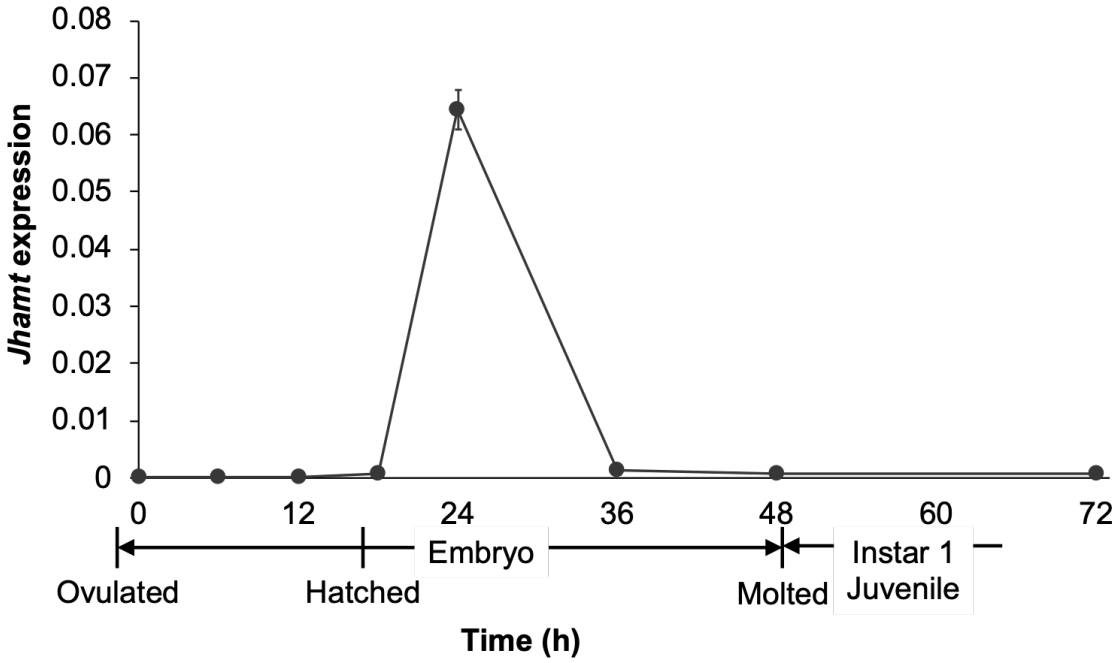


Figure 16 Expression of *Jhamt* during embryogenesis progression
Expression levels were normalized using reference gene expression levels (ribosomal L32). All values are the mean. Error bars represent SD (N=3).

3.3.3. *Jhamt* prevents precocious embryonic development

To determine the function of *Jhamt* during embryogenesis, I performed RNAi-mediated loss-of-function analysis in *D. magna* eggs. To confirm specificity, two siRNAs were used (see section 3.2.6). RNAi injected embryos showed no significant difference in hatching or survival rate relative to control injected, suggesting that *Jhamt* loss of function has little effect on embryo survival (Table 7).

Table 7 Embryo survivability under *Jhamt* knock down

siRNA	Total injected eggs	Relative hatchability (%)	Relative survivability (%)
siJhamt	133	105 ± 3^{a}	102 ± 11^{b}
siJhamt#2	112	104 ± 6^{c}	98 ± 5^{d}
siMet	95	105 ± 5^{e}	86 ± 4^{f}

siControl	104	100 ± 13	100 ± 19

Values are means ± SD. [a]p=0.30; [b]p=0.43; [c]p=0.37; [d]p=0.74; [e]p=0.29; [f]p=0.84 (Welch's *t*-test)

To confirm that the *Jhamt* expression has been efficiently reduced during RNAi treatment, total RNA from 24 h siRNA injected embryos was isolated, and then the *Jhamt* expression level was measured by qRT-PCR. I confirmed the reduced expression of *Jhamt* upon injection of siRNA compared with control as shown in Fig 17, indicating that knock down effectively occurred in the siRNA-injected embryos.

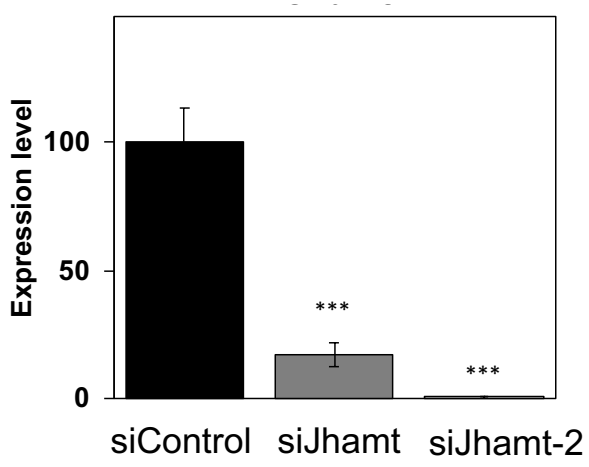


Figure 17 *Jhamt* expression in siRNA-injected embryos
Gene expression level of *Jhamt* in siControl and siSpo embryos at 24 h, as assessed by qRT-PCR. All values are the means. Error bars represent SD (N=3). ***p<0.001 (Student's t-test).

Jhamt is hypothesized as key enzyme for sesquiterpenoid biosynthesis. Because sesquiterpenoids are known as "*status quo"* hormones in insects, I compared the length of embryonic period by examining timing of molting after the end of embryogenesis. SiRNA-*Jhamt*-injected embryos showed a significant reduction of embryonic period compared with the control (Fig 18; siJhamt), suggesting that sesquiterpenoid biosynthesis represses the precocious transition of embryos into the juvenile stage.

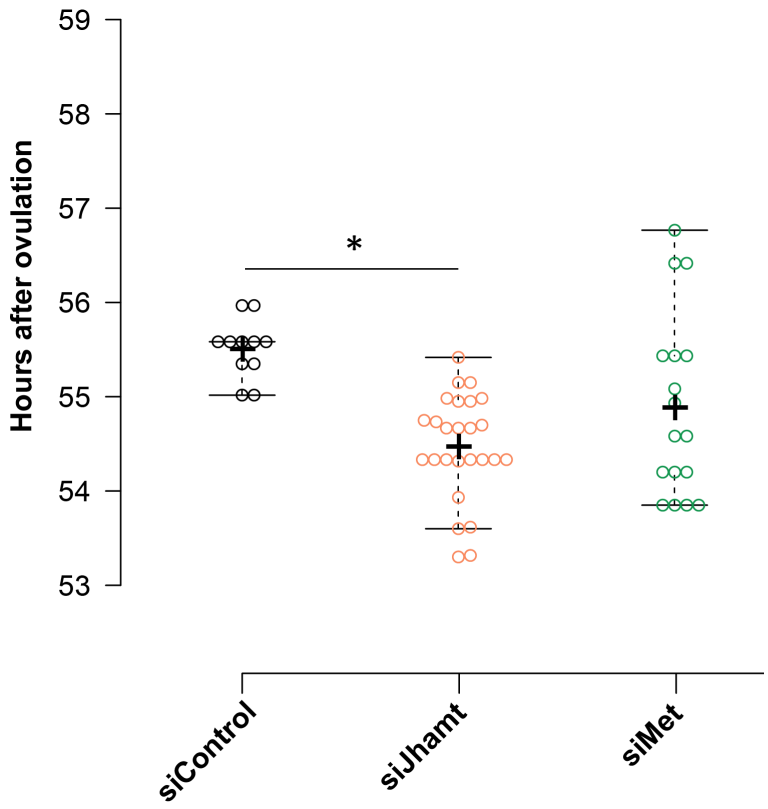


Figure 18 Sesquiterpenoid signaling knock down shortens *D. magna* embryogenesis
Embryonic periods (hours after ovulation, or hao) of siControl, siJhamt, and siMet embryos. *p<0.05 (Kruskal-Wallis followed by Dunn post-hoc test; p-values adjusted using the Benjamini-Hochberg procedure).

To elucidate the regulatory role of sesquiterpenoid signaling in embryos, I silenced *Met*, which codes for the sesquiterpenoid receptor (Miyakawa et al., 2013b). Consistent with siRNA-*Jhamt*, Met RNAi had little effect on embryo survivability (Table 7). Met RNAi injected embryos also exhibit similarities with the *Jhamt* RNAi embryos during embryogenesis, although there was no significant difference between the control and Met RNAi treatment (Fig18; siMet).

I also examined the effect of exposure to the sesquiterpenoid analog Fenoxycarb (FC), on this phenotype. FC increased the length of the embryonic period in both control and Jhamt siRNA-injected embryos (Fig 19; siControl+FC, siJhamt+FC). In contrast, FC treatment of siRNA-Met injected embryos did not significantly elongate the

embryonic period (Fig 19; siMet+FC), possibly due to the silencing of sesquiterpenoid signaling. These results suggest that *Jhamt* functions in sesquiterpenoid biosynthesis and the regulation of the embryonic period.

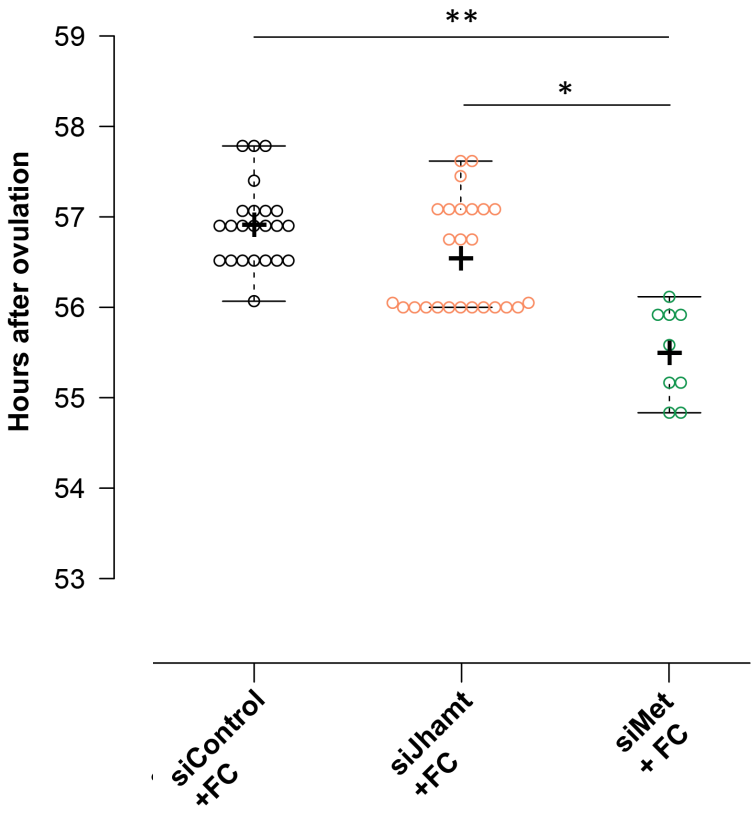


Figure 19 Supplementation of fenoxycarb rescue *Jhamt* knock down phenotype
Embryonic periods (hours after ovulation, or hao) of siControl, siJhamt, and siMet embryos with fenoxycarb (FC) supplementation. *p<0.05 (Kruskal-Wallis followed by Dunn post-hoc test; p-values adjusted using the Benjamini-Hochberg procedure).

3.3.4. Sesquiterpenoid signaling regulates the expression of ecdysteroid metabolism genes

Unexpectedly, significant upregulation of *Jhamt* expression was found to be coincided with lowest levels of *Spo* transcript expression in hatched embryos at 24 h (Fig 20). This antagonistic pattern of *Spo* and *Jhamt* expression suggested the hypothesis of cross-talk between ecdysteroid and sesquiterpenoid biosynthesis at this stage.

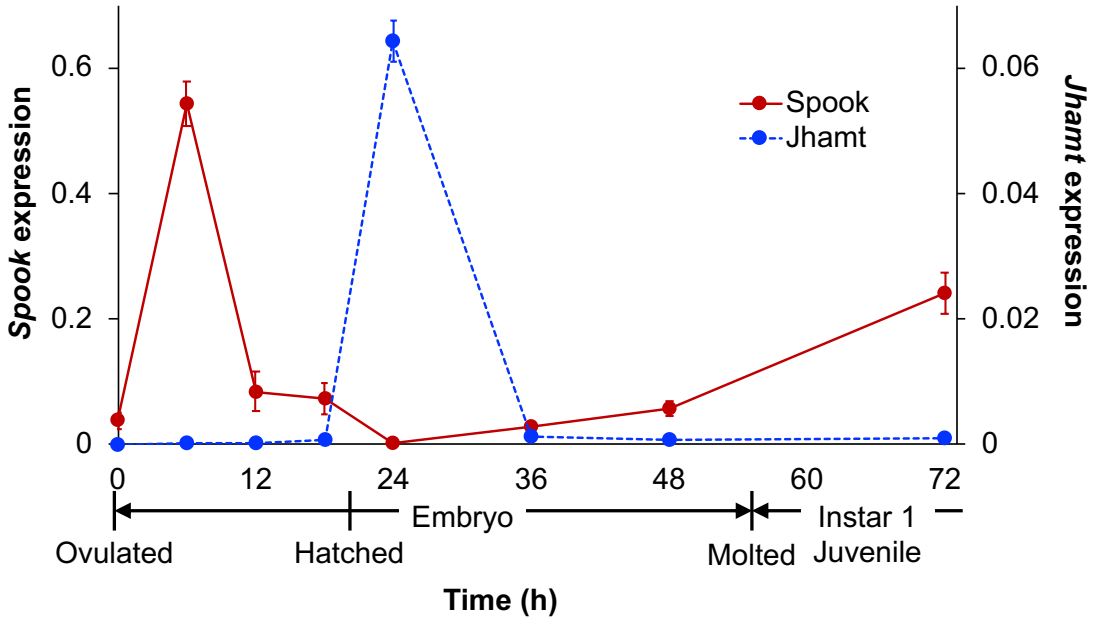


Figure 20 Temporal expression of *Spo* and *Jhamt* during embryogenesis
Spo expression is dropped to the lowest level coincide with the only expression surge of *Jhamt* at 24 h. Expression levels were normalized using reference gene expression levels (ribosomal L32). All values are the mean. Error bars represent SD (N=3).

To investigate this hypothesis, I measured *Spo* expression in *Jhamt* RNAi embryos. I found a significant increase of *Spo* transcript at 24 h after siRNA injection (Fig 21A). To confirm whether this upregulation is orchestrated by sesquiterpenoid signaling pathway, I examined the effect of *Met* RNAi on Spo expression. As predicted, *Spo* expression was also increased similarly to *Jhamt* knock down result (Fig 21A). The level of *Jhamt* mRNA expression was not affected by *Met* knockdown (Fig 21B), suggesting that the upregulation of *Spo* is resulted from sesquiterpenoid signal transduction.

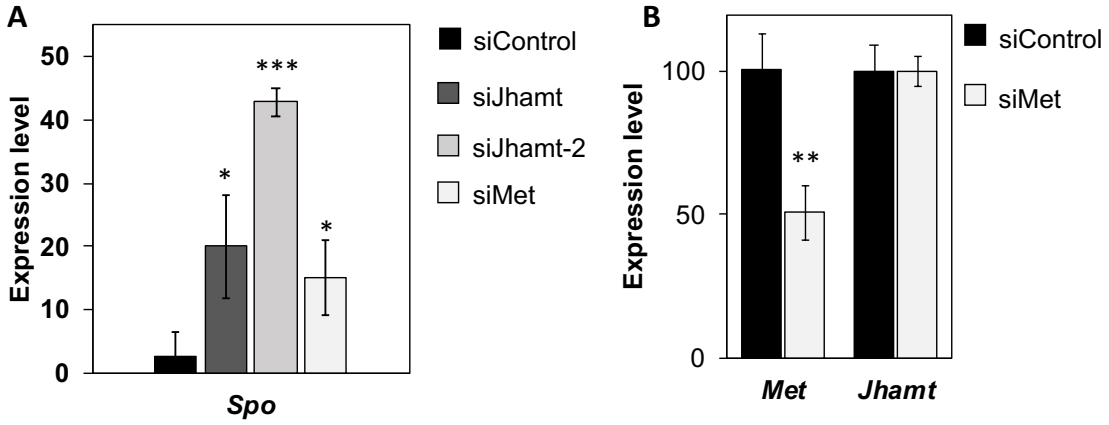


Figure 21 Effect of sesquiterpenoid signaling knock down to *Spo* expression at 24 h
(A) Expression of *Spo* in siJhamt and siMet injected embryos as measured by qRT-PCR. **(B)** Gene expression level of *Met* and *Jhamt* in Met RNAi injected embryo at 24 h as assessed by qRT-PCR. Expression levels are normalized to those of ribosomal *L32*, then shown relative to expression levels of siRNA-ctrl. Values are means. Error bars represent SD (N=3). *p<0.05; **p<0.01; ***p<0.001 (Student's t-test).

I then analyzed the expression of other genes functioning in ecdysteroidogenesis pathways in insects (see section 1.2.1, Fig 1) and found that two ecdysteroidogenesis genes located upstream and downstream of *Spo*, *Nvd2* and *Dib* were upregulated following similar trend with *Spo* (Fig 22). Furthermore, I also measured the expression of *Cyp18a1*, which is responsible for the degradation of ecdysteroid (Rewitz et al., 2010; Sumiya et al., 2016). In contrast to ecdysteroidogenesis genes, *Jhamt* RNAi and *Met* RNAi (Fig 22) reduced *Cyp18a1* expression. Taken together, these results suggest cross-talk between the sesquiterpenoid in regulating ecdysteroid metabolic pathways in hatched embryos.

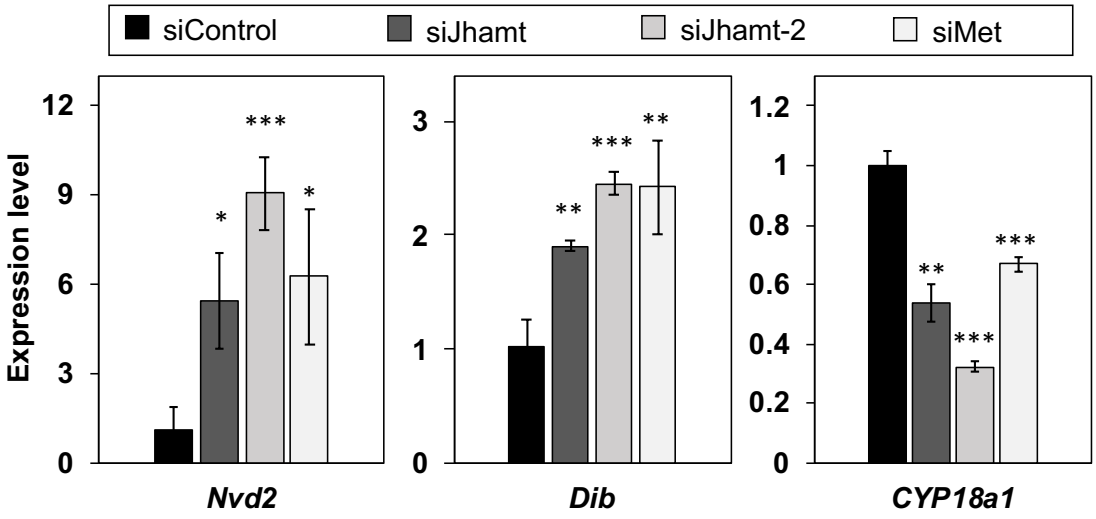


Figure 22 Effect of sesquiterpenoid signaling knock down to other ecdysteroid metabolism genes expression at 24 h
Jhamt and *Met* knock down upregulated two other ecdysteroidogenesis genes and downregulated *Cyp18a1* which responsible in ecdysteroid degradation pathway. Expression levels are normalized to those of ribosomal *L32*, and then shown relative to expression levels of siRNA-ctrl. Values are means. Error bars represent SD (N=3). *p<0.05; **p<0.01; ***p<0.001 (Student's t-test).

3.4. Discussion

Result in previous chapter have shown that *Spo* is essential for progression of embryogenesis. Loss of function analysis combined with hormonal rescue indicated that *Spo* play vital role in *de novo* ecdysteroidogenesis. Expression analysis of *Spo* during embryogenesis indicate its fluctuation throughout developmental time. *Spo* is highly expressed in the early embryogenesis, but the expression dropped shortly after hatching. The modulation of *Spo* expression and subsequently ecdysteroid level is considered essential in controlling embryogenesis progression. However, there are limited studies that investigate the control of *Spo* and ecdysteroidogenesis gene expression level.

In this Chapter, I aimed to clarify the possible interaction of sesquiterpenoid in regulating ecdysteroidogenesis, by analyzing *Jhamt*, the rate-limiting gene for

sesquiterpenoid biosynthesis. First, I confirmed the role of *Jhamt* in sesquiterpenoid biosynthesis in *D. magna*. Expression level of analysis indicated *Jhamt* activation at 24 h time point. Interestingly, this activation coincides with expression drop of *Spo*. Finally, I discover that expression surge of *Jhamt* is responsible in suppressing *Spo* expression down at 24 h timepoint. My results suggest the cross-talk between two hormones in regulating ecdysteroid gene biosynthesis expression in *D. magna* embryogenesis.

The interaction between ecdysteroid and sesquiterpenoid biosynthesis has been previously reported in insects (Liu et al., 2017; Ono, 2014). A recent study on the *Drosophila* ring gland showed that JH induces *Kr-h1* expression, whose gene product inhibits ecdysteroid biosynthesis by suppressing EcR/USP and ecdysone-induced early transcription factors (TFs) (Broad complex, E75, and E73), resulting in reduced PG size and subsequently the inhibition of metamorphosis. *Vice versa*, 20E via EcR/USP action suppress the JH biosynthesis genes *Jhamt* and *HMG-CoA reductase* (*Hmgcr*) in CA, thereby lowering the JH level and allowing the larva to proceed to metamorphosis (Liu et al., 2017).

In this study, I discover interaction between the ecdysteroid and sesquiterpenoid biosynthesis mechanisms during *D. magna* embryogenesis. The regulation of ecdysteroidogenesis by sesquiterpenoid is likely to be orchestrated by a sesquiterpenoid signaling cascade, since the *Met* knockdown caused similar ecdysteroid biosynthesis gene upregulation. In insect, neuropeptide PTTH stimulates ecdysteroidogenesis by specifically activate and regulate transcription of *Spo* gene (Rewitz et al., 2009). As study about neuropeptide PTTH in *Daphnia* is lacking, it is currently unknown whether *Daphnia* PTTH also shares similar mechanism as in insect. Nonetheless, in this study, I found that *Spo* expression also regulated by another component, which is *Jhamt*/sesquiterpenoid signaling. In future, it will be important to clarify which

downstream components of sesquiterpenoid signaling inhibit ecdysteroid biosynthesis genes in *Daphnia*.

Interestingly, a significant downregulation of *Cyp18a1*, which is responsible for ecdysteroid inactivation (Rewitz et al., 2010; Sumiya et al., 2016), was also observed in *Jhamt*/*Met* knock down embryos (Fig 22). To the best of my knowledge, this study is the first to demonstrate a correlation between ecdysteroid inactivation with the action of sesquiterpenoid, indicating that sesquiterpenoid plays a broader role in the regulation of ecdysteroid metabolism.

In insect, knockout and knockdown of *Jhamt* are known to have diverse effect on their development. For instance, *Jhamt* and *Met* knock down resulted in impaired hatchability of embryos in the hemimetabolous insect *Blattella germanica* (Fernandez-Nicolas and Belles, 2017). Meanwhile, in the holometabolous insect *B. mori*, TALEN-mediated *Jhamt* knockout showed no effect on embryonic development, but only slightly led to a decreased embryo hatching rate (Daimon et al., 2015). In *D. magna*, neither *Jhamt* nor *Met* RNAi severely affect hatching of embryos or the survival of juveniles. This may suggest that sesquiterpenoid signaling in *D. magna* embryogenesis is less important than it is in insect embryos. This may be due to the short duration of *Jhamt* expression in the *Daphnia* mid-embryonic stage (Fig16) compared with a longer duration from mid- to late embryogenesis in *B. germanica* and *B. mori*. In *D. pulex*, *Jhamt* was reported to be expressed during the juvenile and adult molting cycles (Miyakawa et al., 2010; Toyota et al., 2015). Moreover, in *Daphnia*, sesquiterpenoid has been found to regulate phenotypic plasticity in predator defense (Miura, 2019; Miyakawa et al., 2013a; Oda et al., 2011) and environmental sex determination (Olmstead and Leblanc, 2002; Tatarazako et al., 2003). Therefore, the role(s) of sesquiterpenoid in *Daphnia* may change in later life stages, as observed in insects

(Jindra, 2019). To disrupt *Jhamt* function completely, generation of homozygous mutant in *D. magna* should be conducted in the future

My results indicated that *Jhamt* and sesquiterpenoid signaling via *Met* play a role in the repression of precocious development, as their knockdown reduced the duration of the embryonic period, while supplementation with a sesquiterpenoid analog elongated embryogenesis. This phenotype is in concordance with the well-known effect of sesquiterpenoid, a "*status quo"* action. In contrast, a *B. mori Jhamt* mutant showed an increased embryonic period, while a sesquiterpenoid analog-exposed cricket showed precocious embryonic development, demonstrating that *D. magna* may have uniquely co-opted sesquiterpenoid signaling for the timing of embryogenesis.

A possible advantage of the repression of precocious embryonic development may be the synchronization of embryogenesis with ovarian development. In *D. magna*, embryos are laid inside the mother's brood chamber and develop here until their first juvenile instar stage. Interestingly, the whole embryo development process is almost perfectly synchronized with the maturation of the mother's ovaries. Shortly after the release of the first instar juveniles from the brood chamber, the mother molts and lays the next batch of eggs. A shortening of embryonic development leads to the earlier release of juveniles from the brood chamber. In contrast, when embryonic development is delayed and embryos are kept longer, they will be released together with the mother's carapace, possibly increasing the risk of predation.

In summary, this chapter demonstrates the roles and cross-talk of ecdysteroid and sesquiterpenoid biosynthesis during embryogenesis in a branchiopod crustacean, *D. magna*. These results illuminate the conserved and specific functions of the sesquiterpenoid in *Daphnia* embryos, as well as interaction between ecdysteroid and sesquiterpenoid.

Chapter 4 Spatio-temporal visualization and detection of ecdysteroid

4.1. Introduction

Ecdysteroid has been known to involve in various processes during growth and development. In Chapter 1, I elucidate the role of ecdysteroid and its key biosynthetic gene, *Spo*, during *D. magna* embryogenesis. Denying embryo from ecdysteroid by disrupting *Spo* at this stage, resulted in immediate cessation of embryogenesis. In Chapter 2, I showed the interaction of other hormone, sesquiterpenoid, to regulate the expression of *Spo* and other ecdysteroidogenesis genes, which demonstrating the cross-talk between two hormones in hormone biosynthesis. Both chapters indicate that ecdysteroid experiencing dynamic changes in time and localization (spatio-temporal) manner. Classical functional analysis through loss-of-function is useful to elucidate the role of genes or hormone. However, such method could not provide spatio-temporal information, as hormone function in complex and time dependent manner.

Ecdysteroid action is exerted by the binding of its receptor to Ecdysteroid response element (EcRE) and initiate gene expression (Antoniewski et al., 1996; Henrich, 2012) (see section 1.2.2). Recently, several EcREs has been identified from ecdysteroid responsive genes in insects (Henrich, 2012; Nishita, 2013). One of the widely used EcRE is *hsp27* EcRE which is derived from *D. melanogaster* heat shock protein promoter (Henrich, 2012; Lucy and Cherbas, 1991). The EcRE sequence is widely used to drive inducible gene expression by attaching a gene of interest downstream (Lee et al., 2016). In application, such system can be switched on/off by adding or removing ecdysteroid into system. Previous *in vitro* study indicates that this EcRE is also compatible with EcR and USP system from *Daphnia*. This suggest that EcRE-inducible system can be used to visualize ecdysteroid in *D. magna* (Kato et al., 2007).

Recently, an ecdysteroid reporter plasmid containing *D. melanogaster hsp27* EcRE followed by *eGFP* reporter gene was constructed and introduced into *D. magna* eggs via microinjection (Asada et al., 2014a). This method succeeded in visualizing ecdysteroid activity in live *D. magna* embryo. Nonetheless, this approach suffered from some limitations. Firstly, the plasmid is transient, therefore reporter plasmid needs to be re-injected into *Daphnia* embryos every single time. This is not practical, as microinjection requires advanced technical skills. Secondly, due to the transient nature and non-homogenous distribution of the injected plasmid inside the eggs, the fluorescence pattern and localization may not stable, which limit the reproducibility of the observation. In order to stably visualize ecdysteroid activity, permanent integration of reporter gene into the genome is necessary. The advantages of genetic manipulation techniques recently developed in *Daphnia* could enable the integration of foreign gene into the genome.

Therefore, in this chapter I aimed to develop a specialized transgenic *Daphnia* containing an EcRE-controlled reporter gene. To assist genome integration, I utilized targeted gene editing technique by CRISPR/Cas9 system. Development of ecdysteroid reporter transgenic *Daphnia* could facilitate the better understanding of spatiotemporal ecdysteroid activity. In addition, this *Daphnia* could be deployed as a tool for monitoring ecdysteroid activities in environmental water.

4.2.Material and methods

4.2.1. *Daphnia* strain and culture condition

The *D. magna* (NIES clone) was obtained from the National Institute for Environmental Studies (NIES; Tsukuba, Japan) and cultured under the same laboratory condition mentioned in Chapter 2 (see 2.2.1 *Daphnia* strain and culture condition).

4.2.2. Construction of donor plasmid

To generate the donor plasmid, I replaced the *H2B-eGFP* gene of the 4xEcRE-H2B-eGFP plasmid (Asada et al., 2014a) with the *mCherry* gene, resulting in the 4xEcRE-mCherry plasmid. The *D. magna scarlet* gene was selected as a target site for knock-in. The *scarlet* gene encodes for the Family G ATP-binding Cassette Transporter (ABC transporter) protein, which plays a key role in the ommochrome pigment synthesis of *D. magna* compound eye. A loss-of-function mutation in this gene was distinctly characterized by the loss of the eye pigmentation phenotype (Ismail et al., 2018). For its integration into the *scarlet* (*st*) locus, a 200 nt fragment of the *st* gene harboring the *st*-targeting gRNA sequence (Ismail et al., 2018) was amplified from *D. magna* genome, and then cloned into the 4xEcRE-mCherrry plasmid to generate the 4xEcRE-mCherrry-st plasmid. The PCR and cloning experiments were performed using the KOD Plus DNA Polymerase (Toyobo) and In-Fusion HD Cloning Kit (Clontech). The PCR was performed under the following condition: initial denaturation at 98°C, followed by a total of 25 three-temperature cycles (denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 68°C for 4 min) and a final extension step at 68°C for 7 min. The donor plasmid was purified using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) and then subjected to a sequencing reaction (BigDye Terminator v3.1, Life Technologies, CA, USA) and sequenced using an ABI 3100 Genetic Analyzer (Life Technologies, CA, USA).

4.2.3. Guide RNA (gRNA) synthesis

The *st*-targeting gRNA was synthesized using a cloning-free method (Gagnon et al., 2014). The sense synthetic oligonucleotide contains three main parts: a T7 promoter (shown in bold), a *st*-targeting sequence (underlined nucleotides), and the first 20 nt of the Cas9 binding scaffold sequence. The full sequence is as follow: (5′ -

GAAA**TTAATACGACTCACTATAGG**TTCACTCGTCGCCTTAAT*GTTTTAGA GCTAGAAATAGC* -3′). The anti-sense oligonucleotide contains an 80-nt full sequence of Cas9 binding scaffold: (5′- AAAAGCACCGACTCGGTGCCACTTTTTCAAG TTGATAACGGACTAGCCTTATTTTAACTT*GCTATTTCTAGCTCTAAAAC*-3′) where italic nucleotides are the complementary nucleotides between the two oligonucleotides. A PCR reaction was performed using the PrimeStar DNA Polymerase (TaKaRa) under the following reaction conditions: initial denaturation at 98°C for 5 min, followed by a total of 15 three-temperature cycles (denaturation at 98 °C for 10 s, annealing at 55°C for 30 s, and extension at 68°C for 15 s) and a final extension step at 68°C for 5 min. The PCR product was subjected to *in vitro* transcription using the MegaScript T7 Transcription Kit (Invtrogen). The synthesized gRNAs were purified using the mini Quick Spin RNA Columns (Roche Diagnostics GmbH, Mannheim, Germany), two times phenol/chloroform extraction, and ethanol precipitation before finally dissolving them in DNase/RNase-free water (Merck Millipore, MA, USA).

For knock-in, 1 μM Cas9 protein and 2 μM gRNA mixture were incubated for 5 min at 37°C to make the Cas9-gRNA ribonucleoprotein (RNP) as described previously (Kumagai et al., 2017). Subsequently, the Cas9-gRNA RNP was mixed with 50 ng/μL donor plasmid and 1 mM Lucifer Yellow fluorescence dye (Invitrogen). The resulting solution was co-injected in the wild type *D. magna* eggs after briefly centrifuging for 5 min at 20,500 × g.

4.2.4. Generation of transgenic *Daphnia*

Transgenic *Daphnia* were generated using the non-homologous end joining (NHEJ) knock-in method as previously described (Kumagai et al., 2017). In brief, Cas9 nuclease, single guide RNA (gRNA), and donor plasmid containing the corresponding gRNA sequence were injected into the embryos. The gRNA-guided Cas9 nuclease

introduces double-strand break (DSB) on both donor plasmid and genome. After DNA cleavage, the break was repaired using the NHEJ pathway, allowing the donor plasmid to be integrated into the genome's DSB site.

For knock-in, 1 μM Cas9 protein and 2 μM gRNA mixture were incubated for 5 min at 37°C to make the Cas9-gRNA ribonucleoprotein (RNP) as described previously (Kumagai et al., 2017). Subsequently, the Cas9-gRNA RNP was mixed with 50 ng/μL donor plasmid and 1 mM Lucifer Yellow. The resulting solution was co-injected in the wild type *D. magna* eggs after briefly centrifuging for 5 min at 20,500 × g.

4.2.5. Genotyping of transgenic *Daphnia*

For genotyping, *Daphnia* was collected and homogenized using the MicroSmash homogenizer (TOMY) at 3000 rpm for 90 s in 500 μL lysis buffer (50 mM Tris-HCl pH 7.5; 20 mM EDTA; 100 mM NaCl; 1% SDS) and 7.5 μL of 10 mg/mL Proteinase K (Nacalai). Subsequently, the homogenized samples were incubated overnight at 55°C. To obtain a clean genomic extract, the lysate was purified by phenol/chloroform extraction, ethanol precipitation, and dissolved in TE buffer. A PCR reaction was performed using the PrimeStar DNA Polymerase (TaKaRa) and the primers corresponding to the 5′ and 3′ predicted integrated junction regions (Table 8). The PCR was performed under the following conditions: initial denaturation at 94°C for 2 min, followed by a total of 30 three-temperature cycles (denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 66°C for 1 min) and a final extension step at 66°C for 5 min. The PCR product was purified and cloned into a plasmid using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) for sequencing.

Table 8 Primer pairs for genotyping

Amplified region	Primer sequence (5′−3′)
5′ junction	Forward: CGTTATACCTCTTGCCGTAC Reverse: TACCGGGTTGGACTCAAG
3′ junction	Forward: GACATCACCTCCCACAACGA Reverse: TGATAAACGTAGCCGCTC
Scarlet allele	Forward: TTCCATTGCTCATACAACC Reverse: GCAAAGTAGATTTCCCTGC

4.2.6. 20-hydroxyecdysone (20E) exposure

To examine the response of transgenic *Daphnia* to ecdysteroid in water, 20-hydroxyecdydone (20E; Enzo Life Science, NY, USA) was dissolved in dimethylformamide (DMF) and was kept at −30 °C. A serial dilution of the stock solution (104 mM) was performed to prepare the working solutions of various concentrations for exposure.

4.3.Results

4.3.1. Checking the functionality of reporter plasmid

Donor plasmid containing four tandem repeats of EcRE sequences followed by mCherry fluorescent gene was evaluated *in vivo* by microinjection. Plasmid concentration was determined to 62.5 ng/μL following previous study (Asada et al., 2014a).

Injected eggs expressed mCherry red fluorescence after 12 h of injection (Fig 23). mCherry expression was mostly detected around posterior (p) region of embryos in the later stage (Fig 23b). There was no specific pattern of signal localization between individual embryo in which some embryo only exhibits –or at least had brighter-fluorescence signal in one side of embryo. It was hypothesized that due to exogenous nature of reporter gene introduced, distribution of the reporter plasmid was not

homogenous. It was also thought that the plasmid copy number that was introduced and expressed might vary from one embryos to another.

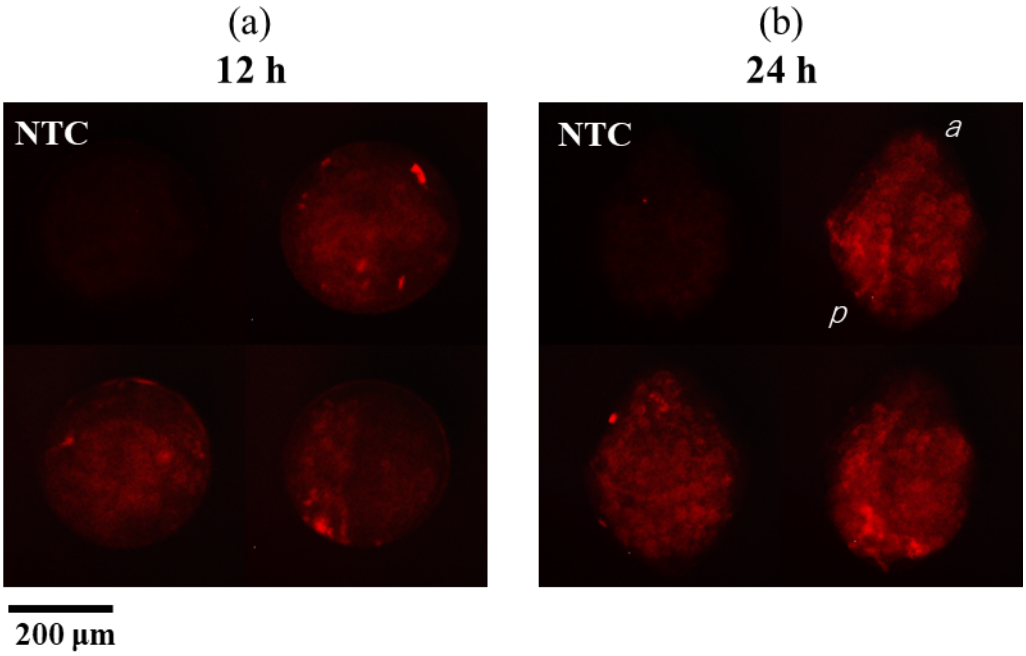


Figure 23 Functionality of reporter plasmid in *D. magna* developing embryos
Photograph shows 4 independent embryos with non-injected control located at uppermost left (NTC). Figure **(a)** shows fluorescent signal at 12 h after injection. Head (anterior, *a*) and tail (posterior, *p*) position cannot be determined at this stage. Figure **(b)** shows growing fluorescent signal at 24 h after injection. Brighter signal observed in posterior region.

Further evaluation of reporter gene functionality was conducted by co-injection of reporter plasmid and 20-hydroxyecdysone (20E) ligand. After 24 h, signal from mCherry gene was detected from the injected embryos. Increment of 20E ligand showed positive correlation with observed mCherry signal (Fig 24). These results altogether may suggest the reporter plasmid could respond to presence of both endogenous and exogenous ecdysteroid.

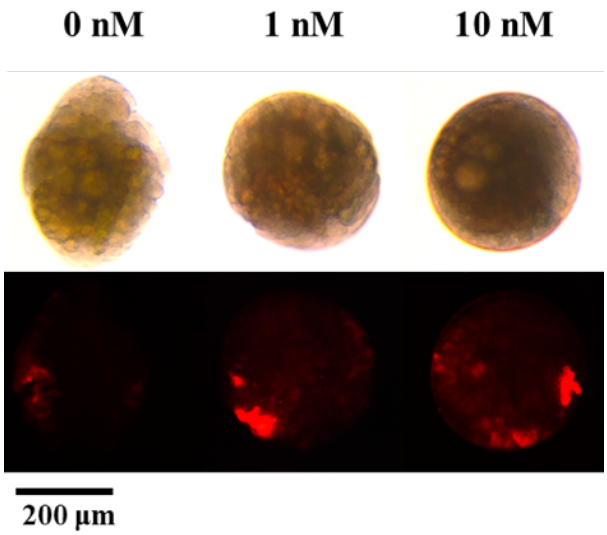


Figure 24 Co-microinjection of 20-hydroxyecdysone and reporter plasmid
After 24 h, fluorescent expression increased in embryo injected using higher concentration (1 nM and 10 nM). Embryo without 20E injection showed normal development as anterior and posterior section could already distinguishable. Embryos injected with higher concentration of 20E showed developmental delay.

4.3.2. Generation of transgenic *Daphnia*

The donor reporter plasmid along with the Cas9-gRNA RNP was microinjected in 98 eggs. Of these, 27 survived and 22 (81%) of them reached the reproductive age. The heritable knockout (K.O) mutation, which could be observed from white-eye phenotype, was observed in 14 embryos (64%), of which the heritable knock-in (K.I) occurred only in one individual (4.5%), which was designated as EcRE-mCh line (Table 9).

Table 9 Summary of knock-in microinjection experiment

Replicate	Injected eggs	Juvenile	Adult	Heritable K.O	Heritable K.I
1	19	6	4	2	0
2	24	6	5	4	0
3	31	10	8	4	1
4	24	5	5	4	0
Total	**98**	**27**	**22**	**14**	**1**
Percentage			**81%**	**64%**	**4.5%**

To determine indel mutations along the junction regions, genotyping was performed to EcRE-mCh line. Initially, integration direction was hypothesized in the same orientation as the endogenous *scarlet* gene, as illustrated in Fig 25. Therefore, primers were designed to amplify the upstream (5′) and downstream (3′) junctions in sense orientation (Fig 25, annotated as *a* and *b*). Gel electrophoresis result showed *a* and *b* amplified correct predicted size of junction region, as shown in Figure 25B, i and ii. Integrated plasmid copy number was determined by designing primer at 5' and 3' junction, which will amplify the whole plasmid (Fig 25A, annotated as *c*). Gel result showed amplicon size similar with one intact copy of linearized donor plasmid (Fig 25B, ii). Slightly lower size was thought to be caused by indel mutations. These results conclude that a single copy of reporter plasmid was integrated in the same orientation with scarlet target gene.

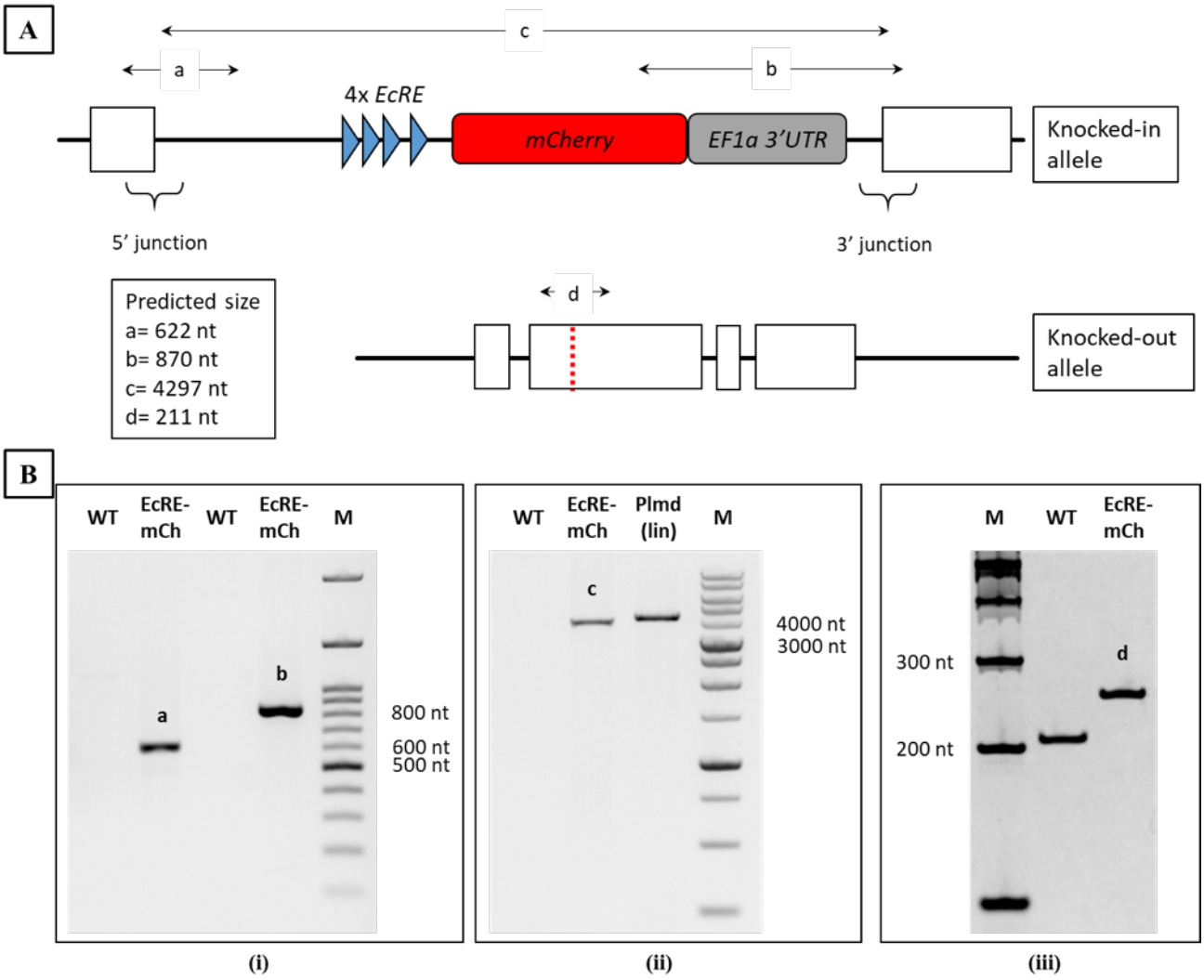


Figure 25 Genotyping of EcRE-mCh transgenic *Daphnia*
(A) Simplified diagram of reporter plasmid integration in *scarlet* locus. One copy of reporter plasmid was integrated in sense orientation similar with *scarlet* gene. White boxes indicate genomic region surrounding integrated reporter plasmid. Alphabet a, b, c, d indicate amplified fragments with corresponding expected size listed in the left. Red-dashed line indicates gRNA-st2 target site.
(B) Gel electrophoresis and native PAGE result of fragments a, b, c, and d. (i) Fragment a and b were within expected size, confirming integration direction. (ii) Fragment c amplified whole integrated plasmid and has similar size with linearized donor plasmid indicating one copy was integrated. (iii) Insertion mutation in second allele disrupt scarlet gene causing loss of eye pigmentation in EcRE-mCh line.

The sequences of PCR products for each junction region showed various indel mutations and were typical of the error-prone NHEJ repair activity (Figure 26). The 5′ junction region showed a 3-nt deletion just before the proto-adjacent motif (PAM) sequence (underlined nucleotides) of the gRNA target (Figure 26B), while the 3′

junction showed a 16-nt deletion and a 1-nt insertion just before the PAM sequence of the gRNA target (Fig 26B). Since EcRE-mCh exhibits colorless eye, a bi-allelic mutation was expected to occur in the *scarlet* locus. The sequence of the knocked-out allele showed a 59-nt random insertion and a 13-nt deletion mutation along the gRNA target (Fig 26C).

(a) 5' junction

```
TTCGCGGAGCTTTACAACCCGGTTCACTCGTCGCCTTAATGGGCGCTAGGTAACAACGATGAAG      (WT)
TTCGCGGAGCTTTACAACCCGGTTCACTCGTCGCCTT---GGGCGCTAGGTAACAACGATGAAG      (EcRE) +0/Δ3
```

(b) 3' junction

```
TTCGCGGAGCTTTACAACCCGGTTCACTCGTCGCCTTAATGGGCGCTAGGTAACAACGATGAAG      (WT)
TTCGCGGAGCTTTACAACCCGGTT--------G-------GGGCGCTAGGTAACAACGATGAAG      (EcRE) +1/Δ16
```

(c) second allele

```
CAACCCGGTT-----C--------A---CT-CGTCG-------------CCT-----T-----------AATGGGCGCTAGG   (WT)
CAACCCGGTTgtaagCcgggttgtAaagCTaCcTaGggctgtaaagctaCCTaaggtTacctagcgcccAATGGGCGCTAGG   (EcRE) +59/Δ13
```

Figure 26 Insertion and deletion mutation in junction regions and second allele
Red, black, and underlined characters indicate gRNA-st2 sequence, proto adjacent motif (PAM) sequence, and genomic sequence respectively.
(a) 5′ junction region showed small deletion in gRNA sequence just before PAM.
(b) 3′ junction showed larger deletion and insertion within gRNA sequence before PAM.
(c) More severe deletion and insertion mutation occurred in second allele.

4.3.3. EcRE-mCh expression pattern during embryogenesis

The expression of EcRE-mCh's mCherry was observed during the early stages of embryogenesis starting from 12 h after ovulation, consistent with result of transiently injected plasmid in Section 4.3.1. however, mCherry signal from EcRE-mCh line showed more restricted and consistent expression than in plasmid-injected embryos. As shown in Fig 27, from 24 h, mCherry signal intensity intensifies, and clustered in developing thoracic appendages area. By 48 h, the intensity was decreasing as clusters of mCherry expression cells dispersed as the body elongates. Finally, at the end of embryogenesis, mCherry signal compacted around digestive track.

For detailed observation of mCherry signal migration, time-lapse imaging was performed. The time-lapse imaging during 12–24 h showed enhancing expression of the mCherry signal originating from the posterior section of embryo and then migrating towards the anterior section (Fig 28). The signal was originated from the bilateral region of the posterior end starting from 16 h. In the next 4 h, the mCherry signal increased in intensity and localized around the developing thoracic appendages (Figure 3, white arrows). The localization continued until 25 h, when all three pairs of thoracic appendages showed the mCherry fluorescence. During later stages, the mCherry signal dispersed around the central part of embryo and decreased in intensity. At 60 h, the fluorescence signal seemed to be localized around the digestive tract region.

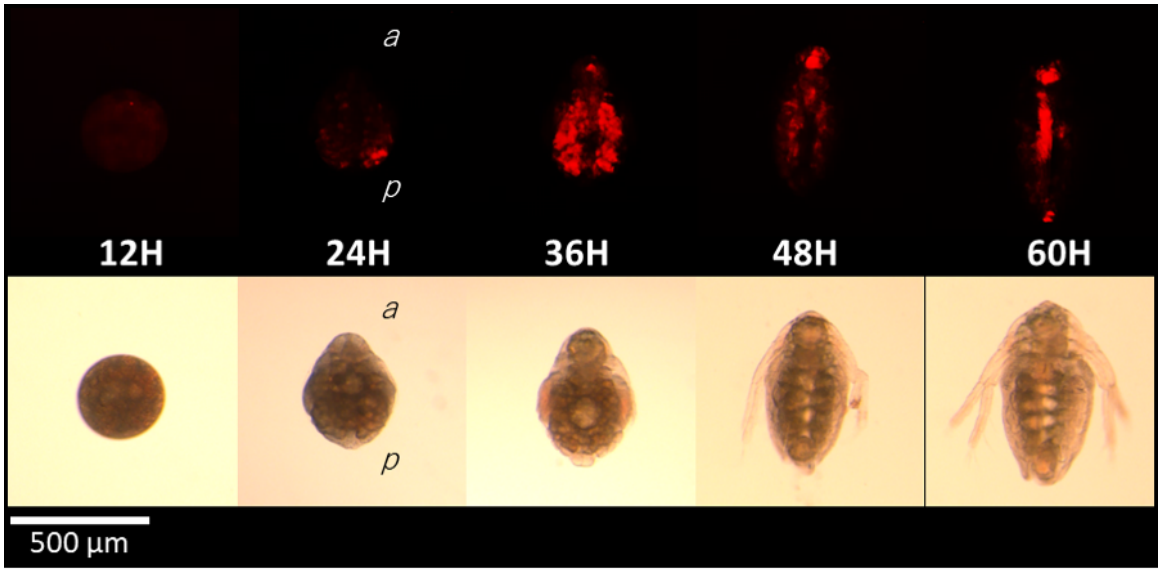


Figure 27 EcRE-mCh's mCherry expression during embryogenesis
At 12 h, mCherry was faintly expressed and barely visible. At 24 h, embryo's head (anterior, *a*) and tail (posterior, *p*) was distinguishable and mCherry signal was observed at posterior region. At 36-48 h, signal expanded in surrounding developing thoracic appendages before finally compacted around digestive tract at 60 h.

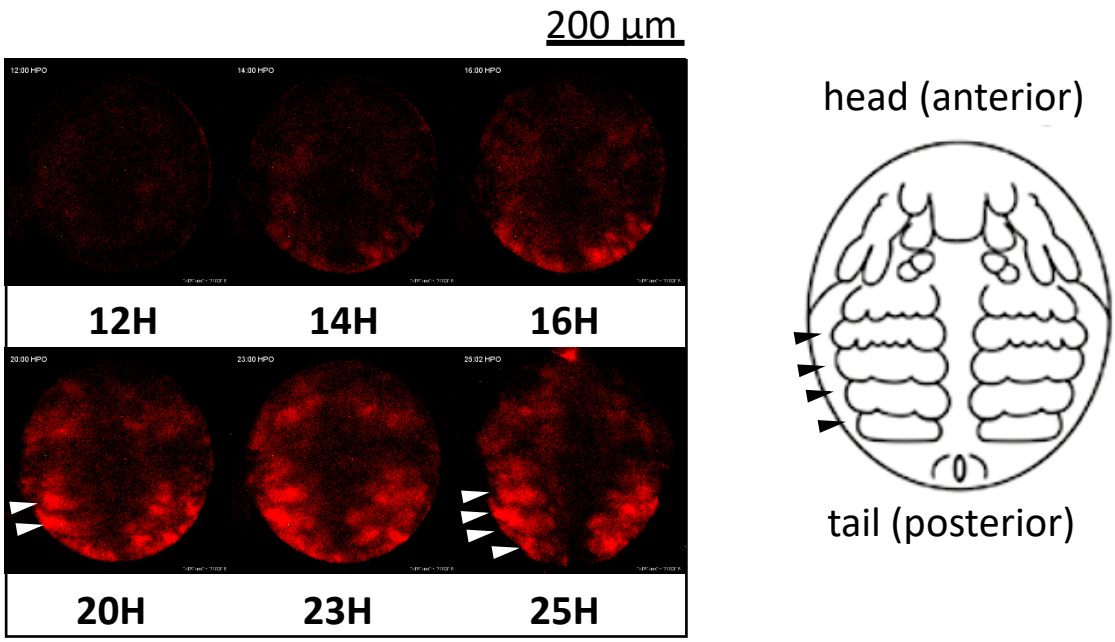


Figure 28 Detailed mCherry expression pattern during 12-24 h embryogenesis
Snapshot of time lapse snapshot from 12-24 h showed movement of mCherry signal from posterior end of embryo.

4.3.4. Detecting ecdysteroid presence in surrounding water

The EcRE-mCh embryos were externally exposed to the ligand (20E) to check whether EcRE can activate the downstream expression of the *mCherry* gene. Upon 24 h of exposure, the mCherry signal could be detected from embryo at 10 µg/L concentration of 20E (Fig 29). The embryos exposed to a higher concentration (50 µg/L) showed higher fluorescence intensity relative to the unexposed control. These results suggest that the EcRE-mCh line could be used to monitor ecdysteroid activities in environmental and waste water.

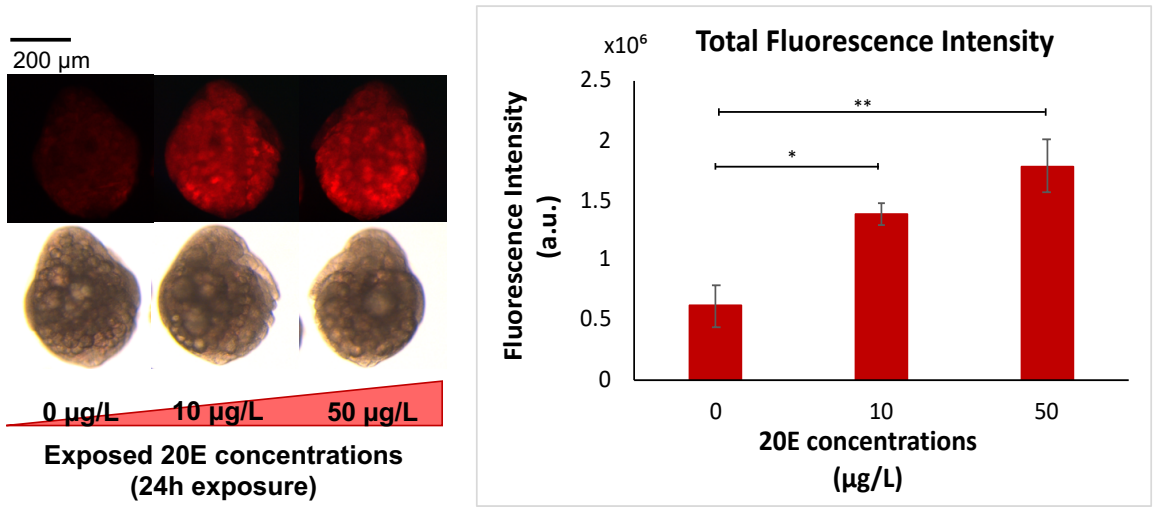


Figure 29 Exposure of 20-hydroxyecdysone increases mCherry fluorescence
Newly ovulated embryos were dissected and cultured in M4 medium with various concentration of 20E. After 24 h, fluorescent signal showed increment in accordance with concentration.

4.4.Discussion

In this chapter, I reported establishment of a transgenic *Daphnia* which contain ecdysteroid reporter gene integrated into the genome. The ecdysteroid activity inside developing embryo can be conveniently visualized through mCherry fluorescence signal. This transgenic reporter could aid spatio-temporal study of ecdysteroid and development of ecdysteroid biosensor.

The expression of EcRE-mCh's mCherry was observed during the early stages of embryogenesis starting from 12 h after ovulation. In the previous study, I injected the plasmids encoding the *H2B-GFP* gene under the control of four tandemly repeated EcREs and observed the GFP signal at 6 h after microinjection into the one-celled embryos (Asada et al., 2014a). This delayed detection of fluorescence in EcRE-mCh might be due to the difference in copy number between the plasmid injected into the one-cell embryos (Asada et al., 2014a) and the plasmid integrated into the genome of the EcRE-mCh. In addition, the difference in nature between the two fluorescent

proteins might affect the temporal fluorescence pattern because GFP is brighter than mCherry (Heppert et al., 2016).

The time-lapse imaging during 12–24 h showed enhancing expression of the mCherry signal originating from the posterior section of embryo and then migrating towards the anterior section (Fig 28). The signal was originated from the bilateral region of the posterior end starting from 16 h. In the next 4 h, the mCherry signal increased in intensity and localized around the developing thoracic appendages (Fig 28, white arrows). The localization continued until 25 h, when all three pairs of thoracic appendages showed the mCherry fluorescence. Since the mCherry signal originated from the ecdysteroid activity, therefore a presumption that ecdysone and posterior growth are related to each other cannot be ruled out. It was hypothesized that the activation of ecdysone correlated with the sequential growth of posterior section.

Posterior growth is a conserved feature among bilaterians, especially in short germ-band organisms (Martin and Kimelman, 2009). During embryonic development, a short germ-band organism adds blocks of muscle tissues (somites) in an anterior-posterior fashion, which accounts for the formation of most of the body posterior to the head. This phenomenon is known to be regulated by *Wnt-Delta-Notch* signaling (McGregor et al., 2009). A previous report showed the presence of 12 *Wnt* genes in *D. pulex*. (Janssen et al., 2010). However, no studies have shown the activation of *Wnt* gene to explain ecdysteroid activity. This phenomenon might suggest the novel role of ecdysteroid activity in posterior growth regulatory signaling of *D. magna*. Upon thorough time-lapse observation in the first 24 h of embryogenesis, the mCherry-expressing region seemed to correspond to the developing thoracic appendages in the posterior to anterior direction. The role of ecdysteroid has not been studied in relation

with the posterior growth signaling. Further confirmation of the ecdysteroid-posterior growth relationship should be studied and could be a focus of the future research.

Chapter 5 General discussion and conclusion

5.1. General discussion

This study aimed to elucidate the role of ecdysteroid during embryogenesis progression of *Daphnia magna*. Ecdysteroid has been known to regulate growth and development. Since most of studies are done in insect model animal, our knowledge in non-insect arthropod are limited, despite the vast diversity and potential economic and ecological significance of other arthropod species. *D. magna* is member of crustacean, one of arthropod clades members, which also include many economically significant species (shrimp, crabs, prawn). *D. magna* was used in this study because 1) being a crustacean, it has close evolutionary relationship with insect, 2) they are easy to handle and reproduce quickly, and 3) they are the only crustacean with a sequenced genome that can be easily manipulated.

To achieve the objective of this study, I performed several approaches. First, I elucidated the biosynthesis process by performing characterization and functional analysis of *Spook* (*Spo*), the rate-limiting gene for ecdysteroid biosynthesis. I found that *Spo*, through ecdysteroid biosynthesis is necessary for embryogenesis progression. *Spo* expression fluctuates during the course of embryogenesis, which suggest interaction from another factor may regulate this expression. In the next chapter, I investigated the possible interaction between ecdysteroid and the other hormone, sesquiterpenoid. I found that sesquiterpenoid control the expression of *Spo* and other ecdysteroidogenesis genes expression, which demonstrate a cross-talk between two hormones. Result from chapter 1 and 2 illustrated the role of ecdysteroid in time dependent manner. However, spatial information of hormone activity is missing. To further understanding the spatio-temporal manner of ecdysteroid, I established an ecdysteroid reporter *Daphnia* by utilizing gene-editing technique. This transgenic *Daphnia* enable me to visualize

ecdysteroid activity during embryogenesis progression. Moreover, this transgenic could also respond to exogenous ecdysteroid, which suggest its potential for development of animal-based biosensor.

Spo is known as rate-limiting enzyme in ecdysteroid biosynthesis. Therefore, functional analysis of this gene in may provide overall insight of ecdysteroid biosynthesis in *D. magna* embryogenesis. Temporal gene expression analysis of *Spo* showed it highly expressed, and its loss of function resulted in early developmental arrest. The role of *Spo* in ecdysteroidogenesis is demonstrated by the fact that development retardation of *Spo* knocked down embryo can be rescued to some extend by supplementation of ecdysteroid agonist. In *D. magna* embryogenesis, as indicated by GFP expression, cell differentiation and body segmentation occurred shortly after gastrulation (Fig 7). However, in *Spo* deficient embryo, such processes failed to occur. This indicate that ecdysteroid is required in regulation of embryonic cell differentiation. The progression of embryogenesis may require careful fluctuation of ecdysteroid level. This is apparent as in 20E-rescued knock down embryos, development could only partially resume, as artificial 20E exposure could not mimic the endogenous ecdysteroid titer.

The fluctuation of ecdysteroid in embryogenesis indicate that there is another component, which interact with its biosynthesis process. Sesquiterpenoid is another major arthropod hormone, which together with ecdysteroid, known to regulates developmental milestone in insects. Therefore, in chapter 3, I investigated the possible interaction between ecdysteroid and sesquiterpenoid in *D. magna* embryogenesis. To begin with, I analyzed the function of *Juvenile hormone acid o-methyltransferase* (*Jhamt*), a key gene for sesquiterpenoid biosynthesis. Interestingly, *Jhamt* is rarely expressed during embryogenesis. RNAi mediated knock down of this gene showed that,

in contrast to *Spo* and ecdysteroid, *Jhamt* and sesquiterpenoid are not essential for *D. magna* embryogenesis.

During embryogenesis, *Spo* and *Jhamt* expression pattern at 24 h time point suggests an antagonistic pattern between two genes (Fig 18). Suppressing *Jhamt* or sesquiterpenoid signaling at this time point resulted in de-repression of *Spo* and other ecdysteroidogenesis genes. This indicate that ecdysteroid biosynthesis is regulated by sesquiterpenoid action. To my knowledge, this is the first study that shows the interaction/cross-talk between two main arthropod hormones during embryogenesis in non-insect species. It is currently unclear why *Spo* and other ecdysteroid biosynthesis genes need to drop, and what are the consequences if they overexpressed at this time point. Previous studies has indicated that decrease of ecdysteroid level is important for hatching (Sumiya et al., 2016). This is also consistent with 20E exposure result in Chapter 2 (Fig 11) where high 20E exposure led to reduced hatchability. However, in this study, knock down of *Jhamt* or *Met* resulted in no significant decrease of hatching rate. This maybe because siRNA mediated knock down is not enough to disrupt *Jhamt* or *Met* expression completely. Therefore, future studies will be benefitted by generation of *Jhamt*/*Met* knock out mutant to clarify these phenomena. In this regard, it is also currently unknown whether ecdysteroid also have role in the regulation of sesquiterpenoid biosynthesis or metabolism in *D. magna* since the currently available approaches for the impairment of ecdysteroid signaling or biosynthesis resulted in embryonic lethality during early embryogenesis (Sumiya et al., 2016). Nevertheless, my results suggest that ecdysteroid and sesquiterpenoid hormonal cross-talk was established before the evolutionary divergence of *Daphnia* from insects over 400 million years ago.

In chapter 4, I established an ecdysteroid reporter *Daphnia* by utilizing targeted gene editing technique. This transgenic is named EcRE-mCh and contains a *mCherry* fluorescence protein gene under the EcRE promoter. Using this reporter *Daphnia*, ecdysteroid activity is visualized by expression of mCherry fluorescence, thus for the first time, facilitating spatio-temporal study of this hormone. EcRE-mCh's mCherry expression during embryogenesis showed localization around developing body segments such as thoracic appendages (Fig 28). In chapter 2, embryos injected with *Spo* RNAi failed to develop thoracic appendages at 16-22 h (Fig 11B), which indicate that ecdysteroid presence required for body segment formation at this time range. While this is information is difficult to obtain from gene expression level analysis, by using EcRE-mCh, I could observe localized mCherry signal in thoracic appendages from 12-24 h. This indicate that spatio-temporal visualization using EcRE-mCh could provide more information than that in temporal gene expression analysis.

Study of ecdysteroid in *D. magna* may be beneficial for the development of ecdysteroid biosensor. As previously described, ecdysteroid analog has commonly used as active substance for pesticide, such as tebufenozide, methoxyfenozide, and halofenozide (Doucet et al., 2008; Smagghe and Degheele, 1998) and may adversely affect non-target arthropods, such as crustacean as they share similar hormonal pathway. Therefore, a monitoring system for evaluating the presence of those chemicals is necessary. The EcRE-mCh embryo can respond to the presence of ecdysteroid in water thus may be developed further for animal-based biosensor in the future.

5.2. Conclusion

In this study, 1) I elucidated the role of ecdysteroid during embryogenesis by analyzing *Spo*, the rate limiting gene in ecdysteroid biosynthesis. 2) I discovered the

interaction between sesquiterpenoid in controlling the ecdysteroid biosynthesis during embryogenesis. This is the first report about cross-talk between two main endocrine hormones in non-insect species. 3) Lastly, I generated an ecdysteroid reporter transgenic *Daphnia* which can visualize ecdysteroid activity spatio-temporally. The establishment of this reporter *Daphnia* have potential to be developed as ecdysteroid biosensor to detect presence of ecdysteroid agonist in the water. Overall, I anticipate that my findings would contribute to further understanding the evolution and diversity of endocrinology in the ecologically and economically important arthropod species.

References

Antoniewski, C., Mugat, B., Delbac, F., Lepesant, J. a, 1996. Direct repeats bind the EcR/USP receptor and mediate ecdysteroid responses in *Drosophila melanogaster*. Mol. Cell. Biol. 16, 2977–86. https://doi.org/10.1128/MCB.16.6.2977

Asada, M., Kato, Y., Matsuura, T., Watanabe, H., 2014a. Visualization of ecdysteroid activity using a reporter gene in the crustacean, *Daphnia*. Mar. Environ. Res. 93, 118–122. https://doi.org/10.1016/j.marenvres.2013.11.005

Asada, M., Kato, Y., Matsuura, T., Watanabe, H., 2014b. Early embryonic expression of a putative ecdysteroid-phosphate phosphatase in the water flea, *Daphnia magna* (Cladocera: *Daphniidae*). J. Insect Sci. 14, 1–6. https://doi.org/10.1093/jisesa/ieu043

Baltaev, U., Shangaraeva, G., 2000. Zooecdysteroids: Distribution and Role in Arthropod Life Cycles. Chem. Nat. Compd. 36, 433–446. https://doi.org/10.1023/A:1017545222062

Barton, N.H., 2009. Why sex and recombination?, in: Cold Spring Harbor Symposia on Quantitative Biology. pp. 187–195. https://doi.org/10.1101/sqb.2009.74.030

Bellés, X., Martín, D., Piulachs, M.-D., 2005. the Mevalonate Pathway and the Synthesis of Juvenile Hormone in Insects. Annu. Rev. Entomol. 50, 181–199. https://doi.org/10.1146/annurev.ento.50.071803.130356

Betts, M.J., Guigó, R., Agarwal, P., Russell, R.B., 2001. Exon structure conservation despite low sequence similarity: A relic of dramatic events in evolution? EMBO J. 20, 5354–5360. https://doi.org/10.1093/emboj/20.19.5354

Butenandt, A., Karlson, P., 1954. Über die Isolierung eines Metamorphose-Hormons der Insekten in kristallisierter Form. Zeitschrift fur Naturforsch. - Sect. B J. Chem. Sci. 9, 389–391. https://doi.org/10.1515/znb-1954-0601

Chávez, V.M., Marqués, G., Delbecque, J.P., Kobayashi, K., Hollingsworth, M., Burr, J., Natzle, J.E., Connor, M.B.O., 2000. The *Drosophila disembodied* gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. Development 4126, 4115–4126.

Colbourne, J.K., Pfrender, M.E., Gilbert, D., Thomas, W.K., Tucker, A., Oakley, T.H., Tokishita, S., Aerts, A., Arnold, G.J., Basu, M.K., Bauer, D.J., Cáceres, C.E., Carmel, L., Casola, C., Choi, J.H., Detter, J.C., Dong, Q., Dusheyko, S., Eads, B.D., Fröhlich, T., Geiler-Samerotte, K.A., Gerlach, D., Hatcher, P., Jogdeo, S.,

Krijgsveld, J., Kriventseva, E. V., Kültz, D., Laforsch, C., Lindquist, E., Lopez, J., Manak, J.R., Muller, J., Pangilinan, J., Patwardhan, R.P., Pitluck, S., Pritham, E.J., Rechtsteiner, A., Rho, M., Rogozin, I.B., Sakarya, O., Salamov, A., Schaack, S., Shapiro, H., Shiga, Y., Skalitzky, C., Smith, Z., Souvorov, A., Sung, W., Tang, Z., Tsuchiya, D., Tu, H., Vos, H., Wang, M., Wolf, Y.I., Yamagata, H., Yamada, T., Ye, Y., Shaw, J.R., Andrews, J., Crease, T.J., Tang, H., Lucas, S.M., Robertson, H.M., Bork, P., Koonin, E. V., Zdobnov, E.M., Grigoriev, I. V., Lynch, M., Boore, J.L., 2011. The ecoresponsive genome of *Daphnia pulex*. Science. 331, 555–561. https://doi.org/10.1126/science.1197761

Daimon, T., Uchibori, M., Nakao, H., Sezutsu, H., Shinoda, T., 2015. Knockout silkworms reveal a dispensable role for juvenile hormones in holometabolous life cycle. Proc. Natl. Acad. Sci. U. S. A. 112, E4226–E4235. https://doi.org/10.1073/pnas.1506645112

Doucet, D., Cusson, M., Retnakaran, A., 2008. Insect endocrinology and hormone-based pest control products in IPM. Integr. Pest Manag. Concepts, Tactics, Strateg. Case Stud. 286–297. https://doi.org/10.1017/CBO9780511626463.023

Elendt, B., Bias, W., 1990. Trace nutrient deficiency in *Daphnia magna* cultured in standard medium for toxicity testing: Effects of the optimization of culture conditions on life history parameters of *Daphnia magna*. Water Res. 24, 1157–1167. https://doi.org/10.1016/0043-1354(90)90180-E

Enya, S., Ameku, T., Igarashi, F., Iga, M., Kataoka, H., Shinoda, T., Niwa, R., 2014. A Halloween gene noppera-bo encodes a glutathione S-transferase essential for ecdysteroid biosynthesis via regulating the behaviour of cholesterol in *Drosophila*. Sci. Rep. 4. https://doi.org/10.1038/srep06586

Fernandez-Nicolas, A., Belles, X., 2017. Juvenile hormone signaling in short germ-band hemimetabolan embryos. Development 144, 4637–4644. https://doi.org/10.1242/dev.152827

Feyereisen, R., 1999. Insect P450 enzymes. Annu. Rev. Entomol. 44, 507–533. https://doi.org/10.1146/annurev.ento.44.1.507

Gagnon, J.A., Valen, E., Thyme, S.B., Huang, P., Ahkmetova, L., Pauli, A., Montague, T.G., Zimmerman, S., Richter, C., Schier, A.F., 2014. Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. PLoS One 9, 5–12. https://doi.org/10.1371/journal.pone.0098186

Gilbert, L.I., 2004. Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*, in: Molecular and Cellular Endocrinology. pp. 1–10. https://doi.org/10.1016/j.mce.2003.11.003

Gilbert, L.I., Rybczynski, R., Warren, J.T., 2002. Control and Biochemical Nature of the Ecdysteroidogenic Pathway. Annu. Rev. Entomol. 47, 883–916. https://doi.org/10.1146/annurev.ento.47.091201.145302

Glenner, H., Thomsen, P.F., Hebsgaard, M.B., Sørensen, M.V., Willerslev, E., 2006. The origin of insects. Science. 314, 1883–1884. https://doi.org/10.1126/science.1129844

Guittard, E., Blais, C., Maria, A., Parvy, J.P., Pasricha, S., Lumb, C., Lafont, R., Daborn, P.J., Dauphin-Villemant, C., 2011. CYP18A1, a key enzyme of *Drosophila* steroid hormone inactivation, is essential for metamorphosis. Dev. Biol. 349, 35–45. https://doi.org/10.1016/j.ydbio.2010.09.023

Hebert, P.D.N., 1978. the Population Biology of *Daphnia* (Crustacea, *Daphnidae*). Biol. Rev. 53, 387–426. https://doi.org/10.1111/j.1469-185x.1978.tb00860.x

Henrich, V.C., 2012. The Ecdysteroid Receptor, in: Gilbert, L.I.B.T.-I.E. (Ed.), Insect Endocrinology. Academic Press, San Diego, pp. 177–218. https://doi.org/10.1016/B978-0-12-384749-2.10005-6

Heppert, J.K., Dickinson, D.J., Pani, A.M., Higgins, C.D., Steward, A., Ahringer, J., Kuhn, J.R., Goldstein, B., 2016. Comparative assessment of fluorescent proteins for in vivo imaging in an animal model system. Mol. Biol. Cell 27, 3385–3394. https://doi.org/10.1091/mbc.E16-01-0063

Ismail, N.I.B., Kato, Y., Matsuura, T., Watanabe, H., 2018. Generation of white-eyed *Daphnia magna* mutants lacking *scarlet* function. PLoS One 13, 1–21. https://doi.org/10.1371/journal.pone.0205609

Janssen, R., Le Gouar, M., Pechmann, M., Poulin, F., Bolognesi, R., Schwager, E.E., Hopfen, C., Colbourne, J.K., Budd, G.E., Brown, S.J., Prpic, N.M., Kosiol, C., Vervoort, M., Damen, W.G., Balavoine, G., McGregor, A.P., 2010. Conservation, loss, and redeployment of Wnt ligands in protostomes: Implications for understanding the evolution of segment formation. BMC Evol. Biol. 10, 1–21. https://doi.org/10.1186/1471-2148-10-374

Jindra, M., 2019. Where did the pupa come from? The timing of juvenile hormone signalling supports homology between stages of hemimetabolous and holometabolous insects. Philos. Trans. R. Soc. B Biol. Sci. 374, 20190064.

https://doi.org/10.1098/rstb.2019.0064

Jockusch, E.L., Smith, F.W., 2015. Hexapoda: Comparative Aspects of Later Embryogenesis and Metamorphosis BT - Evolutionary Developmental Biology of Invertebrates 5: Ecdysozoa III: Hexapoda, in: Wanninger, A. (Ed.), . Springer Vienna, Vienna, pp. 111–208. https://doi.org/10.1007/978-3-7091-1868-9_3

Kato, Y., Kobayashi, K., Oda, S., Tatarazako, N., Watanabe, H., Iguchi, T., 2007. Cloning and characterization of the ecdysone receptor and ultraspiracle protein from the water flea *Daphnia magna*. J. Endocrinol. 193, 183–194. https://doi.org/10.1677/JOE-06-0228

Kato, Y., Matsuura, T., Watanabe, H., 2012. Genomic Integration and Germline Transmission of Plasmid Injected into Crustacean *Daphnia magna* Eggs. PLoS One 7, 0–6. https://doi.org/10.1371/journal.pone.0045318

Kato, Y., Perez, C.A.G., Mohamad Ishak, N.S., Nong, Q.D., Sudo, Y., Matsuura, T., Wada, T., Watanabe, H., 2018. A 5′ UTR-Overlapping LncRNA Activates the Male-Determining Gene doublesex1 in the Crustacean *Daphnia magna*. Curr. Biol. 28, 1811-1817.e4. https://doi.org/10.1016/j.cub.2018.04.029

Kato, Y., Shiga, Y., Kobayashi, K., Tokishita, S.I., Yamagata, H., Iguchi, T., Watanabe, H., 2011. Development of an RNA interference method in the cladoceran crustacean *Daphnia magna*. Dev. Genes Evol. 220, 337–345. https://doi.org/10.1007/s00427-011-0353-9

Kleiven, O.T., Larsson., P., Hobk, A., 1992. Sexual reproduction in Daphnia magna requires three stimuli. Oikos 65, 197–206.

Klüttgen, B., Dülmer, U., Engels, M., Ratte, H.T., 1994. Adam , an Artificial Freshwater for the Culture of Zooplankton. Water Res. 28, 743–746. https://doi.org/doi.org/10.1016/0043-1354(94)90157-0

Kozlova, T., Thummel, C.S., 2003. Essential roles for ecdysone signaling during Drosophila mid-embryonic development. Science. 301, 1911–1914. https://doi.org/10.1126/science.1087419

Kumagai, H., Nakanishi, T., Matsuura, T., Kato, Y., Watanabe, H., 2017. CRISPR/Cas-mediated knock-in via nonhomologous end-joining in the crustacean Daphnia magna. PLoS One 12, 1–12. https://doi.org/10.1371/journal.pone.0186112

Lafont, R., Dauphin-Villemant, C., Warren, J.T., Rees, H., 2012. Ecdysteroid Chemistry and Biochemistry, in: Gilbert, L.I. (Ed.), Insect Endocrinology. Academic Press, San Diego, pp. 106–176. https://doi.org/10.1016/B978-0-12-

384749-2.10004-4

Lafont, R., Koolman, J., 2009. Diversity of Ecdysteroids in Animal Species, in: Smagghe, G. (Ed.), Ecdysone: Structures and Functions. Springer Netherlands, Dordrecht, pp. 47–71. https://doi.org/10.1007/978-1-4020-9112-4_2

Lafont, R., Mathieu, M., 2007. Steroids in aquatic invertebrates. Ecotoxicology 16, 109–130. https://doi.org/10.1007/s10646-006-0113-1

LeBlanc, G.A., 2007. Crustacean endocrine toxicology: A review. Ecotoxicology 16, 61–81. https://doi.org/10.1007/s10646-006-0115-z

Lee, B.Y., Choi, B.S., Kim, M.S., Park, J.C., Jeong, C.B., Han, J., Lee, J.S., 2019. The genome of the freshwater water flea *Daphnia magna*: A potential use for freshwater molecular ecotoxicology. Aquat. Toxicol. 210, 69–84. https://doi.org/10.1016/j.aquatox.2019.02.009

Lee, S., Sohn, K.C., Choi, D.K., Won, M., Park, K.A., Ju, S.K., Kang, K., Bae, Y.K., Hur, G.M., Ro, H., 2016. Ecdysone Receptor-based Singular Gene Switches for Regulated Transgene Expression in Cells and Adult Rodent Tissues. Mol. Ther. - Nucleic Acids 5, e367. https://doi.org/10.1038/mtna.2016.74

Liu, S., Li, K., Gao, Y., Liu, X., Chen, W., Ge, W., Feng, Q., Palli, S.R., Li, S., 2017. Antagonistic actions of juvenile hormone and 20-hydroxyecdysone within the ring gland determine developmental transitions in *Drosophila*. Proc. Natl. Acad. Sci. 115, 201716897. https://doi.org/10.1073/pnas.1716897115

Lucy, C., Cherbas, P., 1991. Identification of ecdysone response elements by analysis of the *Drosophila Eip28/29* gene 120–131.

Martin, B.L., Kimelman, D., 2009. Wnt Signaling and the Evolution of Embryonic Posterior Development. Curr. Biol. 19, R215–R219. https://doi.org/10.1016/j.cub.2009.01.052

McGregor, A.P., Pechmann, M., Schwager, E.E., Damen, W.G.M., 2009. An ancestral regulatory network for posterior development in arthropods. Commun. Integr. Biol. https://doi.org/10.4161/cib.7710

Mittmann, B., Ungerer, P., Klann, M., Stollewerk, A., Wolff, C., 2014. Development and staging of the water flea *Daphnia magna* (Straus, 1820; Cladocera, Daphniidae) based on morphological landmarks. Evodevo 5. https://doi.org/10.1186/2041-9139-5-12

Miura, T., 2019. Juvenile hormone as a physiological regulator mediating phenotypic plasticity in pancrustaceans. Dev. Growth Differ. 61, 85–96.

https://doi.org/10.1111/dgd.12572

Miyakawa, H., Gotoh, H., Sugimoto, N., Miura, T., 2013a. Effect of juvenoids on predator-induced polyphenism in the water flea, *Daphnia pulex*. J. Exp. Zool. Part A Ecol. Genet. Physiol. 319, 440–450. https://doi.org/10.1002/jez.1807

Miyakawa, H., Imai, M., Sugimoto, N., Ishikawa, Y., Ishikawa, A., Ishigaki, H., Okada, Y., Miyazaki, S., Koshikawa, S., Cornette, R., Miura, T., 2010. Gene up-regulation in response to predator kairomones in the water flea, *Daphnia pulex*. BMC Dev. Biol. 10. https://doi.org/10.1186/1471-213X-10-45

Miyakawa, H., Sato, T., Song, Y., Tollefsen, K.E., Iguchi, T., 2017. Ecdysteroid and juvenile hormone biosynthesis, receptors and their signaling in the freshwater microcrustacean *Daphnia*. J. Steroid Biochem. Mol. Biol. 0–1. https://doi.org/10.1016/j.jsbmb.2017.12.006

Miyakawa, H., Toyota, K., Hirakawa, I., Ogino, Y., Miyagawa, S., Oda, S., Tatarazako, N., Miura, T., Colbourne, J.K., Iguchi, T., 2013b. A mutation in the receptor Methoprene-tolerant alters juvenile hormone response in insects and crustaceans. Nat. Commun. 4, 1856–1857. https://doi.org/10.1038/ncomms2868

Miyakawa, H., Toyota, K., Sumiya, E., Iguchi, T., 2014. Comparison of JH signaling in insects and crustaceans. Curr. Opin. Insect Sci. 1, 81–87. https://doi.org/10.1016/J.COIS.2014.04.006

Mu, X., Leblanc, G.A., 2002. *Environmental antiecdysteroids alter embryo development in the crustacean Daphnia magna.* J. Exp. Zool. 292, 287–292. https://doi.org/10.1002/jez.10020

Mykles, D.L., 2011. Ecdysteroid metabolism in crustaceans. J. Steroid Biochem. Mol. Biol. https://doi.org/10.1016/j.jsbmb.2010.09.001

Naitou, A., Kato, Y., Nakanishi, T., Matsuura, T., Watanabe, H., 2015. Heterodimeric TALENs induce targeted heritable mutations in the crustacean *Daphnia magna*. Biol. Open 4, 364–369. https://doi.org/10.1242/bio.20149738

Nakanishi, T., Kato, Y., Matsuura, T., Watanabe, H., 2016. TALEN-mediated knock-in via non-homologous end joining in the crustacean *Daphnia magna*. Sci. Rep. 6, 1–7. https://doi.org/10.1038/srep36252

Nakanishi, T., Kato, Y., Matsuura, T., Watanabe, H., 2015. TALEN-mediated homologous recombination in *Daphnia magna*. Sci. Rep. 5, 1–10. https://doi.org/10.1038/srep18312

Nakanishi, T., Kato, Y., Matsuura, T., Watanabe, H., 2014. CRISPR/Cas-mediated

targeted mutagenesis in *Daphnia magna*. PLoS One 9. https://doi.org/10.1371/journal.pone.0098363

Nishita, Y., 2013. Identification of a DNA sequence critical for ecdysone receptor binding to the distal promoter of the *Bombyx* Broad-Complex gene. J. Insect Biotechnol. Sericology 82, 71–78.

Niwa, R., Matsuda, T., Yoshiyama, T., Namiki, T., Mita, K., Fujimoto, Y., Kataoka, H., 2004. *CYP306A1*, a cytochrome P450 enzyme, is essential for ecdysteroid biosynthesis in the prothoracic glands of *Bombyx* and *Drosophila*. J. Biol. Chem. 279, 35942–35949. https://doi.org/10.1074/jbc.M404514200

Niwa, R., Namiki, T., Ito, K., Shimada-Niwa, Y., Kiuchi, M., Kawaoka, S., Kayukawa, T., Banno, Y., Fujimoto, Y., Shigenobu, S., Kobayashi, S., Shimada, T., Katsuma, S., Shinoda, T., 2010. *Non-molting glossy*/*shroud* encodes a short-chain dehydrogenase/reductase that functions in the "Black Box" of the ecdysteroid biosynthesis pathway. Development 137, 1991–1999. https://doi.org/10.1242/dev.045641

Niwa, R., Niimi, T., Honda, N., Yoshiyama, M., Itoyama, K., Kataoka, H., Shinoda, T., 2008. Juvenile hormone acid O-methyltransferase in *Drosophila melanogaster*. Insect Biochem. Mol. Biol. 38, 714–720. https://doi.org/10.1016/j.ibmb.2008.04.003

Niwa, Y.S., Niwa, R., 2016. Transcriptional regulation of insect steroid hormone biosynthesis and its role in controlling timing of molting and metamorphosis. Dev. Growth Differ. 58, 94–105. https://doi.org/10.1111/dgd.12248

Noriega, F.G., 2014. Juvenile Hormone Biosynthesis in Insects: What Is New, What Do We Know, and What Questions Remain? Int. Sch. Res. Not. 2014, 1–16. https://doi.org/10.1155/2014/967361

Oda, S., Kato, Y., Watanabe, H., Tatarazako, N., Iguchi, T., 2011. Morphological changes in *Daphnia galeata* induced by a crustacean terpenoid hormone and its analog. Environ. Toxicol. Chem. 30, 232–238. https://doi.org/10.1002/etc.378

Olesen, J., 2004. On the ontogeny of the Branchiopoda (Crustacea): Contribution of development to phylogeny and classification, in: Crustacean Issues Vol 15. pp. 217–269.

Olmstead, A.W., Leblanc, G.A., 2002. Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. J. Exp. Zool. 293, 736–739. https://doi.org/10.1002/jez.10162

Ono, H., 2014. Ecdysone differentially regulates metamorphic timing relative to 20-hydroxyecdysone by antagonizing juvenile hormone in *Drosophila melanogaster*. Dev. Biol. 391, 32–42. https://doi.org/10.1016/j.ydbio.2014.04.004

Ono, H., Rewitz, K.F., Shinoda, T., Itoyama, K., Petryk, A., Rybczynski, R., Jarcho, M., Warren, J.T., Marqués, G., Shimell, M.J., Gilbert, L.I., O'Connor, M.B., 2006. *Spook* and *Spookier* code for stage-specific components of the ecdysone biosynthetic pathway in Diptera. Dev. Biol. 298, 555–570. https://doi.org/10.1016/j.ydbio.2006.07.023

Orsini, L., Orsini, L., Gilbert, D., Podicheti, R., Jansen, M., Brown, J.B., 2016. Data Descriptor : *Daphnia magna* transcriptome by RNA-Seq across 12 environmental stressors 1–15. https://doi.org/10.1038/sdata.2016.30

Panfilio, K.A., 2008. Extraembryonic development in insects and the acrobatics of blastokinesis. Dev. Biol. 313, 471–491. https://doi.org/10.1016/J.YDBIO.2007.11.004

Petryk, A., Warren, J.T., Marqués, G., Jarcho, M.P., Gilbert, L.I., Kahler, J., Parvy, J.P., Li, Y., Dauphin-Villemant, C., O'Connor, M.B., 2003. Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. Proc. Natl. Acad. Sci. U. S. A. 100, 13773–13778. https://doi.org/10.1073/pnas.2336088100

Qu, Z., Kenny, N.J., Lam, H.M., Chan, T.F., Chu, K.H., Bendena, W.G., Tobe, S.S., Hui, J.H.L., 2015. How Did Arthropod Sesquiterpenoids and Ecdysteroids Arise? Comparison of Hormonal Pathway Genes in Noninsect Arthropod Genomes. Genome Biol. Evol. 7, 1951–1959. https://doi.org/10.1093/gbe/evv120

Rewitz, K.F., Gilbert, L.I., 2008. Daphnia Halloween genes that encode cytochrome P450s mediating the synthesis of the arthropod molting hormone: Evolutionary implications. BMC Evol. Biol. 8, 1–8. https://doi.org/10.1186/1471-2148-8-60

Rewitz, K.F., Larsen, M.R., Lobner-Olesen, A., Rybczynski, R., O'Connor, M.B., Gilbert, L.I., 2009. A phosphoproteomics approach to elucidate neuropeptide signal transduction controlling insect metamorphosis. Insect Biochem. Mol. Biol. 39, 475–483. https://doi.org/https://doi.org/10.1016/j.ibmb.2009.04.005

Rewitz, K.F., O'Connor, M.B., Gilbert, L.I., 2007. Molecular evolution of the insect Halloween family of cytochrome P450s: Phylogeny, gene organization and functional conservation. Insect Biochem. Mol. Biol. 37, 741–753. https://doi.org/https://doi.org/10.1016/j.ibmb.2007.02.012

Rewitz, K.F., Rybczynski, R., Warren, J.T., Gilbert, L.I., 2006. Identification, characterization and developmental expression of Halloween genes encoding P450 enzymes mediating ecdysone biosynthesis in the tobacco hornworm, *Manduca sexta*. Insect Biochem. Mol. Biol. 36, 188–199. https://doi.org/10.1016/j.ibmb.2005.12.002

Rewitz, K.F., Yamanaka, N., O'Connor, M.B., 2010. Steroid Hormone Inactivation Is Required during the Juvenile-Adult Transition in *Drosophila*. Dev. Cell 19, 895–902. https://doi.org/10.1016/j.devcel.2010.10.021

Schumann, I., Kenny, N., Hui, J., Hering, L., Mayer, G., 2018. Halloween genes in panarthropods and the evolution of the early moulting pathway in Ecdysozoa. R. Soc. Open Sci. 5. https://doi.org/10.1098/rsos.180888

Schwentner, M., Combosch, D.J., Pakes Nelson, J., Giribet, G., 2017. A Phylogenomic Solution to the Origin of Insects by Resolving Crustacean-Hexapod Relationships. Curr. Biol. 27, 1818-1824.e5. https://doi.org/10.1016/j.cub.2017.05.040

Shinoda, T., Itoyama, K., 2003. Juvenile hormone acid methyltransferase: A key regulatory enzyme for insect metamorphosis. Proc. Natl. Acad. Sci. U. S. A. 100, 11986–11991. https://doi.org/10.1073/pnas.2134232100

Sin, Y.W., Kenny, N.J., Qu, Z., Chan, K.W., Chan, K.W.S., Cheong, S.P.S., Leung, R.W.T., Chan, T.F., Bendena, W.G., Chu, K.H., Tobe, S.S., Hui, J.H.L., 2015. Identification of putative ecdysteroid and juvenile hormone pathway genes in the shrimp *Neocaridina denticulata*. Gen. Comp. Endocrinol. 214, 167–176. https://doi.org/10.1016/j.ygcen.2014.07.018

Smagghe, G., Degheele, D., 1998. Ecdysone Agonists: Mechanism and Biological Activity, in: Ishaaya, I., Degheele, Danny (Eds.), Insecticides with Novel Modes of Action. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 25–39. https://doi.org/10.1007/978-3-662-03565-8_2

Smirnov, N.N., 2017. Reproduction, in: Smirnov, N.N.B.T.-P. of the C. (Second E. (Ed.), Physiology of the Cladocera: Second Edition. Academic Press, pp. 151–174. https://doi.org/10.1016/B978-0-12-805194-8.00011-8

Smith, W., Rybczynski, R., 2012. Prothoracicotropic Hormone, in: Gilbert, L.I.B.T.-I.E. (Ed.), Insect Endocrinology. Academic Press, San Diego, pp. 1–62. https://doi.org/10.1016/B978-0-12-384749-2.10001-9

Song, M.Y., Stark, J.D., Brown, J.J., 1997. Comparative toxicity of four insecticides, including imidacloprid and tebufenozide, to four aquatic arthropods. Environ.

Toxicol. Chem. 16, 2494–2500. https://doi.org/10.1002/etc.5620161209

Stecher, G., Tamura, K., Kumar, S., 2020. Molecular Evolutionary Genetics Analysis (MEGA) for macOS. Mol. Biol. Evol. 37, 1237–1239. https://doi.org/10.1093/molbev/msz312

Sumiya, E., Ogino, Y., Toyota, K., Miyakawa, H., Miyagawa, S., Iguchi, T., 2016. Neverland regulates embryonic moltings through the regulation of ecdysteroid synthesis in the water flea *Daphnia magna*, and may thus act as a target for chemical disruption of molting. J. Appl. Toxicol. 36, 1476–1485. https://doi.org/10.1002/jat.3306

Tarkowská, D., Strnad, M., 2016. Plant ecdysteroids: plant sterols with intriguing distributions, biological effects and relations to plant hormones. Planta. https://doi.org/10.1007/s00425-016-2561-z

Tatarazako, N., Oda, S., Watanabe, H., Morita, M., Iguchi, T., 2003. Juvenile hormone agonists affect the occurrence of male *Daphnia*. Chemosphere 53, 827–833. https://doi.org/10.1016/S0045-6535(03)00761-6

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680. https://doi.org/10.1093/nar/22.22.4673

Törner, K., Nakanishi, T., Matsuura, T., Kato, Y., Watanabe, H., 2018. Genomic integration and ligand-dependent activation of the human estrogen receptor α in the crustacean *Daphnia magna*. PLoS One 13. https://doi.org/10.1371/journal.pone.0198023

Törner, K., Nakanishi, T., Matsuura, T., Kato, Y., Watanabe, H., 2014. Optimization of mRNA design for protein expression in the crustacean *Daphnia magna*. Mol. Genet. Genomics 289, 707–715. https://doi.org/10.1007/s00438-014-0830-8

Toyota, K., Miyakawa, H., Hiruta, C., Furuta, K., Ogino, Y., Shinoda, T., Tatarazako, N., Miyagawa, S., Shaw, J.R., Iguchi, T., 2015. Methyl farnesoate synthesis is necessary for the environmental sex determination in the water flea *Daphnia pulex*. J. Insect Physiol. 80, 22–30. https://doi.org/10.1016/j.jinsphys.2015.02.002

Truman, J.W., Riddiford, L.M., 1999. The origins of insect metamorphosis. Nature 401, 447–452. https://doi.org/10.1038/46737

Wang, Y.H., Wang, G., LeBlanc, G.A., 2007. Cloning and characterization of the retinoid X receptor from a primitive crustacean *Daphnia magna*. Gen. Comp.

Endocrinol. 150, 309–318. https://doi.org/10.1016/j.ygcen.2006.08.002

Yoshiyama, T., Namiki, T., Mita, K., Kataoka, H., Niwa, R., 2006. Neverland is an evolutionally conserved Rieske-domain protein that is essential for ecdysone synthesis and insect growth. Development 133, 2565–2574. https://doi.org/10.1242/dev.02428

Zitnan, D., Adams, M.E., 2012. Neuroendocrine Regulation of Ecdysis, in: Gilbert, L.I.B.T.-I.E. (Ed.), Insect Endocrinology. Academic Press, San Diego, pp. 253–309. https://doi.org/10.1016/B978-0-12-384749-2.10007-X

List of publications

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