# Analyses of regulative strategies of moulting, reproduction and embryo development by ecdysteroids in a crustacean *Daphnia magna*

Sumiya, Eri

Doctor of Philosophy

Department of Basic Biology
School of Life Science
SOKENDAI (The Graduate University for Advanced Studies)

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脱皮ホルモンによるオオミジンコ脱皮、生殖、胚発生制御機構の解析

Sumiya, Eri

**SOKENDAI (The Graduate University for Advanced Studies)**

**School of Life Science**
**Department of Basic Biology**

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**List of Abbreviations**

CYP: Cytochrome P450

dsRNA: double-stranded RNA

EcR: ecdysone receptor

ELISA: Enzyme-linked immunosorbent assay

EPPase: ecdysteroid-phosphate phosphatase

GVBD: germinal vesicle breakdown

HAI: hours after injection

HAO: hours after oviposition

Nvd: neverland

PG: prothoracic gland

RNAi: RNA interference

Shd: shade

USP: ultra spiracle

7dC: 7-dehydrocholesterol

20E: 20-hydroxyecdysone

20Eoic: 20-hydroxyecdysonoic acid

## General Introduction

Reproduction is a fundamental process for the survival of a species. Successful reproduction definitely facilitates the fitness and abundance of species on our planet (Bliss, 1983). The most successful species on land and in aquatic habitats are the Arthropoda with arthropods encompassing 75% of all animal species described to date (Pechenik, 2005). Their success in reproduction is driven not only by their flight ability to exploit different niches, but also endocrine signaling cascades that enable them to cope with dynamic environments (LeBlanc, 2007). Arthropod species can respond to environmental cues, and subsequently change their reproductive strategy. For example, some insects commit into diapause, a genetically programmed arrest in development, by which they can wait until conditions turn favourable (Nijhout, 1994). The aquatic crustacean species *Daphnia* produce resting eggs by adjusting their reproductive styles. Reproductive modes in other crustaceans vary, but the precise regulation of the reproductive cycle is equally important for all crustacean species. This is because crustaceans, specifically females, need to manage egg production (oogenesis) as well as cuticle deposition for moulting (Subramoniam, 2000). In species such as the fresh water crab *Paratelphusa hydrodromus* and penaeid shrimps, oogenesis and cuticle deposition alternates. In the case of the mole crab *Emerita asiatica* and a freshwater prawn *Macrobrachium nipponense*, the reproductive and moulting cycles must be concurrently regulated because oogenesis and cuticle deposition overlap (Subramoniam, 2000). Since daphnids produce eggs immediately after they moult (Hiruta *et al*., 2010), they synchronously regulate reproduction and moulting events (Figure GI-1). Daphnids

are unique in that they are able to switch between sexual and parthenogenetic reproduction and the sexes of their offspring (Figure GI-2). Such a phenomenon ensures that the next generation survives when the environment becomes favourable or maximizes the abundance of offspring that can utilize available resources as much as possible. Daphnids are an important form of plankton in the freshwater food web, and are also valuable laboratory animals for toxicological research (Mu and LeBlanc, 2002). Their unique features related to their lifecycle, environmental sex determination, and laboratory-friendly characteristics have provided the impulse for a much needed to understanding of the basic biology of daphnids.

The management of embryogenesis is also important for successful reproduction. Embryonic development requires a series of precisely regulated processes. Various arthropod species show developmental strategies to increase embryonic survival rate. In scorpions, the mother carries new hatchlings on her back for as long as two weeks (Truman and Riddiford, 2002). Many aquatic crustaceans spend their larval stages as free-living larval forms such as nauplius and zoea (Pechenik, 2005). Similarly, daphnids maintain the embryos inside the mother's "brood pouch" until the neonates are released into the surrounding water as free-swimming juveniles.

Steroid hormones are central to how all steps, from reproduction to embryonic development, are regulated. Steroid hormones play important roles in vertebrates and invertebrates. Vertebrates have several kinds of steroid hormones such as the adrenal hormone, cortisol and aldosterone. They also have sex hormones such as estradiol and testosterone. On the other hand, ecdysteroid is the only invertebrate-specific steroid

hormone. Ecdysteroids are only present in arthropods (LaFont, 2000), and their *de novo* synthesis in other invertebrates remains questionable (LaFont and Mathieu, 2007). While many aspects differ between the steroid hormones of vertebrates and invertebrates, they share some basic features. Therefore, these hormones are thought to have the same ancestry (LaFont, 2000). In both cases, steroid hormones are synthesized from cholesterol, and the enzymes catalyzing the steps are the cytochrome P450 family (CYP450) (Payne and Hales, 2004; Yoshiyama *et al.*, 2006). The CYP450 proteins are engaged in steroid hormone synthesis, and hydroxylate and cleave the steroid substrate. They modulate NADPH as the electron donor to reduce molecular oxygen (Payne and Hales, 2004). The activation of the synthesized steroid hormones follows the following steps. The steroid hormone passively or actively diffuses into a cell where it binds to a specific receptor. The receptor-steroid complex undergoes a conformational change allowing the complex to bind to selective sites on chromatin. The activated steroid receptor complex recognizes the response element of DNA. Finally, the receptor-ligand complex works as a transcription factor operating during mRNA synthesis and subsequent translation (Lachase *et al.*, 1993; Revelli *et al*., 1998). The ecdysteroid that binds to the receptor is 20-hydroxyecdysone, 20E (Petryk *et al.*, 2003; Guittarda *et al.*, 2011). Synthesis, inactivation, excretion and sequestration are factors that determine the ecdysteroids titre (Gilbert *et al.*, 1980). Ecdysteroids serve as the moulting hormone for all arthropods, and are multifunctional regulators of yolk production, ovarian maintenance, larval development, and other processes in insects (König *et al*., 2011; Gilbert, 2012). The synthetic pathway of ecdysteroids is largely understood in insects.

The synthesis involves an oxygenase enzyme for the first conversion step, and the following conversions employ the CYP450 enzymes (Figure GI-3). Unlike insects, the roles of ecdysteroids in crustaceans are mostly drawn from correlations of data than from direct molecular experiments (LaFont and Mathieu, 2007). The molecular mechanisms of ecdysteroids underlying the regulation of moulting, reproduction and development in crustaceans are still unclear, even more so in non-decapod crustaceans. Ecdysteroidogenic tissues are prothoracic glands and follicle cells in insects, and the Y-organ in decapods (crabs and shrimps), but are unknown in other crustaceans. In daphnids, recent studies have revealed five CYP450 genes in the ecdysteroids synthesis pathway in *D. pulex* (Rewitz and Gilbert, 2008), and have identified an ecdysteroid receptor that was actively bound by 20E in *D. magna* (Kato *et al.*, 2007). However, large parts of the synthesis, regulation and roles of ecdysteroids remain unknown in daphnids despite the growing importance of *Daphnia* as an experimental animal. This is especially true for daphnids that regulate moulting and reproduction concurrently, and studies on ecdysteroids would shed light on the multifunctional and multitasking nature of ecdysteroids.

The purpose of my study is to understand the endocrine system underlying the reproductive features that facilitate the adaptive strategies of daphnids. In *D. magna*, previous studies reported a fluctuation of the ecdysteroids titre during embryogenesis and the moulting cycle in adults (Mu and LeBlanc, 2004; Martin-Creuzburg *et al.*, 2007), and a CYP450 inhibitor induced developmental abnormalities (Mu and LeBlanc, 2002). However, no genes for ecdysteroidogenesis were identified and the

consequences of these fluctuations of the ecdysteroids titre remained unknown.

In chapter I, I cloned the potential genes for ecdysteroidogenesis and analyzed how gene expression changed during the reproductive cycle. I also determined correlations between gene expression and ecdysteroids titre during moulting and as the reproductive cycle progressed. In chapter II, I performed a functional analysis of the genes identified in chapter I to understand the roles of these genes in embryo development. The results indicated that *neverland1* is required for embryonic moultings apparently by regulating the ecdysteroids titre. I also showed that the ecdysteroids titre itself initiated the onset of the moultings. I further analyzed the spatial expression of the *neverland* genes in the adult and embryo to show possible ecdysteroidogenic tissues. Collectively, I discuss how *D. magna* manages to moult, reproduce and develop by utilizing ecdysteroids.

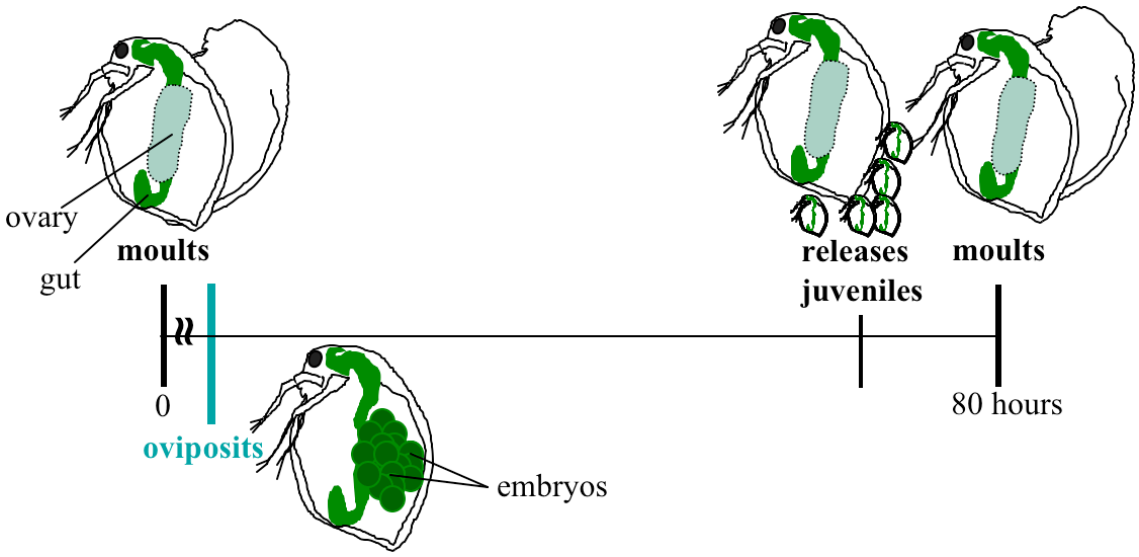



**Figure GI-1.** Coordinated moulting and parthenogenetic reproductive cycles of *D. magna*. The cycle repeats on average once every 80 hours at 20 ˚C.

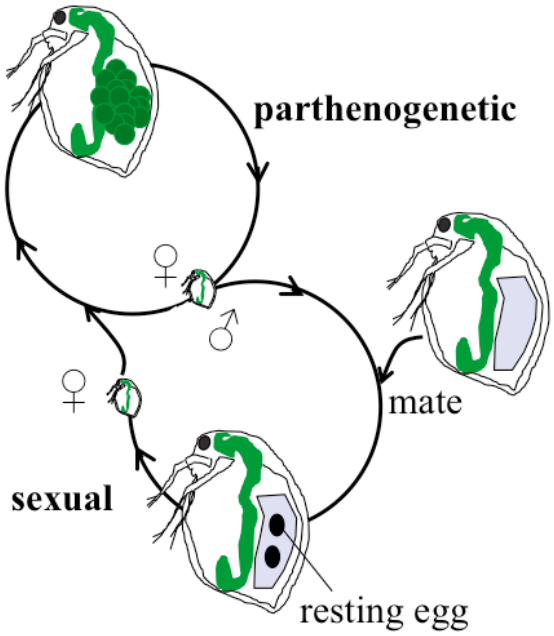



**Figure GI-2.** Life cycle of *Daphnia* showing parthenogenetic and sexual reproduction. Daphnids produce parthenogenetic diploid female offspring under favourable conditions. When conditions turn hostile, they produce a parthenogenetic haploid male, and then switch to sexual reproduction to form a resting egg.

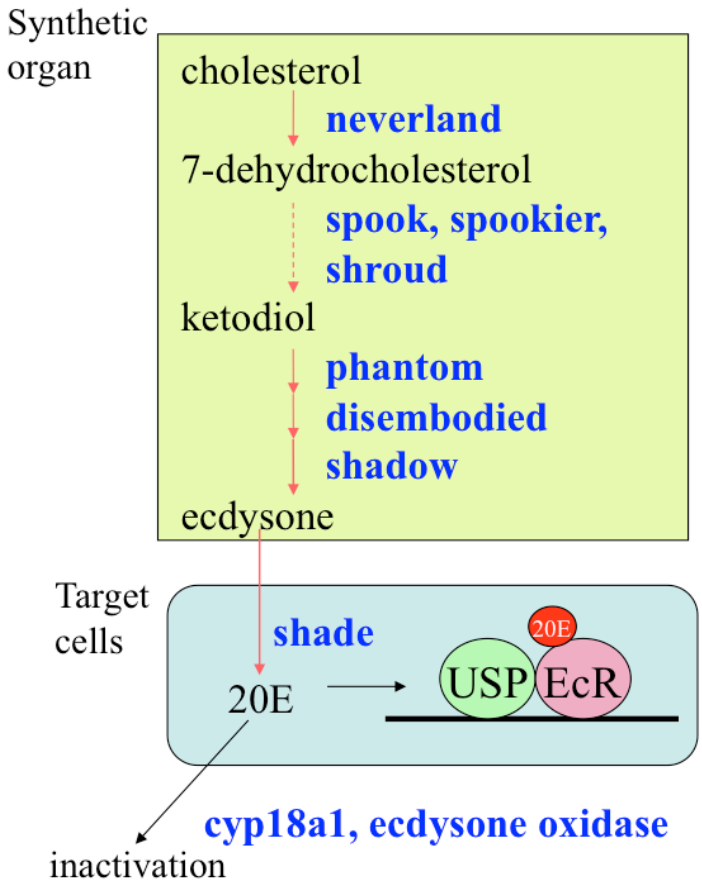



**Figure GI-3.** Synthetic and inactivation pathways, and a heterodimeric receptor of ecdysteroids revealed in insects. The enzymes in each pathway are shown in blue. The dashed arrow represents the conversion steps that are not fully understood. USP: ultra spiracle, EcR: ecdysone receptor, 20E: 20-hydroxyecdysone.

# Chapter I

# Roles of ecdysteroids for progression of reproductive cycle in the fresh water crustacean *Daphnia magna*

**Abstract**

*Daphnia magna* exhibits a parthenogenetic reproductive cycle linked to a moulting cycle, but regulatory mechanisms of neither moulting nor reproductive cycle are understood in daphnids. Moulting is regulated by ecdysteroids in insects. A previous study showed that a titre of ecdysteroids changed during the reproductive cycle in *D. magna*; however, no clear correlation between this titre and moulting and reproductive cycles has been proved in daphnids. To understand endocrine mechanisms underlying the coordinated reproductive cycle, I analyzed the expression of genes coding for enzymes in ecdysteroids synthesis or inactivation pathways and the effects of 20-hydroxyecdysone on moulting and oviposition in *D. magna*. Additionally, ecdysteroids titre was measured during the moulting cycle, and found that the titre has a single peak in the mid-moulting cycle. I cloned orthologues of *neverland* and *shade* in the ecdysteroids synthesis pathway, and *Cyp18a1* in the ecdysteroids inactivation pathway previously identified in insects. Expression analyses of these genes in adult females during the reproductive cycle indicated that Cyp18a1 possibly regulates the decline of ecdysteroid titre before moulting and oviposition. Furthermore, *nvd* was strongly expressed in the gut indicating that ecdysone might be synthesized in the gut. Treatment with 20E resulted in the failure of not only moulting, but also oviposition, suggesting that a low level of ecdysteroids before moulting is required for moulting and oviposition in *D. magna*.

## Introduction

Crustacea and Insecta comprising monophyletic group, the Pancrustacea, are the most diverse animal group in aquatic habitats (Kenny *et al*., 2013). Reproductive diversity as an adaptation to environmental conditions may have facilitated the radiation of crustaceans (Bliss, 1983). Reproduction of decapod crustaceans is regulated by complex endocrine mechanisms involving ecdysteroids, neuropeptides, biogenic amines, opioids, juvenile hormones, and vertebrate-type steroids (Swetha *et al*., 2011). Daphnids show characteristic features in reproduction. They reproduce parthenogenetically every three days under favourable conditions, and the reproductive cycle occurs synchronously with the moulting cycle. After moulting, mother daphnids oviposit new eggs from the ovary into the brood pouch (oviposition). At the end of each reproductive cycle, the mother releases the brood from the pouch to the surrounding water. After this release, the mother moults again and repeats the cycle. These events occur in a very systematic order with intervals that span approximately three days depending on the condition. Consequently, a closely related system might regulate the moulting and reproductive cycle. A similar association of moulting with a reproductive cycle has also been reported in other crustaceans such as amphipods, isopods, and decapods (Shyama, 1987; Gunamalai *et al*., 2004). However, regulation of the reproductive cycle remains largely unknown in most crustaceans.

Ecdysteroids (ecdysone and 20-hydroxyecdysone; 20E) are steroid hormones that regulate moulting and metamorphosis in insects. These have been extensively studied in insects, and are commonly called “moulting hormones” as their major role

implies. Recent studies in insects revealed that ecdysteroids have a variety of functions besides moulting in the control of reproduction and embryogenesis (Nijhout, 1994). In *Drosophila melanogaster*, ecdysteroids are required for differentiation of germ line stem cells (Ables and Drummond-Barbosa, 2010; König, 2011) and prepupal development (Agawa *et al*., 2007; Akagi and Ueda, 2011). A pioneering study reported that a titre of ecdysteroids increased at the premoult stage and decreased back to the basal level before moulting in *Daphnia magna* (Martin-Creuzburg *et al*., 2007), indicating that ecdysteroids are predominant moulting hormones. However, in crustaceans, reports on ecdysteroidal regulation in both moulting and reproduction are scarce (Gunamalai *et al*., 2004). The synchronized moulting and reproductive cycle in daphnids implies a possibility that ecdysteroids may regulate not only moulting, but also the reproductive cycle.

In insects, biosynthesis of ecdysteroids starts with dietary cholesterol. Rieske oxygenase DAF-36 (neverland, nvd) converts it to 7-dehydrocholesterol (Yoshiyama *et al*., 2006). After several steps mediated by enzymes belonging to CYP450, it is converted to ecdysone. Then, Cyp314A1 (shade, shd) finally converts ecdysone into 20E, which is the biologically active form binding to an ecdysone receptor (EcR) (Petryk *et al*., 2003; Guittarda *et al*., 2011). The complex of 20E and EcR recruits ultraspiracle (USP) in insects and daphnids or retinoid X receptor (RXR) in decapods, and acts as a heterodimer (Hopkins, 2009). EcR and USP have been cloned in *D. magna* (Kato *et al*., 2007). Three EcR paralogues with five distinct N-terminus sequences and one USP were identified (Kato *et al*., 2007). These genes in the ecdysteroids

biosynthesis pathway except for *nvd* belong to CYP450 encoding hydroxylases. *D. pulex* possesses five CYP450 genes: *spook*, *phantom*, *disembodied*, *shadow* and *shd* (Rewitz and Gilbert, 2008). In decapod crustaceans, *Cyp306A1* (*phantom*) in kuruma prawn (*Marsupenaeus japonicus*) (Asazuma *et al*., 2009), and four CYP450s (*spook*, *phantom*, *disembodied* and *shadow*) and *nvd* in cray fish (*Pontastacus leptodactylus*) (Tom *et al*., 2013) have been identified to date. However, neither gene expression nor functional analyses have been explored in microcrustaceans such as copepoda, phyllopoda and cladocera (Hopkins, 2009).

The synthesis and secretion of ecdysone take place in a specialized organ in insects and decapods. Insect larvae synthesize ecdysone in the prothoracic gland although it is destined to degenerate in the adult of most species (Marchal *et al*., 2011). The synthetic organ in decapods is the Y-organ. However, the organ that synthesizes and secretes ecdysone has not been identified in microcrustaceans, including daphnids. Some reports have speculated that these species may synthesize ecdysone in the epidermis (Lachaise *et al*., 1993; Lafont and Mathieu, 2007; Hopkins, 2009) since ecdysone production in the epidermis was observed *in vitro* in the tick *Ornithodoros parkeri* (Zhu *et al*., 1991). Identification of the endocrine organ is essential to understand the endocrine system of daphnids.

As an entry point to understand the roles of ecdysteroids in the reproductive cycle in *D. magna*, I cloned genes of three key enzymes for 20E synthesis or inactivation: *nvd*, s*hd* and *Cyp18a1*. Spatio-temporal expression analyses of these genes during the reproductive cycle indicated that ecdysone is possibly synthesized in the gut.

The ecdysteroids titre during the moulting cycle fluctuated with a single peak in the middle, and was low at the beginning and the end. Cyp18a1 might regulate the decline of the ecdysteroids titre before moulting. In addition, exogenous 20E treatment during the reproductive cycle resulted in a failure of moulting and oviposition, suggesting that a low level of ecdysteroids before moulting is required for successful moulting and oviposition in *D. magna*. The present study provides insight into the endocrine system that regulates the highly coordinated reproductive system in crustaceans.

## Materials and Methods

### Animals

*Daphnia magna* (NIES strain) obtained from the National Institute for Environmental Studies (NIES; Tsukuba, Japan) were maintained in M4 medium (Elendt and Bias, 1990) at 20±1 °C under a 14-hour:10-hour light:dark photoperiod. *Chlorella vulgaris* was added daily into each culture. For experiments, each animal was incubated under the same conditions mentioned above but in 50 ml of M4 medium individually. The culture medium was substituted every three days. Under these conditions, *D. magna* produces female offspring every three days. All procedures and protocols were approved by the institutional animal care and use committee at the National Institute for Basic Biology.

### Cloning and phylogenetic analysis

Total RNA extracted from adult female *D. magna* using the RNAqueous Kit (Life Technologies, Carlsbad, CA, USA) was reverse transcribed with Super Script III (Life Technologies). To obtain gene fragments of *nvd* and *Cyp18a1*, oligonucleotide primers were designed based on the sequences of the *D. magna* EST database (Kato *et al*., 2011) (Table 1-1). PCR was performed with Platinum Taq DNA Polymerase High Fidelity (Life Technologies). The amplified products were cloned into pGEM-T easy (Promega, Tokyo, Japan), and sequenced using the BigDye Terminator Cycle Sequencing Kit (Life Technologies). Based on the sequence information of the obtained

PCR products, the 5′ and 3′ ends of *nvd1* and *Cyp18a1* sequences were amplified using a rapid amplification cDNA ends (RACE) kit (Gene Racer Kit, Life Technologies) with Platinum Taq High Fidelity (Life Technologies). The obtained two *nvd* and *Cyp18a1* sequences were assigned the following accession numbers (DDBJ: *nvd1*: AB839171, *nvd2*: AB839172 and *Cyp18a1*: AB839173). Sequence of *shd* of *D. magna* was available in the NCBI database (AB257771.1). Sequences of *EcR* and *USP* were defined in a previous study (AB274819-24) (Kato *et al*., 2007).

The amino acid sequences of *nvd*, *shd* and *Cyp18a1* in other arthropod species were identified in euGenes/Arthropod genomes (Indiana University, http://arthropods.eugenes.org/arthropods/). The amino acid sequences were aligned with MEGA5 software using MUSCLE method with the default options (Tamura *et al*., 2011). Neighbour joining trees were constructed from these alignments using Poisson model with bootstrap analyses of 1000 replicates along with complete deletion options.

**Quantitative PCR**

Whole body adult *D. magna* samples were prepared at nine time points with 10-hour intervals during an 80-hour intermoulting period. For the tissue-specific gene expression analysis, adult *D. magna* at 70 hours after moulting (HAM) was dissected, and ovary, gut and the remaining tissues were separately obtained. Three replicates were prepared for each time point for each experiment. Total RNA was extracted using the RNAqueous-Micro Kit (Life Technologies). cDNA was synthesized from 0.5 μg of total RNA using Super Script III (Life Technologies) with random primers. PCR was

conducted with SYBR-Green PCR Master Mix (Life Technologies) using the 7000 real-time PCR system (Life Technologies). The primer sequences used for the quantitative PCR analyses are shown in Table 1-2. To detect *EcR*, a common sequence region of three *EcR* genes was utilized to design the primers. Among four housekeeping genes (*cyclophilin*, *Ltd4ds*, *LBT* and *L32*), *D. magna cyclophilin* was selected as a reference gene calculated with geNorm (Vandesompele *et al*., 2002) and NormFinder (Andersen *et al*., 2004). Statistical analyses of gene expression at each time point were preformed by ANOVA followed by the Tukey-Kramer post-hoc test using Excel 2004 (Microsoft Corp., Redmond, WA) with an add-in software Statcel 2 (Yanai, 2004).

**Enzyme-linked immunosorbent assay (ELISA)**

Ecdysteroids titre in adult female *D. magna* was measured by ELISA following procedures described previously (Pankotai *et al*., 2010; Shimada-Niwa and Niwa, 2014) with a slight modification. *D. magna* were collected at each 10-hour time point during the 0-80 HAM moulting cycle, and the embryos in a brood pouch were removed. The samples were quickly frozen in liquid nitrogen and kept at -80 °C. The samples were homogenized in 100 µl methanol, and then centrifuged at 15000 rpm for one minute. The supernatant was collected into a glass vial. These processes were repeated three times, and the supernatants were combined in a single glass vial for each replicate. The contents were dried by vacuum-centrifugation, and stored at -80 °C. Five replicates of one adult *D. magna* were used for each time point. 20-Hydroxyecdysone (Sigma, St. Luis, MO, USA) (20E) was used as the standard. An EIA 96-well strip plate

precoated with mouse anti-rabbit IgG was used for each assay (Cayman Chemical, Ann Arbor, MI, USA). 20E Acetylcholinesterase Tracer (Cayman Chemical) and 20E EIA Antiserum (Cayman Chemical) were used in plate preparation, and Ellman's Reagent (Cayman Chemical) was used to develop the plate. In all cases, the manufacturer's instructions were followed. The absorbance at 412 nm was- read with an SH-9000Lab Multigrading Micro Plate Reader (Corona Electric Co., Ltd, Ibaraki, Japan).

**Treatment with exogenous ecdysteroid**

A stock solution of 10 mM 20E (Sigma, St. Luis, MO, USA) dissolved in ethanol was kept at -20 °C until use. This stock solution was directly added to each 50 ml of M4 medium containing one adult female *D. magna* at two days after moulting ($n$ = 128). The final concentration was 0.5 μM 20E. Ethanol (0.0001%) served as the control ($n$ = 85). The number of individuals was counted for three phenotypes in the following order of severity: normal moulting and oviposition (no effect), normal moulting following no oviposition (moderate effect), or neither moulting nor oviposition (severe effect). Differences in the incidence between the control and 20E-treated animals were analyzed with the Mann-Whitney U test using Excel 2004 (Microsoft Corp.) with Statcel 2 (Yanai, 2004). For tissue sectioning, the 20E-treated and control animals were fixed in Bouin's fluid overnight. The samples dehydrated in ethanol and xylene were embedded in paraffin. Serial sections of 5 μm thicknesses were subjected to haematoxylin and eosin staining. The sections were studied under a microscope (Axioplan2, ZEISS, Oberkochen, Germany) and the images were processed

with callSens Standard, version 1.3 (Olympus, Tokyo, Japan). Six individuals of the 20E-treated animals were observed for each of the three phenotypes. Three control animals were examined at three stages: before moulting (sampled after the release of a brood), soon after moulting and before oviposition (sampled within 15 minutes after moulting) and soon after oviposition (sampled when oviposition finished).

## Results

### Molecular cloning and phylogenetic analysis of genes in 20E synthesis or inactivation pathway in *D. magna*

I cloned full-length of *nvd* encoding 444 amino acids (a.a.), *shd* encoding 526 a.a. and *Cyp18a1* encoding 551 a.a. from *D. magna*. Furthermore, I identified the partial cDNA sequence of a distinct *nvd*. Aligning amino acids of these genes with *D. pulex* and two insect species, *D. melanogaster* and *Bombyx mori*, revealed that both *D. magna* nvd contain a characteristic domain, Rieske (2Fe-2S), and a non-heme Fe (II) motif (Figure 1-1*a*) (Yoshiyama *et al*., 2006). The obtained shd and Cyp18a1 belonging to the CYP450 family have a P/G-rich domain and five conserved P450 motifs: Helix-C, Helix-I, Helix-K, PERF and Heme-binding (Figure 1-1*b*) (Iga and Smagghe, 2010).

Phylogenetic analysis was conducted by comparing the amino acid sequences of the obtained nvd, shd and Cyp18a1 with other available arthropod sequences. In each obtained tree, *D. magna* and *D. pulex* clustered together with other arthropods, forming a monophyletic group. This suggests that the orthologous genes of nvd, shd and Cyp18a1 were identified in *D. magna*. Two of the *D. magna* nvd were assembled in the *Daphnia* nvd cluster. These nvd paralogoues were therefore designated nvd1 (full length, AB839171) and nvd2 (partial cDNA sequence, AB839172) (Figure 1-2*a*). I further found a duplication of nvd1, nvd1a (sequence ID in *D. pulex* genome database, NCBI_GNO_67514) and nvd1b (NCBI_GNO_462084).

In the case of shd, no paralogous genes were identified in the *D. magna*

database. In agreement with the arthropod phylogenetic trend, tick shd is the most ancestral followed by daphnids shd, and insects' shd being the most descendant (Figure 1-2*b*). As seen in nvd and shd, the obtained *D. magna* Cyp18a1 pairs with *D. pulex* Cyp18a1 (Figure 1-2*c*) showing a close relationship.

**Time-course and tissue-specific expression analyses of *neverland*, *shade*, *Cyp18a1*, *EcR* and *USP* during intermoulting period**

Adult *D. magna* has a moulting cycle of three days under current conditions, which takes 80 hours on average between two consecutive moultings. Investigation of the expression profiles of the genes in the ecdysteroids synthesis or inactivation pathway during the intermoulting period was performed by quantitative real-time PCR. *Nvd1*, *nvd2* and *shd* were highly expressed in the early intermoulting period at 0-10 hours after moulting (HAM) (Figure 1-3*a-c*). The lowest expression level of *nvd1* was reached at 20 HAM, and it increased at 70 HAM. In contrast, the expression level of *nvd2* did not change significantly after the abrupt drop at 20 HAM. The expression level of *shd* gradually decreased until 20 HAM. Thereafter, it stayed at a rather constant level. *Cyp18a1* was also highly expressed at 0 HAM, and its expression declined to the lowest level by 30 HAM (Figure 1-3*d*). At the end of the moulting cycle, *Cyp18a1* expression showed a steep rise at 80 HAM. The expression level of *EcR* was relatively high until 10 HAM and after 50 HAM (Figure 1-3*e*). The expression level of *USP* was highest at 0 HAM but decreased to the lowest level by 10 HAM after which it no longer shifted (Figure 1-3*f*).

A synthetic and target organs/tissues for ecdysteroids are unknown in daphnids. Therefore, tissue-specific expression of genes in the ecdysteroids synthesis or inactivation pathway was analyzed in the major organs such as the ovary, gut and remaining tissues (i.e., tissues other than the ovary and gut) by quantitative real-time PCR. *Nvd1* was strongly expressed in the gut (Figure 1-4*a*). *Nvd2* expression was also observed primarily in the gut, but there was no significant difference in expression levels among tissues (Figure 1-4*b*). *Shd* was predominantly expressed in the gut, and its expression was also detected in the ovary and remaining tissues (Figure 1-4*c*). Alternatively, the expression level of *Cyp18a1* was relatively low in the gut (Figure 1-4*d*). The expression level of *Cyp18a1* was the highest in the remaining tissues, but not significantly higher than in the ovary or gut. *EcR* and *USP* expression were detected in all tissues examined, and the expression levels were highest in the remaining tissues (Figure 1-4*e*,*f*).

**Fluctuation of ecdysteroids titre during the moulting cycle of *D. magna***

A profile of ecdysteroids titre was measured every 10 hours during 80-hour moulting cycle (Figure 1-5). The result showed the ecdysteroids titre was low during the 0-30 HAM. Some of the samples were below the lowest point of the standard curve ($9.6 \times 10^{-6}$ pg). In the middle of the moulting cycle, it increased giving a peak at 40 HAM and 50 HAM. The ecdysteroids titre decreased again towards the end of the moulting cycle.

**Exogenous 20E treatment on adult *D. magna* in the mid-reproductive cycle**

To understand the importance of fluctuation of the ecdysteroids titre on moulting and oviposition, adult female *D. magna* (*n*=128) were treated with exogenous 20E (0.5 μM) in the middle of the reproductive cycle, which is a day before moulting and oviposition occur. After this treatment, unusual phenotypes were observed at a significantly higher rate ($P < 0.001$): normal moulting without oviposition or neither moulting nor oviposition (Figure 1-6). In the 20E-treated group, only 34% of 128 individuals underwent normal moulting followed by oviposition whereas 99% of 85 control individuals moulted and oviposited normally. The most frequent phenotype appeared in 53% of the 20E-treated individuals. Those individuals neither moulted nor oviposited. In addition, 13% of the 20E-treated individuals moulted normally but failed to oviposit. The toxic influence seemed minimal as none of the individuals died within 48 hours after the treatment.

The ovary and carapace were then analyzed histologically. The whole ovary was full of yolk granules in control individuals (Figure 1-7*a*,*b*). The meiotic phase of the eggs was also examined. In the ovarian eggs of *D. pulex*, the nuclear envelope degenerates and becomes invisible at the point of moulting (Hiruta *et al*., 2010). This was confirmed in *D. magna*. The nuclear envelope was invisible in control individuals before moulting (Figure 1-7*c*). After moulting, a meiotic apparatus was prominently visible in the ovarian egg (Figure 1-7*d*) and oviposited egg (Figure 1-7*e*). In comparison, yolk granules were also present in the ovary of 20E-treated individuals (Figure 1-7*f*,*g*). However, the nuclear envelope did not degenerate in the 20E-treated individuals that

failed to moult (Figure 1-7*h*). Interestingly, the meiotic apparatus was observed in the ovarian egg of those 20E-treated individuals that failed to oviposit after normal moulting (Figure 1-7*i*). As a result, treatment with 20E caused *D. magna* to be unable to moult and/or oviposit, and it ceased meiotic division at the time when the nuclear envelope breaks or meiotic metaphase. A new carapace, which was present in the control before moulting (Figure 1-7*j*,*k*), was also formed in the 20E-treated individuals (Figure 1-7*l*,*m*). Developing eggs and undifferentiated germ cells in an ovary of a normal *D. magna* are also shown (Figure 1-7*n*).

## Discussion

### Genes in ecdysteroids synthesis or inactivation pathway are evolutionary conserved in *D. magna*

The moulting hormones, ecdysone and 20E, have long been known to play critical roles in moulting and development of insects and crustaceans (Subramoniam, 2000). The final product of the ecdysteroids synthesis pathway, 20E, actively interacts with EcR/USP heterodimeric receptor in *D. magna* (Kato *et al*., 2007). This indicates that the binding of 20E to EcR in arthropod species can activate the ecdysteroids signalling pathway. Although there is physiological evidence indicating that crustaceans synthesize 20E from cholesterol, the molecular basis of 20E synthesis in crustaceans is not fully understood. Recently, orthologues of the insect Halloween genes encoding CYP450 enzymes for ecdysteroids biosynthesis such as spook, disembodied, phantom, shadow and shade were obtained by searching the *D. pulex* database (Rewitz *et al*., 2008). I newly identified genes covering the ecdysteroids synthesis or inactivation pathway, i.e. not only these CYP450 genes but also *nvd* and *Cyp18a1* in the *D. magna* EST database. The present study focused on nvd, which catalyses the first step in the biosynthesis of ecdysteroids (Yoshiyama *et al*., 2006), shade mediating the hydroxylation of ecdysone to 20E (Petryk *et al*., 2003), and Cyp18a1, a key enzyme for 20E inactivation (Guittarda *et al*., 2011) in insects. These cloning and phylogenetic analyses showed that nvd and shd as well as Cyp18a1 genes are evolutionary conserved in *D. magna* (Figure 1-1, 1-2). This suggests that *D. magna* possesses ecdysteroids

synthesis and inactivation pathways similar to those conserved in insects. This result is in agreement with recent phylogenetic analysis suggesting that these genes appeared in arthropods before the radiation of insects. Interestingly, I identified two different paralogues of the *nvd* gene in *D. magna*. The present phylogenetic analysis indicates that gene duplication gave rise to these two different nvd paralogues, nvd1 and nvd2 in daphnids. This duplication is thought to have occurred after the divergence of daphnids and insects but before the split of *D. magna* and *D. pulex*. I also identified the two nvd1 paralogues, nvd1a and nvd1b, in *D. pulex*. In the *D. pulex* linage, a number of gene duplications occurred (Colbourne *et al*., 2011). These duplication events are thought to have resulted in a complex lifecycle of daphnids.

**Ecdysteroids titre peaks in the middle of the moulting cycle, and Cyp18a1 might regulate the decline of ecdysteroids titre before moulting**

In shrimp and crab species, ecdysteroids titre shows a single peak during the intermoulting period (Soumoff and Skinner, 1983; Huberman, 2000; Gunamalai *et al*., 2004). The present study also demonstrates the ecdysteroids titre peaked at 40 and 50 HAM of 80-hour moulting cycle of adult *D. magna* (Figure 1-5). In contrast, ecdysteroids titre in insect larvae fluctuates in more complex patterns during embryogenesis and larval development. These rises and falls of ecdysteroids at specific times are mandatory for normal moulting and metamorphosis in insects (Gilbert, 2012). Cyp18a1 inactivates 20E through 26-hydroxylation in insects to lower the ecdysteroid titre (Guittarda *et al*., 2011). Gene expression analysis during the intermoulting period

in *D. magna* showed that the relative expression of *Cyp18a1* changed conversely with the fluctuation in ecdysteroids titre (Figure 1-3*d*). In other words, the ecdysteroids titre is high in the mid-moulting cycle when *Cyp18a1* expression is low, but is low at the end of the moulting cycle when *Cyp18a1* expression is high. This result suggests that Cyp18a1 possibly regulates the decline of the ecdysteroids titre before moulting in *D. magna*.

Relative expression analysis of *shd* and *Cyp18a1* showed different patterns (Figure 1-3*c*,*d*). Each CYP450 gene in the ecdysteroids synthesis pathway exhibits a different expression pattern in insect larvae, and it varies among species (Subramoniam 2000; Rewitz *et al*., 2006; Ono *et al*., 2006; Yamanaka *et al*., 2007; Niwa *et al*., 2010). Therefore, having a unique expression pattern for each of the genes appears to be a common phenomenon. Such variable expression patterns reflect which gene is responsible for a rate-limiting step or is a target of prothoracicotropic hormone (PTTH), which induces ecdysone synthesis in insects (Yamanaka *et al*., 2007).

**Ecdysone might be synthesized in the gut, as judged by the expression of *neverland***

In insect larvae, expression of *nvd* is exclusively detected in the ecdysone synthetic organ (Yoshiyama *et al*., 2006). On the other hand, *shd* has been observed in a range of 20E-target tissues/organs (Petryk *et al*., 2003; Rewitz *et al*., 2006; Iga and Smagghe, 2010). In adult *D. melanogaster*, *nvd* is expressed in the ovary where ecdysone is produced (Yoshiyama *et al*., 2006). A gene encoding phantom, which participates in ecdysone synthesis, in kuruma prawn is expressed in the Y-organ

(Asazuma *et al*., 2009). *D. magna nvd1* was primarily expressed in the gut of the adult, suggesting that ecdysone is possibly synthesized in the gut (Figure 1-4*a*). The expression of *shd* was detected in the ovary as well as in the gut at 70 HAM when oogenesis had been promoted in the ovary for upcoming oviposition, and the eggs had formed inside (Figure 1-4*c*). Such spatial expression of *shd* indicates that ecdysone targets the ovary besides the gut and remaining tissues. Ecdysteroids might function in ovarian egg maturation in *D. magna*.

**The decline of ecdysteroids level before moulting is required for ensuing moulting and oviposition**

Ecdysteroids stimulate vitellogenesis in most decapod species (Nagaraju, 2010). On the other hand, 20E inhibits vitellogenesis in *D. magna* (Hannas, *et al*., 2011). In support of this, endogenous ecdysteroids titre was low when moulting and oviposition were about to occur in *D. magna* (Figure 1-5). Oviposition occurs immediately after moulting when ecdysteroids titre has decreased. As such a correlation between ecdysteroids titre, moulting and reproductive events exists, I hypothesized that the fluctuation of ecdysteroids titre plays a key role in the endocrine systems regulating the reproductive and moulting cycles. Therefore, I analyzed the effect of 20E-treatment on moulting and oviposition in *D. magna*. Treatment with 20E from the middle of the moulting cycle inhibited moulting and/or oviposition. This indicates that a decrease of ecdysteroids at the end of the reproductive cycle is required for moulting and oviposition. Indeed, *Cyp18a1* was highly expressed at the end of the moulting cycle.

Histological analyses revealed that yolk granules existed in the ovary of both the control and 20E-treated *D. magna*. This implies that suppressed oviposition might not have been caused by inhibition of vitellogenesis by a high 20E titre. In the present study, those individuals that did not moult after treatment with 20E produced a new carapace underneath the old one. Therefore, 20E treatment did not affect the process of cuticle deposition.

Recently, a meiosis-like process of parthenogenetic oogenesis was documented in *D. pulex* (Hiruta *et al*., 2010). In the present study, the nuclei-germinal vesicles broke down (GVBD: germinal vesicle breakdown), chromosomes condensed, and the nuclei were invisible before moulting in the ovarian eggs of control *D. magna*, as observed in *D. pulex*. In the ovary of 20E-treated individuals showing neither moulting nor oviposition, the meiotic process had stopped before GVBD. Although the ovary contained as many yolk granules as the control ovary, 20E inhibited the entry of the ovarian eggs into GVBD, which marks the onset of meiosis.

A meiotic apparatus at metaphase was observed soon after moulting and soon after oviposition in the control eggs in *D. magna* (Figure 1-7*d*,*e*). Such a meiotic apparatus at metaphase was also observed in the eggs of 20E-treated individuals that moulted normally but failed to oviposit (Figure 1-7*i*). In the 20E-treated individuals, however, meiosis stopped at metaphase and did not proceed further even after enough time had passed for meiosis to progress (Figure 1-7*i*). Such a terminated meiotic process might correspond to a failure in meiotic reinitiation. The first reinitiation appears as GVBD and the second as anaphase. Furthermore, meiotic reinitiation is correlated with

the ecdysteroids titre in the prawn *Palaemon serratus* and the locust *Locusta migratoria* (Lanot and Clédon, 1989). The present results suggest that the 20E titre might also be responsible for meiotic reinitiation in *D. magna*. Oviposition never occurs unless moulting precedes it. The time interval between moulting and oviposition is precisely 13 minutes in *D. pulex* at 18 °C (Hiruta *et al*., 2010). Therefore, moulting is expected to act as an oviposition trigger. There must be a coordinated regulation between moulting and oviposition. My results are compatible with this idea, but moulting alone is not enough to initiate oviposition. Some regulatory mechanisms might be present to promote oviposition after moulting. Alternatively, some inhibitory mechanisms might also be present for oviposition of abnormal eggs.

In *Drosophila*, neural signalling with octopamine, which causes oviduct-muscle contraction, controls oviposition (Lee *et al*., 2003; Rodríguez-Valentín *et al*., 2006). In decapod crustaceans, octopamine in the lobster *Homarus americanus* also stimulates muscle contraction of ovarian walls (Swetha *et al*., 2011). Neural signalling besides a direct endocrine pathway could possibly drive the regulation of oviposition in *D. magna*. That is because ovarian eggs likely require some mechanical force, such as muscle contraction, to be released into the brood pouch. This suggests that 20E might affect oviposition through muscle contraction.

In conclusion, I identified genes in the ecdysteroids synthesis or inactivation pathway in *D. magna*. Decline of the ecdysteroids level at the end of the reproductive cycle was necessary for ensuing moulting and oviposition. The gene expression analyses suggest that fluctuation of the ecdysteroids titre during the reproductive cycle

might be regulated by the expression of *Cyp18a1*. Judging by the predominant expression of *nvd*1 in the gut, I also propose that ecdysone might be synthesized in the *D. magna* gut although further studies are required to identify the specific site of ecdysteroidogenesis in the gut. Gene identification and expression analyses in ecdysteroids synthesis or inactivation pathways provide a concrete basis for understanding the contribution of ecdysteroids signals in the highly coordinated reproductive system of crustaceans.

**Table 1-1.**

## Primer sequences used for gene cloning

| genes | forward | reverse |
|---|---|---|
| *neverland1* | ATGGATGTCTTTTTCGTTTTCAAATCC | GGTGTAACCGTCTCGAAG |
| *neverland2* | GGACGCGTATTGCCCTCATTTGGGA | TAATCCCGGCCCGATTTGTTTGGCT |
| *Cyp18a1* | ATGATCGTGTGGAGTTTGGGA | TTTGGCGTTCTGGATCTCCAG |
| **3′ RACE** | | |
| *neverland1* | GCCTCCATCATGAACTACGCCTATTCGG | |
| *Cyp18a1* | GCGACGGCCGTGAGAAAATGGAG | |
| | GCCGTTCACATGAACCCTGCCTTGC | |

**Table 1-2.**

## Primer sequences used for quantitative real-time PCR

| genes | forward | reverse |
|---|---|---|
| *cyclophilin* | GACTTTCCACCAGTGCCATT | AACTTTCCATCGCATCATCC |
| *neverland1* | AGCACAAGGCGGGAAGAGT | GCTTCCCATTTCACCTTCCA |
| *neverland2* | CGTCGGTGACTGCATCGA | TGCCGTCGTTCCCATTG |
| *shade* | GACTGCTGAAGGCGTTGACA | CGGCTGCCACTAGGTCGATA |
| *Cyp18a1* | TACCCGATCGTCGGTTACCT | GAGCGCCGTCAGCTCTTC |
| *EcR* common region | GAGGCGCTGCAGGCTTAC | GAGTTTGGCAAACTCCGTCATC |
| *USP* | GTTGGAGTCAAGGATGGTATCGT | AGCCGAGTTCCGGTGGAT |

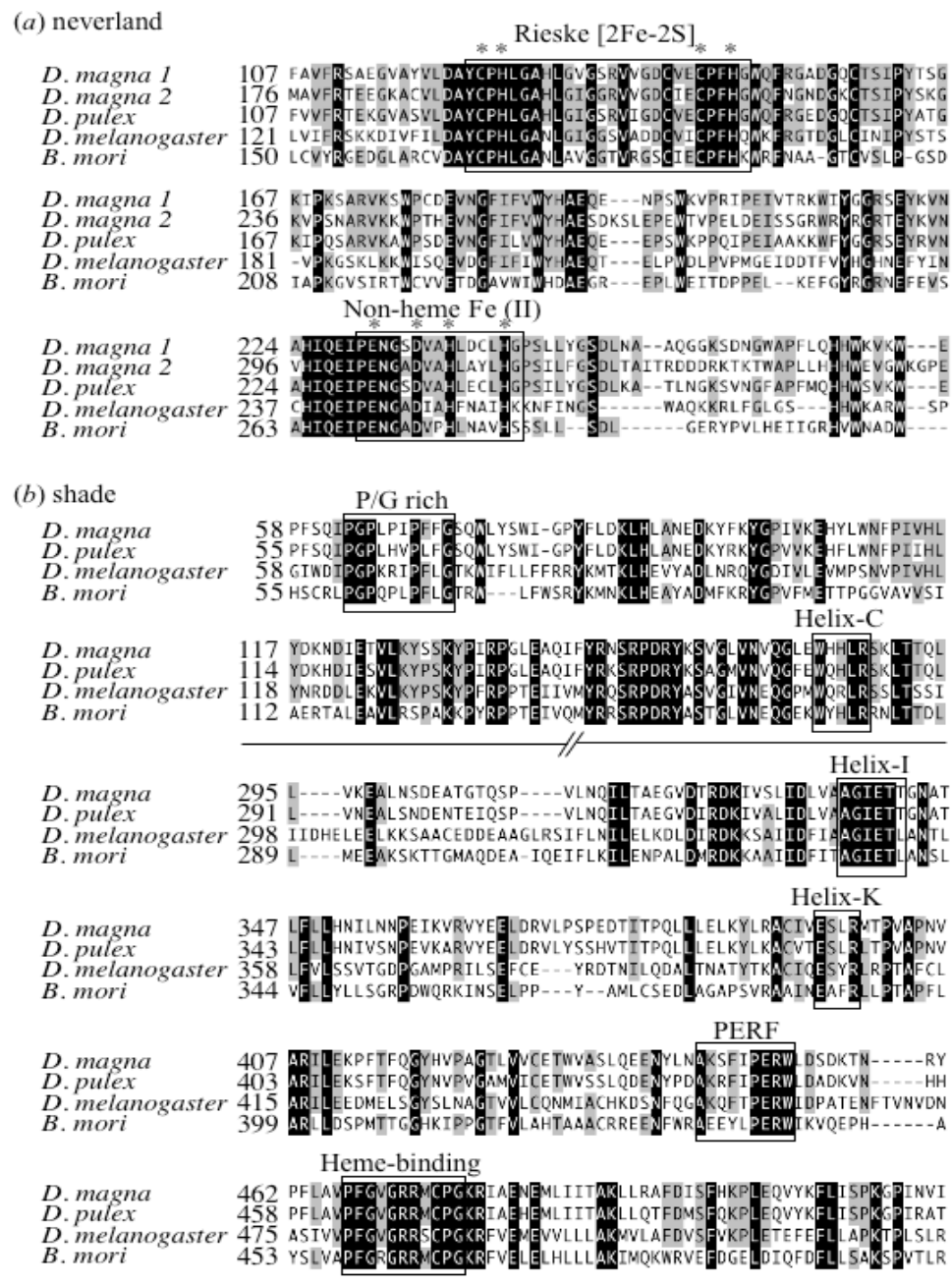

(a) neverland
Rieske [2Fe-2S]
D. magna 1
D. magna 2
D. pulex
D. melanogaster
B. mori
Non-heme Fe (II)
(b) shade
P/G rich
Helix-C
Helix-I
Helix-K
PERF
Heme-binding

**Figure 1-1.** Alignments of predicted amino acid sequences of (*a*) neverland and (*b*) shade of *Daphnia magna* with other arthropod species, *Daphnia pulex*, *Drosophila melanogaster* and *Bombyx mori*. *D. magna 1* and *D. magna 2* indicate the two neverland paralogues, nvd1 and nvd2, respectively. The conserved Rieske (2Fe-2S) domain and the non-heme Fe (II) motif in nvd and conserved domain and motifs of the CYP450 family in shd are shown in boxes. Asterisks show the evolutionally conserved amino acids in nvd. Entries in a black background indicate identities.

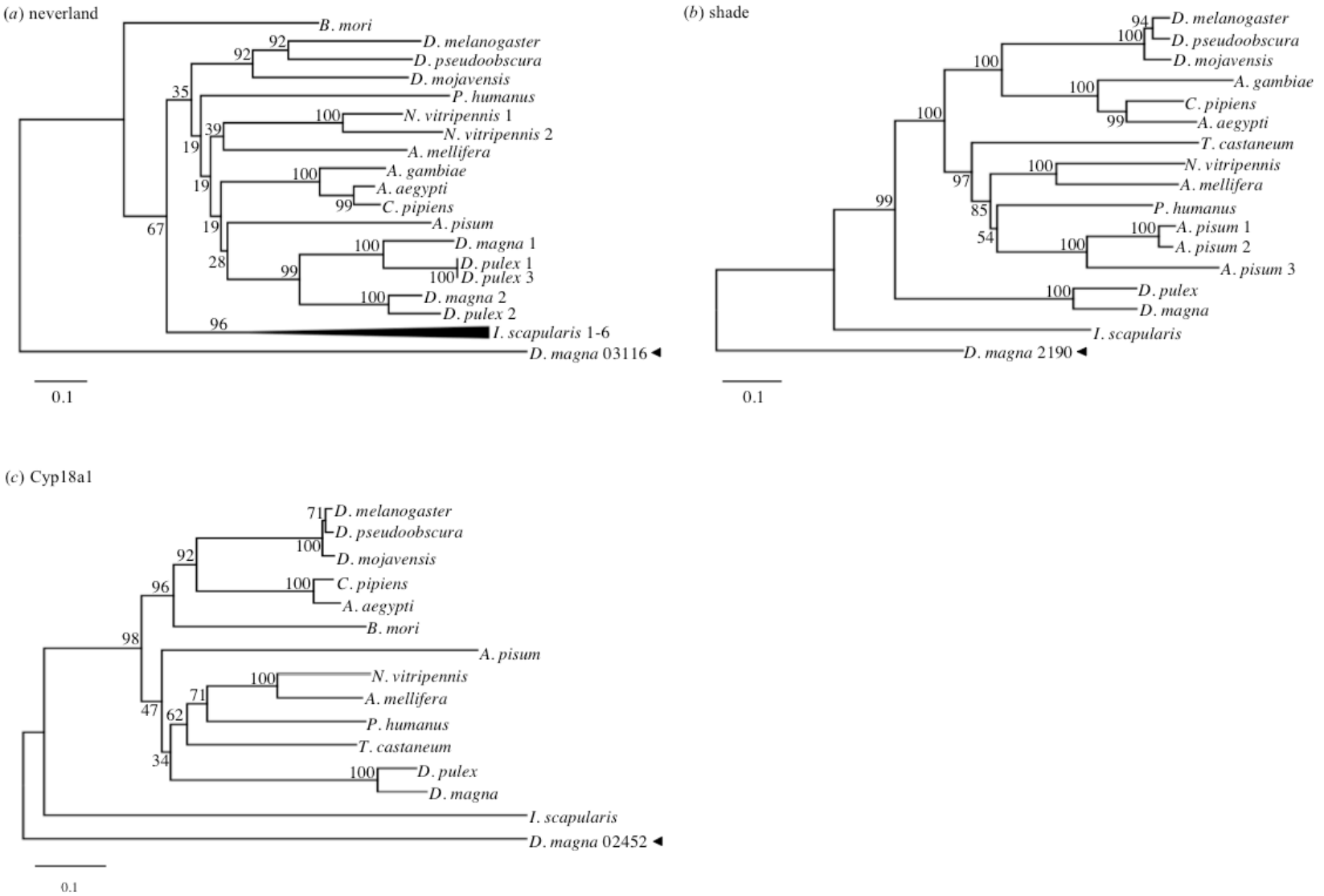

(a) neverland
B. mori
D. melanogaster
D. pseudoobscura
D. mojavensis
P. humanus
N. vitripennis 1
N. vitripennis 2
A. mellifera
A. gambiae
A. aegypti
C. pipiens
A. pisum
D. magna 1
D. pulex 1
D. pulex 3
D. magna 2
D. pulex 2
I. scapularis 1-6
D. magna 03116
0.1
(b) shade
D. melanogaster
D. pseudoobscura
D. mojavensis
A. gambiae
C. pipiens
A. aegypti
T. castaneum
N. vitripennis
A. mellifera
P. humanus
A. pisum 1
A. pisum 2
A. pisum 3
D. pulex
D. magna
I. scapularis
D. magna 2190
0.1
(c) Cyp18a1
D. melanogaster
D. pseudoobscura
D. mojavensis
C. pipiens
A. aegypti
B. mori
A. pisum
N. vitripennis
A. mellifera
P. humanus
T. castaneum
D. pulex
D. magna
I. scapularis
D. magna 02452
0.1

**Figure 1-2.** Neibour joining trees of (*a*) neverland, (*b*) shade and (*c*) Cyp18a1. These trees were constructed with Poisson model and the amino acid sequences excluding gap positions. Bootstrap values (%) show analyses of 1000 trials. Arrowhead represents an outgroup. Scale bar indicates branch length of 0.1 changes per residue. *D. magna* 03116, 2190 and 02452 are sequences in *D. magna* database showing high homology of amino acids with respective genes, and were used as outgroups.

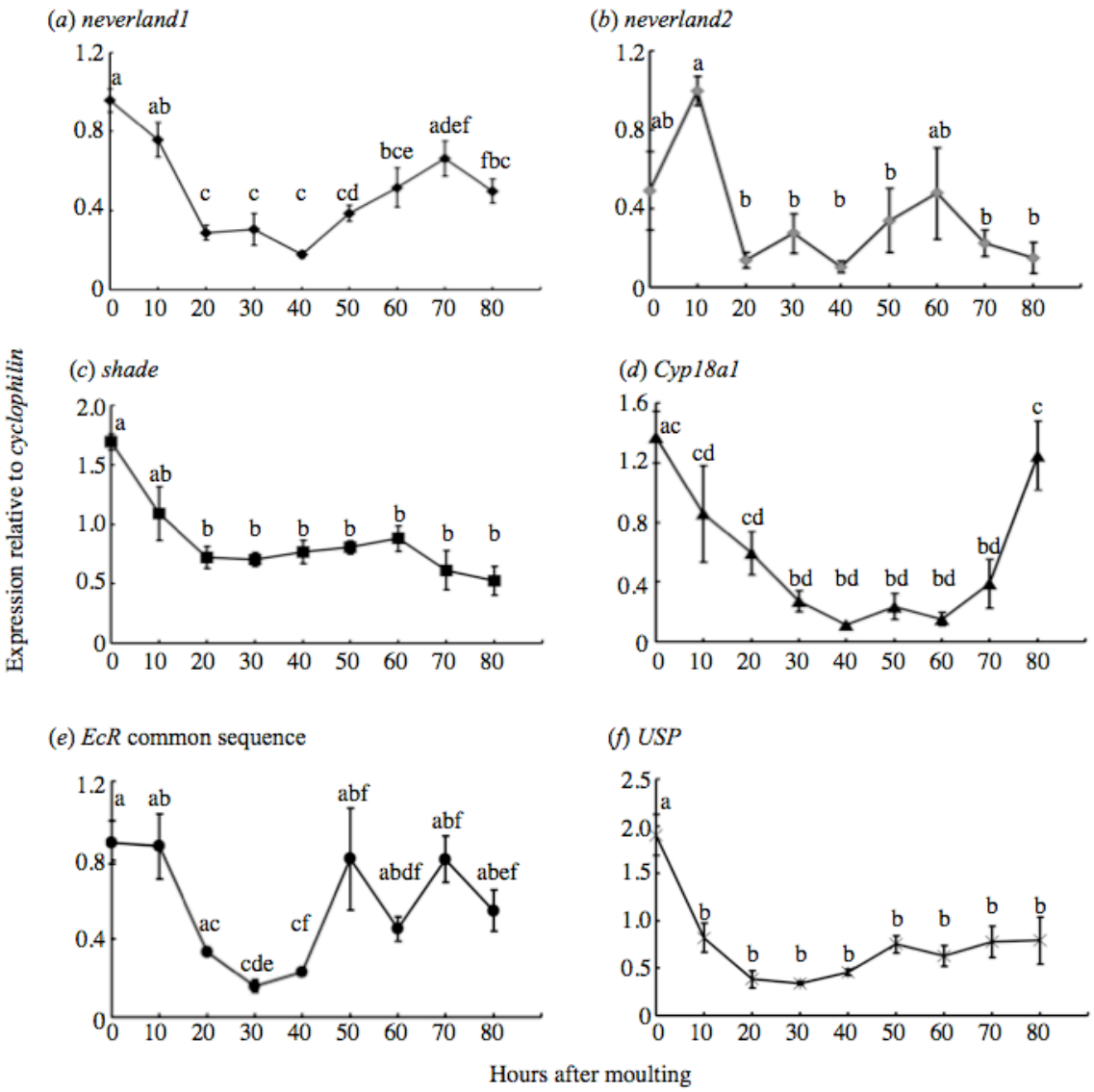



**Figure 1-3.** Gene expression profiles of (*a*) *neverland1*, (*b*) *neverland2*, (*c*) *shade*, (*d*) *Cyp18a1*, (*e*) *EcR* common sequence and (*f*) *USP* in the intermoulting period of adult female *D. magna*. 0 hour after moulting (HAM) refers to the time immediately after moulting and 80 HAM represents the time before the next moulting. Bars represent standard errors. Different letters indicate significant differences among time points. $P < 0.05$ (ANOVA followed by the Tukey-Kramer test).

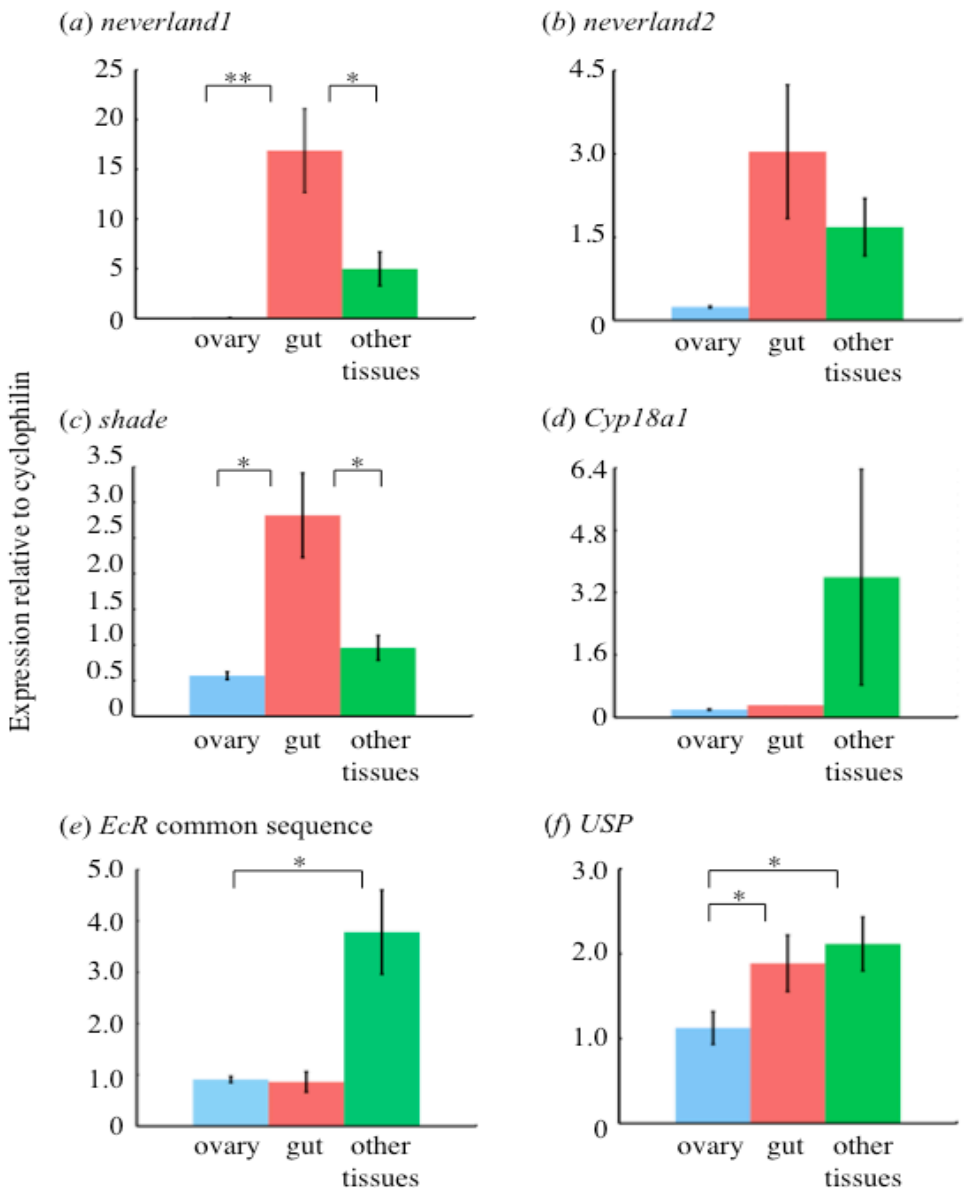



**Figure 1-4.** Relative gene expression to cyclophilin. (*a*) *neverland1*, (*b*) *neverland2*, (*c*) *shade*, (*d*) *Cyp18a1*, (*e*) *EcR* common sequence and (*f*) *USP* in *D. magna* adult female tissues. The ovary, gut and remaining tissues were excised at 70 hours after moulting in the intermoulting period. Bars represent standard errors. $*P < 0.05$ $**P < 0.01$ (ANOVA followed by the Tukey-Kramer test).

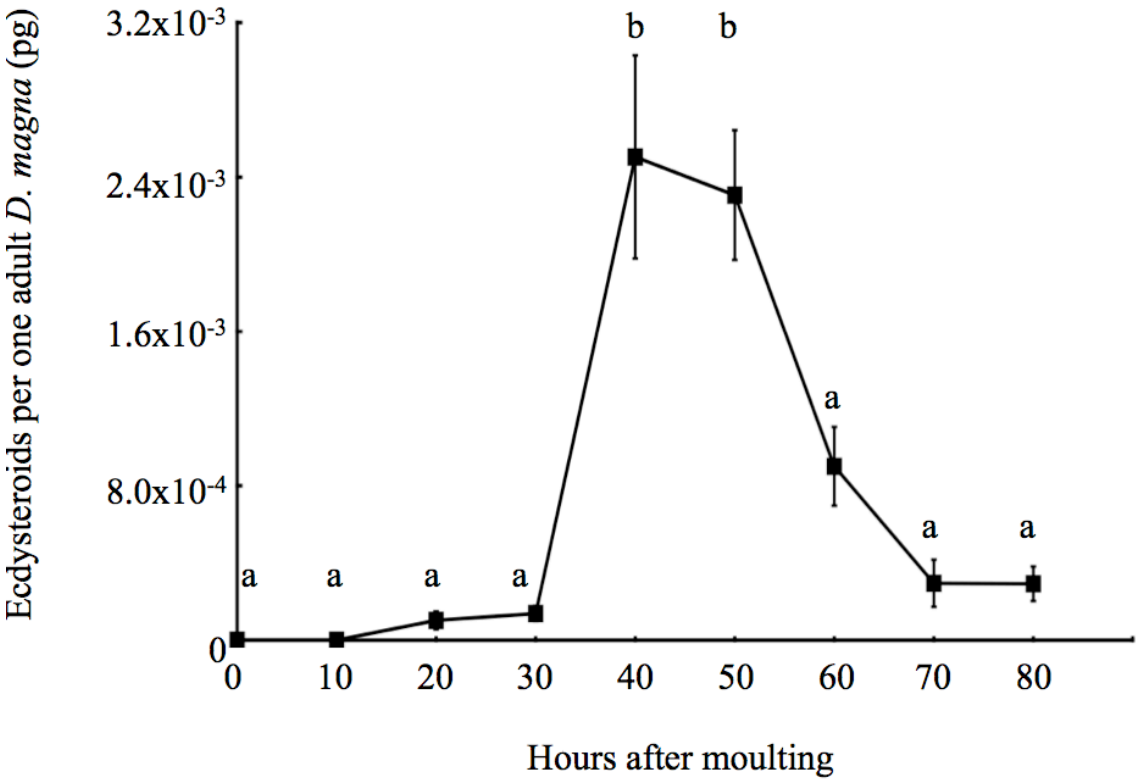



**Figure 1-5.** Ecdysteroids titre during the reproductive cycle of adult female *D. magna*. The titre was determined by ELISA. Each point represents the average ecdysteroids titre per adult *D. magna* of five replicates at each of the nine time points. Any values below the lowest standard point ($9.6 \times 10^{-6}$ pg) were analyzed with this value. Bars represent standard errors. Different letters indicate significant differences among the time points. $P < 0.01$ (ANOVA followed by the Tukey-Kramer test).

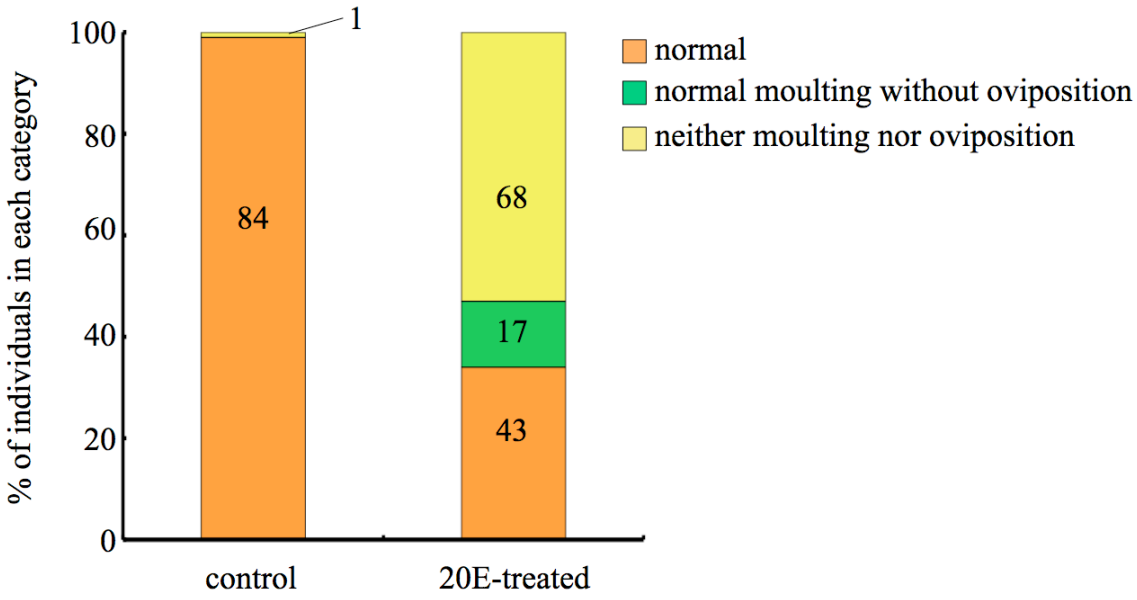
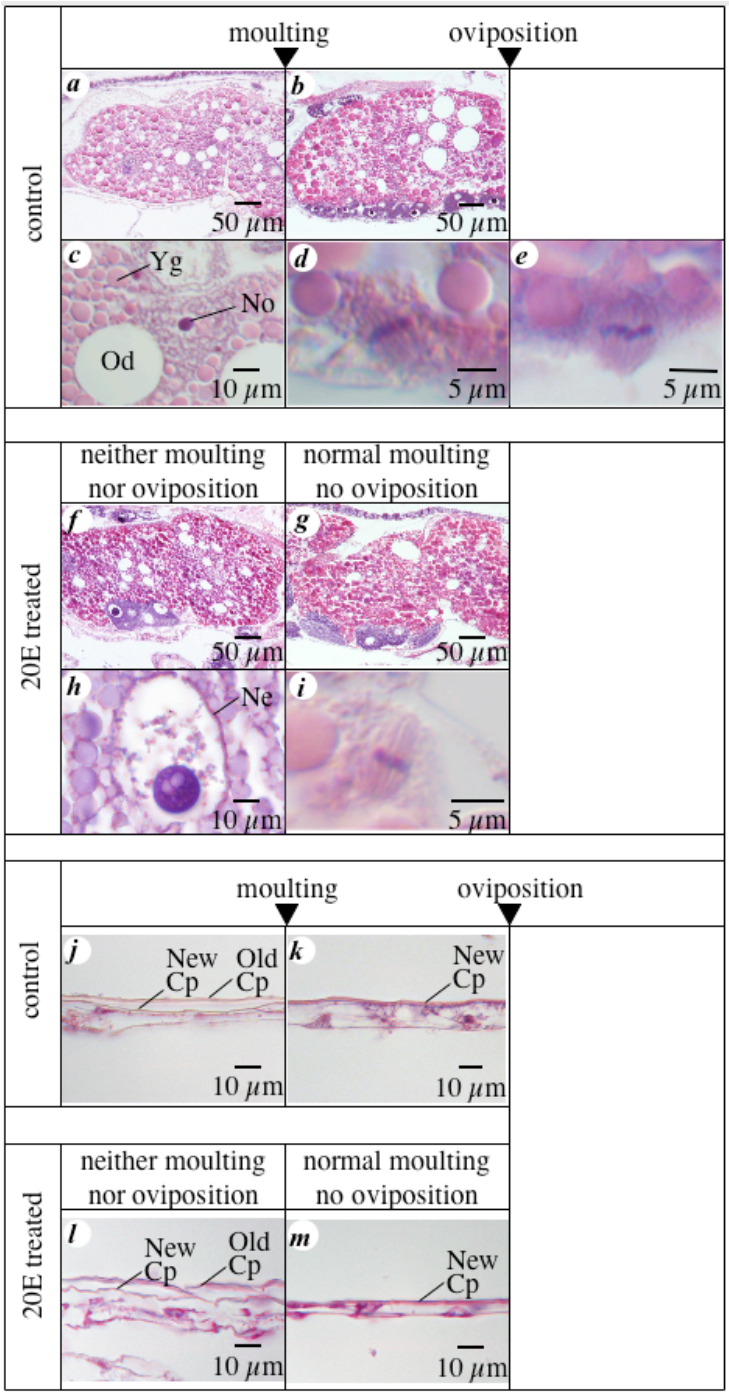



**Figure 1-6.** Percentage of adult female *D. magna* categorized into different phenotypic classes after treatment with exogenous 20-hydroxyecdysone (20E) ($n$ = 128) or ethanol as the control ($n$ = 85). Values in bars represent the number of *D. magna* used.


control
moulting
oviposition
a
b
50 μm
50 μm
c
Yg
No
Od
10 μm
d
5 μm
e
5 μm
20E treated
neither moulting
nor oviposition
normal moulting
no oviposition
f
50 μm
g
50 μm
h
Ne
10 μm
i
5 μm
control
moulting
oviposition
j
New
Cp
Old
Cp
10 μm
k
New
Cp
10 μm
20E treated
neither moulting
nor oviposition
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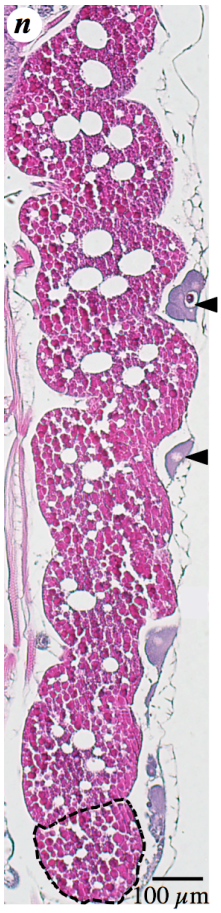
New
Cp
Old
Cp
10 μm
m
New
Cp
10 μm


n
100 μm

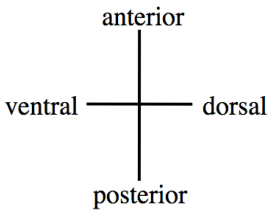

anterior
ventral
dorsal
posterior

**Figure 1-7.** Histological analysis of the ovary and carapace. (*a*,*b*) Ovarian eggs in a control individual. (*c*) The nuclear envelope was invisible in controls before moulting. (*d*,*e*) A meiotic apparatus found in ovarian and oviposited eggs of controls, respectively, and (*f*,*g*) ovarian eggs of a 20E-treated individual. (*h*) The nuclear envelope had not degenerated in the ovarian egg in the 20E-treated individual that neither moulted nor oviposited. (*i*) A division apparatus appeared and chromosomes aligned at the metaphase plate in the 20E-treated individual that moulted normally but did not oviposit. This meiotic apparatus at metaphase was also seen in the control eggs released into the brood pouch immediately after oviposition. However, in the 20E-treated individuals, meiosis stopped at metaphase and did not proceed. (*j*,*k*) Carapace formation in controls and (*l*,*m*) 20E-treated individuals. (*j*) A carapace layer with a newly formed carapace underneath the old one formed before moulting in the control. (*k*) A new carapace was observed immediately after moulting in the control. (*l*) A new carapace was also formed in the 20E-treated individual that neither moulted nor oviposited, and (*m*) the 20E-treated individual that moulted normally but did not oviposit. (*n*) A longitudinal section of a normal *D. magna* ovary. Dashed line: a single egg. Arrowheads: undifferentiated germ cells.

Yg: yolk granule, No: nucleolus, Od: oil droplet, Ne: nuclear envelope, Cp: carapace. Tissue sections of exogenous 20E-treated and the control adult female *D. magna* were stained with hematoxylin and eosin.

# Chapter II

# *Neverland* regulates embryonic moults through the regulation of ecdysteroids synthesis in *Daphnia magna*

**Abstract**

Embryo development in arthropods is accompanied with a series of moultings. A cladoceran crustacean *Daphnia magna* moults three times before reaching first instar neonate during embryogenesis. Previous studies argued that ecdysteroids might regulate embryogenesis in *D. magna*. However, no direct evidence between innate ecdysteroids fluctuation and their functions has been forthcoming. Recently, I identified genes involved in ecdysteroid synthesis called, *neverland* (*nvd1* and *nvd 2*) and *shade* (*shd*) and in the degradation of ecdysteroid (*Cyp18a1*). To understand the physiological roles of ecdysteroids in *D. magna* embryos, I performed expression and functional analyses of these genes. I also examined innate ecdysteroids titre during *D. magna* embryogenesis. Two surges of ecdysteroids titre were detected at 41 and 61 hours after oviposition. The first and second embryonic moultings occurred at each ecdysteroid surge. Expression of *neverland1* and *shade* began to increase before the first peak in ecdysteroid. Knockdown of *neverland1* or *shade* by RNAi technique caused defects in embryonic moultings and subsequent development. Furthermore, the ecdysteroids titre seemingly decreased in *nvd1*-knowckdown embryos. Unlike for *nvd1* and *shd*, knockdown of *Cyp18a1* resulted in early embryonic lethality before the first moulting. My *in situ* hybridization analysis revealed that *nvd1* was prominently expressed in embryonic gut epithelium suggesting the site for an initial step of ecdysteroid synthesis, a conversion of cholesterol to 7-dehydrocholesterol, and possibly for ecdysone production. Taken together, *de novo* ecdysteroid synthesis by *nvd1* in the gut epithelial

cells stimulates moulting, which is indispensable for progression of *D. magna* embryo development.

## Introduction

Steroid hormones control diverse physiological events in development, homeostasis, and reproduction of animals (Lafont and Mathieu, 2007). Ecdysteroids are known as moulting hormone and to regulate not only moulting, but also various fundamental processes such as metamorphosis, reproduction and embryogenesis in arthropods. Especially in insects, many studies have demonstrated the physiological roles of ecdysteroids regulating aforementioned biological processes. As an example, the level of ecdysteroids is correlated with the deposition of new cuticle proteins, remodeling of the fat body and prepupal development (Bownes *et al*., 1988; Bond *et al*., 2011; Gilbert, 2012). Moreover, a series of moultings are necessary for larval development such as the larval-larval and pupa-adult transitions in insects (Riddiford and Truman, 1993).

In addition to the established physiological functions of ecdysteroids, their synthetic and degradation pathways have also been well studied. In insects, 20-hydroxyecdysone (20E) is the most active form of ecdysteroids (Li *et al*., 2014). Ecdysteroid synthesis begins with cholesterol and the enzyme Neverland (nvd) catalyzes the first step of the synthetic pathway by converting cholesterol into 7-dehydrocholesterol (7-dC) (Yoshiyama *et al*., 2006). After several conversions, ecdysone is finally converted to 20E by Shade (shd) in peripheral tissues (Petryk *et al*., 2003). The enzymes involved in ecdysteroid synthesis downstream of nvd are encoded by the Cytochrome P450 (CYP) family (except for *shroud*), and termed the Halloween genes (Chávez *et al*., 2000; Petryk *et al*., 2003; Warren *et al*., 2004; Namiki *et al*., 2005;

Niwa *et al*., 2010). 20E binds to the ecdysone receptor (EcR) to form a heterodimer with ultraspiracle (USP) (Gilbert, 2012), and the complex subsequently activates target gene expression. Ecdysteroid signalling is negatively regulated by Cyp18a1, which inactivates 20E through a degradation pathway (Guittarda *et al*., 2011). The biosynthesis and degradation regulate the ecdysteroids titre, and fluctuations of ecdysteroid at specific timing are mandatory for normal moulting and metamorphosis in insects (Gilbert, 2012).

The importance of *nvd* in larval development has been comprehensively analyzed in *Drosophila melanogaster*. A loss-of-function of *nvd* by RNAi prevented the first instar larvae from moulting to the second instar larvae. These larvae spend a prolonged period of time as the first instar until they eventually ceased to feed, leading to death (Yoshiyama *et al*., 2006). The insect mutants of the Halloween genes consistently also lead to embryonic death (Chávez *et al*., 2000; Petryk *et al*., 2003; Warren *et al*., 2004; Namiki *et al*., 2005; Niwa *et al*., 2010). These developmental arrests were caused by a reduction in the ecdysteroids titre, and were rescued by applying 20E (Chávez *et al*., 2000; Petryk *et al*., 2003; Warren *et al*., 2004; Namiki *et al*., 2005; Yoshiyama *et al*., 2006; Niwa *et al*., 2010). Thus, *nvd* plays key roles in regulation of moulting and larval development.

As in insect species, in decapod crustaceans (eg. shrimps and crabs), the ecdysteroids titre fluctuates during embryonic or larval development, suggesting a correlation with developmental events such as cuticle deposition and onset of ecdysteroid synthesis (Charmantier and Charmantier-Daures, 1998; Subramoniam,

2000; Block *et al*., 2003; Mu and LeBlanc, 2004; Techa *et al*., 2014). Furthermore, in the mole crab *Emerita asiatica*, ecdysteroids appear to regulate pleopodal embryogenesis as indicated by the fact that injection of 20E accelerates the developmental rate (Gunamalai *et al*., 2004). However, molecular mechanisms for regulation of development remain largely unknown since few studies have been conducted on the roles of ecdysteroids during embryonic development in crustaceans.

The water flea *Daphnia magna* is a small freshwater crustacean with a continuous reproductive cycle under favorable conditions. The study described in chapter I have revealed that a decline of the ecdysteroids titre is required for the subsequent moulting during parthenogenetic reproductive cycle of *D. magna*. In addition to the regulation of reproductive cycle, ecdysteroids are crucial for embryonic development in *D. magna*. Treating the embryos with an ecdysteroidogenesis inhibitor (fenarimol) induced a deformed carapace and antennae, and severely arrested embryonic development (Mu and LeBlanc, 2002). A previous study on *D. magna* embryos showed that the ecdysteroids titre decreased during early development, and then increased as development proceeded towards the mid and late stages (Mu and LeBlanc, 2004). Notably, embryos of daphnids show a complex development. During the embryogenesis of *D. magna*, the embryonic membranes are shed twice (stages 1 and 4; Fig. 2-1), and the third embryonic moulting results in first instar neonate that looks miniature adult (Fig. 2-1) (Hiruta *et al*., 2014; Toyota *et al*., in press). The first and second embryonic moultings occur inside a brood pouch of the mother daphnid. Finally, just after neonates release from the mother's brood pouch, the third moulting occurs,

and the neonates spend several days as juveniles until sexually mature. Nonetheless, ecdysteroids titre and its contribution for regulating the complex processes of the embryogenesis have not been addressed.

In daphnids, recent studies have identified five Halloween genes in *D. pulex* (Rewitz and Gilbert, 2008), and *nvd*, *shd* and *Cyp18a1* in *D. magna* (chapter I). Additionally, *D. magna* EcR was reported to bind to 20E as in insects (Kato *et al*., 2007; Table 2-1). This information suggests that ecdysteroid synthesis, degradation and signalling pathways are highly conserved in daphnids as well as insects. Interestingly, *D. magna* has two paralogues (*nvd1* and *nvd2*) duplicated solely in the daphnids linage as described in chapter I (Table 2-1). Gene duplication likely occurred after insects and daphnids diverged, and thus *nvd* gene duplication may serve as to give unique roles in daphnids. However, no functional analyses of nvd paralogues to demonstrate each of their roles have been conducted to date. Furthermore, endocrine organ for ecdysteroid synthesis still remains unknown in daphnids. The prothoracic glands (PG) in insect larvae and the Y-organ in decapod crustaceans have been reported as synthetic organs of ecdysone (Lachaise *et al*., 1993; Gilbert, 2012). However, no such equivalent organ has been identified in daphnids. Further information about the ecdysteroid synthetic organ is necessary to better understand the endocrine system in *D. magna*.

In chapter II, I focused on roles of ecdysteroids during the embryonic development in *D. magna*. I found a positive correlation between changes in ecdysteroid titre and expression patterns of *nvd1* and *shd* during embryogenesis. Functional analysis of these genes *in vivo* using RNAi indicated that *nvd1* and *shd* were

required for embryonic moultings. Finally, I identified tissue-specific expression of *nvd1* in the gut epithelium, implying a site for *de novo* ecdysteroid synthesis, which regulates embryo development in *D. magna*.

## Materials and Methods

### Animals

*Daphnia magna* (NIES strain) was obtained from the National Institute for Environmental Studies (NIES; Tsukuba, Japan). They were maintained in M4 medium (Elendt and Bias, 1990) at 20±1 ˚C under a 14-hour:10-hour light:dark photoperiod cycles. *Chlorella vulgaris* was added daily into each culture. Under these conditions, *D. magna* produces only female offspring every three days. All procedures and protocols were approved by the institutional animal care and use committee at the National Institute for Basic Biology.

*D. magna* embryo development at lower temperature (18 ˚C) was monitored to mark time delay at each developmental hallmark. Embryos were taken out from a brood pouch one hour after oviposition (HAO) by dissection in a drop of water on a glass dish. Each of the 12 embryos from the same brood was individually placed in a well of a culture plate containing a medium used for RNAi experiment to duplicate the condition (see below). The embryos were incubated at 18±1 ˚C under a 14-hour:10-hour light:dark photoperiod cycles. The embryos were photographed every two hours from 1 HAO to 73 HAO until all of them developed into first instar neonates (tail spine elongation).

### Enzyme-linked immunosorbent assay (ELISA)

The levels of ecdysteroids present during embryo development were

measured by ELISA, following procedures described previously (Pankotai *et al*., 2010; Shimada-Niwa and Niwa, 2014) with a minor modification. The embryos were removed from a brood pouch by dissection every 10 hours during 1-81 HAO. Each brood of embryos was frozen in liquid nitrogen and stored at -80 ˚C for later processing. The samples were homogenized in 100 µl methanol, and then centrifuged at 15,000 rpm for one minute. The supernatant was collected into a glass vial. These processes were repeated three times, and the supernatants were combined in a single glass vial for each replicate. The contents were centrifuged in vacuum to dry, and stored at -80 ˚C until use. Five broods of embryos (each brood from one mother daphnid) at each time point were used. 20-Hydroxyecdysone (Sigma, St. Louis, MO, USA) was used as the standard. EIA 96-well strip plates precoated with mouse anti-rabbit IgG were used for each assay (Cayman Chemical, Ann Arbor, MI, USA). 20E Acetylcholinesterase Tracer (Cayman Chemical) and 20E EIA Antiserum (Cayman Chemical) were used in plate preparation, and Ellman's Reagent (Cayman Chemical) was used to develop the plate. In all cases, the manufacturer's instructions were followed. The absorbance at 412 nm was read with an SH-9000 Lab Multigrading Micro Plate Reader (Corona Electric Co. Ltd, Ibaraki, Japan). To examine the change of ecdysteroids titre more precisely, the embryos were sampled again every 5 hours from 31 to 46 HAO, and subjected to the ELISA analysis as described above. For analyses of the dsRNA-injected embryos, the embryos were dissected from the mother daphnid kept at 18 ˚C. The embryos were injected immediately after oviposition before hardened. Time after injection is indicated by hours after injection (HAI). The injected embryos were kept at 18 ˚C and homogenized

at stage 3 (Fig. 2-1). Each embryo was homogenized on a sheet of Parafilm with 2 µl of ethanol, and then transferred directly into each well. Further procedures were the same as described above.

**Quantitative PCR**

*D. magna* embryo samples were prepared at nine time points with 10-hour intervals during an 80-hour developmental period. The embryos were dissected out of adult *D. magna* at each time point. The start point was set 1 HAO when the eggs had sufficiently hardened. Three replicates were prepared for each time point. Total RNA was extracted using the RNAqueous-Micro Kit (Life Technologies, Carlsbad, CA, USA). cDNA was synthesized from 0.25 µg of total RNA using SuperScript III (Life Technologies) with random primers. PCR was performed with SYBR-Green PCR Master Mix (Life Technologies) using the 7000 real-time PCR system (Life Technologies). The primer sequences used for quantitative PCR analyses are shown in Table 2-2. *D. magna cyclophilin* was selected as the reference gene out of four housekeeping genes (*cyclophilin*, *Ltd4ds*, *LBT* and *L32*) calculated with geNorm (Vandesompele *et al*., 2002) and NormFinder (Andersen *et al*., 2004). Statistical analyses of gene expression at each time point were performed by ANOVA followed by the Tukey-Kramer post-hoc test using Excel 2004 (Microsoft Corp., Redmond, WA, USA) with an add-in software Statcel 2 (Yanai, 2004). Samples of embryos after dsRNA injections were also prepared as described above. Ten embryos per replicate were used in triplicate. The embryos were sampled and homogenized at 61 HAI when

the target gene expression was high. Further processes were as described above except that 0.20 μg of total RNA and Oligo$(dT)_{20}$ primer were used for reverse transcription. The results were analyzed with a Student's *t*-test.

**Sectioned *in situ* hybridization**

Probes were synthesized from primers listed in Table 2-3. RNA was regenerated using the DIG RNA Labeling Mix (Roche, Mannheim, Germany). Embryo samples were removed from the brood pouch by dissection when they were at the single black eye stage (stage 5, Fig. 2-1). Adult *D. magna* were sampled after moulting but before oviposition. All samples were fixed in 4% paraformaldehyde in PBS and heptane (PFAh) overnight. For the adult samples, the carapace was removed after two-three hours of fixation in PFAh, and then the samples were returned and kept in PFAh overnight. The samples were dehydrated in ethanol and lemosol, and were embedded in paraffin. Serial sections of 5 μm thicknesses were prepared. Paraffin was removed in xylene, and dehydrated in ethanol. Samples were then washed in PBTw (0.1% Tween-20 in PBS) for 10 minutes, and treated with proteinase K (10 μg/ml) for 10 minutes. The sections were fixed with PFAh for 5 minutes, and then washed twice in PBTw for 5 minutes each time. The samples were pre-hybridized with a hybridization buffer (HB) [formamide 50%, 5xSSC, Denhardt's 10%, Tween-20 0.1%, salmon sperm DNA 300 μg/ml, CHAPS 0.1%, yeast RNA 50 μg/ml] for 40 minutes. The probes were incubated at 100 ˚C for 3 minutes, cooled on ice for 10 minutes, and then added to HB at a concentration of 100 ng/ml. The samples were hybridized at 65 ˚C for over 16 hours.

They were washed twice in wash buffer 1 (WB1) [2xSSC, CHAPS 0.1%] for 20 minutes each time, and three times in WB2 [PBS 10%, BSA 0.1%, Tween-20 0.1%] for 15 minutes each time. Samples were then incubated in 0.1% Anti DIG-AP in WB2 for one hour. After washing four times in WB2 for 5 minutes each time, and in WB3 [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM MgCl2, Tween-20 0.1%] for 5 minutes, the samples were developed with BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tetrazoline chloride) (0.018 mg and 0.035 mg/ml of WB3, respectively), or stained with hematoxylin and eosin (HE). The sections were studied under a microscope (Axioplan2, ZEISS, Oberkochen, Germany) and the images were processed with cellSens Standard, version 1.3 (Olympus, Tokyo, Japan).

**RNA interference**

Double-stranded RNA was prepared using the MEGAscript T7 RNAi Kit (Ambion, Austin, TX, USA). DNA templates were synthesized by PCR with primers listed in Table 2-3. *E. coli malE* was used as the negative control. Samples were incubated at 37 ˚C for 4 hours then annealed at 75 ˚C for 5 minutes. Nuclease digestion and purification were then performed. Following ethanol precipitation, the dsRNA was suspended with TE. The dsRNA (2 µg/ml) was then mixed with 2 µg/µl Chromeo 494 fluorescent dye (Active Motif Chromeo GmbH, Tegernheim, Germany). It was then loaded to a column (SUPREC-01, TAKARA BIO INC., Otsu, Shiga, Japan) and centrifuged at 5000g for 10 minutes at 4 ˚C, and kept -20 ˚C until use. A medium was prepared with 2xM4 medium (Elendt and Bias, 1990) mixed with 0.16 M sucrose water

that had passed through 0.45 μm filter (#431220; Corning) beforehand (M4-sucrose). It was kept at 4 ˚C until use, and kept on ice when injecting. A glass tube (GD-1; NARISHIGE, Tokyo, Japan) was pulled using Sutter instrument (Novato, CA) to make a needle, and the tip was cut out with forceps. The embryos were dissected out, immediately after being oviposited, in the ice-cold M4-sucrose in a glass plate, and were kept on ice to slow down the development. The embryos were then transported to a petri dish filled with the ice-cold M4-sucrose, and injected with the glass needle loaded with the dsRNA using an injector (Femtojet, Eppendorf, Hauppauge, NY, USA) and a micro-manipulator (M-152, MMO-220A, Narishige) under a microscope. The injected embryos were incubated in M4-sucrose at 18 ˚C. The phenotypes of developmental abnormalities were observed at 72 HAI and 96 HAI for 7-16 embryos. Statistical analyses were performed by the Steel test using Excel 2004 (Microsoft Corp.) with an add-in software Statcel 3 (Yanai, 2011).

## Results

### Fluctuations of ecdysteroids titre during embryo development

The innate ecdysteroids profile in *D. magna* embryos was analyzed every 10 hours during an 80-hour period of development from newly oviposited embryos to first instar neonates. The titre of some broods was below the lowest point of the standard curve ($9.6x10^{-6}$ pg). Therefore, those were set a level $9.6x10^{-6}$ pg ecdysteroids, and analyzed with this value. The titre during the first 31 HAO was below the detection limit. The titre at 41 HAO and 61 HAO tended to be higher, and dropped below the standard line again at 51 HAO and 71 HAO (Fig. 2-2). The titre then increased slightly at 81 HAO (Fig. 2-2). The appearance of the first peak of the ecdysteroids titre at 41 HAO was assessed more precisely by sampling the embryos every 5 hours during 31-46 HAO. The result showed the ecdysteroids titre increased at 41-46 HAO (Fig. 2-2, inset).

### Time-course expression analyses of *neverland1*, *neverland2*, *shade* and *Cyp18a1* during embryogenesis

A group of genes encoding ecdysteroid receptors and enzymes for ecdysteroid synthesis or inactivation have been reported in insects (Table 2-1). *D. magna* orthologues used in this study (i.e. *nvd1*, *nvd2*, *shd*, *Cyp18a1*, *EcR* and *USP*) have already identified by previous works (chapter I; Kato *et al*., 2007) (Table 2-1).

*D. magna* embryogenesis proceeds in a brood pouch of the mother. It takes three days on average under the current culture conditions. Investigations on the

expression patterns of genes involved in the ecdysteroid synthesis (*nvd1*, *nvd2* and *shd*), inactivation pathway (*Cyp18a1*) or receptor complex (*EcR* and *USP*) were performed every 10 hours during embryogenesis by real-time quantitative PCR. The time-course profiles of *nvd1* and *shd* expression level resembled each other (Fig. 2-3*a* and *b*). The expression levels of *nvd1* and *shd* increased 7.1-fold and 1.5-fold at 31 HAO against the expression right after oviposition at 1 HAO, respectively, showing an initial peak (Fig. 2-3*a* and *b*). Following the temporary decreases, the mRNA levels of these genes again increased and reached levels 18 and 1.8 times higher than the levels just after oviposition, respectively, at 71 HAO showing a second peak (Fig. 2-3*a* and *b*). *Nvd2* expression showed a prominent and sharp peak at 11 HAO (Fig. 2-3*c*), however, expression pattern of *nvd2* after 11 HAO resembled that of *nvd1* (Fig. 2-3*c* inset). In the case of *Cyp18a1*, a peak in expression appeared at 21 HAO (Fig. 2-3d). *D. magna* has several *EcR* subtypes and their splicing isoforms (Kato *et al*., 2007). *EcR* subtypes A1 and A2 each with two different UTRs (*EcR A1-*$\alpha$, *EcR A1-*$\beta$, *EcR A2-*$\alpha$ and *EcR A2-*$\beta$) all show similar expression patterns with peaks at 51 and 71 HAO (Fig. 2-4*a-d*). In contrast, *EcR B* showed peaks at 11 HAO and 51 HAO (Fig. 2-4*e*). *USP* was highly expressed at 1 HAO, and also showed a peak at 51 HAO (Fig. 2-4*f*).

### ***In situ*** **hybridization analyses of *nvd1* and *nvd2***

The tissue-specific expression of *nvd1* in the embryo was analyzed to detect the site for the initial step of ecdysteroidogenesis. I examined the *nvd1* expression in stage 5 embryos where the expression level of *nvd1* was being increased (see Fig. 2-3*a*).

*Nvd1* was predominantly expressed in gut epithelial cells of the embryo (Fig. 2-5*a*-*c*), and was detected lengthwise in the gut epithelium on the anterior-to-posterior plane (Fig. 2-5*d* and *e*). These results were comparable to the expression pattern in adult samples (Fig. 2-6*a*-*e*). *Nvd2* expression was not detected in the embryo while a strong expression was observed in undifferentiated germ cells inside the ovary in the adult (Fig. 2-7*a*, *a*', *b* and *b*'). A positive control *vasa*, known to be expressed in undifferentiated germ cells in *D. magna* (Sagawa *et al*., 2005), was detected in the same location as *nvd2* (Fig. 2-7*c*). These cells develop into either an oocyte or nurse cells (Fig. 2-7*d*). Based on the spatio-temporal expression analysis during embryogenesis, I chose *nvd1* as the primary target of the following RNAi analysis for the ecdysteroids titre manipulation.

**Effects of decline of the innate ecdysteroids titre by *nvd1*-knockdown**

To assess importance of the ecdysteroid synthesis during the embryogenesis, I carried out RNAi analyses of *nvd1*. The *nvd1*-specific silencing was confirmed by quantitative PCR at the single-black eye stage (stage 5 at 18 ˚C; Fig 2-1) (Fig. 2-8). The dsRNA-injected embryos were kept in M4 medium (Elendt and Bias, 1990) containing sucrose at 18 ˚C to avoid a bacterial growth. The embryo development proceeded slower than it does in general condition at 20 ˚C. For an easy contrast of the development at 18 and 20 ˚C, the characteristic developmental events at 18 ˚C are shown (Fig. 2-1).

Decline of the ecdysteroids titre in the *nvd1*-dsRNA injected embryos was determined by ELISA. The dsRNA-injected embryos were kept in M4 medium

containing sucrose at 18 ˚C. I analyzed the injected embryos at stage 3 at 18 ˚C. The developmental period showing this feature at 18 ˚C is equal to 41 HAO at 20 ˚C when the ecdysteroids titre peaks (see Fig. 2-2). Twelve out of 30 in total control embryos injected with *malE*-dsRNA injection showed a ranged ecdysteroids titre though the remaining 18 embryos exhibited the ecdysteroids titre below the lowest detection level in my assay ($9.6x10^{-6}$ pg) (Fig. 2-9). The embryos from the same brood had similar ecdysteroids titre values. On the other hand, all of the 28 *nvd1*-dsRNA-injected embryos from 3 broods showed the ecdysteroids titre below $9.6x10^{-6}$ pg (Fig. 2-9). These results suggest that *nvd1* is essential for ecdysteroidogenesis in *D. magna* embryo. I then carried out RNAi knockdown of *nvd2* using three different dsRNA. However, expression analyses showed *nvd2*-dsRNA injections decreased the expression of *nvd1* as well as *nvd2* (Fig. 2-10). Therefore, I could not demonstrate functional analyses of *nvd2* at this point and further study is required.

The eggs injected with dsRNA of *nvd1* developed normally until 72 HAI, as judged by normal eye development compared to the control embryos. However, 69% of *nvd1*-dsRNA-injected embryos had not completed the second embryonic moulting at 72 HAI (Fig. 2-11*a*). Their second antennae were also not out of the membrane, whereas 82% of the *malE*-dsRNA-injected embryos (control) had moulted and had the second antennae revealed at 72 HAI (Fig. 2-11*a*). Similar defects in the embryonic moulting were observed by RNAi of *shd*. A half of the *shd*-dsRNA-injected embryos had successfully moulted, but the other half had not at 72 HAI (Fig. 2-11*a*). At 96 HAI, all of the control embryos completed to the first instar neonates after the final embryonic

moulting giving fully formed setae on the second antennae and an extended tail spine. *Nvd1*- or *shd*-dsRNA-injected embryos resulted in the significant delay or arrest to moult (Fig. 2-11*b*). Additionally, 25% and 44% of the *nvd1*-dsRNA-injected embryos did not complete the second or third moulting, respectively. Similarly, 40% of *shd*-dsRNA-injected embryos did not complete the second embryonic moulting (Fig. 2-11*b*). The pictures of the control embryo after the second moulting, *nvd1*-dsRNA-injected embryo that failed the second moulting, and the control neonate after the third moulting are shown (Fig. 2-11*c*-*e*). The *nvd1*-dsRNA-injected embryo that failed the second moulting had the second antennae unrevealed whereas the control embryo showed the antennae separated from the body (Fig. 2-11*c*, *d*). The individuals that had not succeeded to the final embryonic moulting eventually died. I finally conducted knockdown of *Cyp18a1*. All of the *Cyp18a1*-dsRNA-injected embryos died at stage 1 ($n$ = 7) (Fig. 2-1, 2-11*f*).

## Discussion

### Defects in embryonic moultings and subsequent development induced by knockdown of *nvd1* or *shd*

Daphnid embryos develop inside the brood pouch, which is a part of the mother's body, but separated from the internal lining and hemolymph. The pouch is open to the surrounding water. Therefore, the oviposited embryos are considered to be unaffected by maternal factors such as endocrine signals although the timing of development coincides with the mother's moulting cycle. Based on this information, the embryos are considered to synthesize ecdysteroids *de novo* when their own endocrine systems become ready. Insect larvae with a mutation in the Halloween gene show a low ecdysteroid titre and defects in cuticle deposition, head involution, dorsal disclosure and gut development (Chávez *et al*., 2000; Petryk *et al*., 2003; warren *et al*., 2004; Namiki *et al*., 2005).

In chapter II, I, for the first time in crustaceans, successfully manipulated the embryonic ecdysteroid titre by knocking down the ecdysteroidogenic genes. The ecdysteroids titre in *nvd1*-dsRNA-injected *D. magna* decreased in contrast to *malE*-dsRNA-injected embryos (Fig. 2-9) when it most likely peaked (Fig. 2-2). Additionally, *nvd1*- or *shd*-dsRNA-injected embryos both failed the embryonic moulting and subsequently died (Fig 2-11). These results suggest that *nvd1* is required for *de novo* synthesis of ecdysteroids, and the conversion to 20E by shd is necessary for the normal development of *D. magna* embryos governing embryonic moultings.

Embryonic moultings and ecdysteroids fluctuation are correlated in decapod crustaceans where the secretion of embryonic or larval envelopes occurs when the level of embryonic ecdysteroids peaks (Subramoniam, 2000). In other crustaceans, an embryo and juvenile copepod *Amphiascus tenuiremis* showed a peak of the ecdysteroids titre during development (Block *et al*., 2003). The peaks of the ecdysteroids titre followed by declines were observed in developing insect species (Thummel, 2001; Yamanak, 2007; Gilbert, 2012) and adult decapod species (Lachaise *et al*., 1993; Charmantier and Charmantier-Daures, 1998; Huberman, 2000; Gunamalai *et al*., 2004; Okumura, 2004). The present study also indicates that the embryonic ecdysteroids titre likely peaked before the timing of moultings at 41 HAO and 61 HAO (Fig. 2-2). Notably, the ecdysteroids titre declined after each peak at 51 HAO and 71 HAO coinciding with the second and third moultings, respectively. These results suggest that embryonic moultings are likely stimulated by a decline of the ecdysteroids titre in *D. magna*, which is consistent with insects and decapods as described above.

The present study showed *nvd1*- or *shd*-dsRNA injection did not immediately inhibit moultings, and all embryos successfully completed the first moulting. The effects appeared later on and eventually led to embryonic lethality. One possible reason why it did not affect the early period of development might because the early embryos contained the maternal ecdysteroids, which are considered to be supplied during the oogenesis in the mother's ovary. In insect species, the eggs enclose ecdysteroids as maternal resources (Bownes *et al*., 1988; Yamada *et al*., 2005). In addition, an ecdysone-deficient mutant fly crossed with wild type males produced progeny with

defects in metamorphosis suggesting maternal ecdysteroids are essential for normal development (Kozlova and Thummel, 2003). *Nvd*-knockdown fly completed embryogenesis and achieved the first instar larvae, but failed to induce further moultings and died as first instar larva (Yoshiyama *et al*., 2006). In both cases, i.e., *D. melanogaster* and *D. magna*, the *nvd*-knockdown embryos succeeded in the first moulting indicating the contribution of maternal ecdysteroids. The expression levels of *EcR* and *USP* were high during the early embryogenesis (Fig. 2-4) although the profile of the ecdysteroids titre during 1-41 hours of development showed levels below the lowest point of the standard curve. This implies the maternal ecdysteroids present in very small fraction, or a different technical approach may give definite results. Furthermore, this maternal resource was possibly in the form of modified, conjugated ecdysteroids such as phosphoric esters (Bownes *et al*., 1988; Yamada *et al*., 2005). These phosphoric forms are converted to free ecdysteroid by ecdysteroid-phosphate phosphatase (EPPase), which shows high expression patterns during early embryogenesis in *D. magna* embryos (Asada *et al*., 2014). A previous study reported *D. magna* newly oviposited eggs (within 4 HAO) contain conjugated forms of ecdysteroids (Martin-Creuzburg *et al*., 2007). The peaks of the *D. magna* embryonic ecdysteroids titre might indicate when their endocrine system fully formed, allowing for the *de novo* synthesis of ecdysteroids. This is true in the case of shrimp *Palaemon seratus* and a crab *C. sapidus* in which an increase in the ecdysteroids titre seemed to coincide with the formation of the Y-organ (Lachaise *et al*., 1993; Techa *et al*., 2014). *D. magna* embryos appeared to start synthesizing ecdysteroids before 41 HAO as indicated by an increase

in the ecdysteroids titre (Fig. 2-2). Furthermore, the expression levels of *nvd* and *shd* gene increased before the ecdysteroids titre peaked (Fig. 2-3*a* and *b*). Overall, these results suggest that the embryos possibly used maternal resources first, and before 41 HAO, they began to synthesize ecdysteroids on their own. Ecdysteroids in *D. magna* embryos regulate embryonic moultings, thus ensuring normal development.

**Inactivation of maternal ecdysteroids by CYP18a1 is required for early embryogenesis**

The fluctuating ecdysteroids titre indicates that ecdysteroid inactivation process also appears to be critical. *Cyp18a1* knockdown or overexpression in *D. melanogaster* and the silk moth, *Bombyx mori*, result in embryonic or larval lethality (Guittarda *et al*., 2011; Li *et al*., 2014). Cyp18a1 inactivates 20E through hydroxylation into 20-hydroxyecdysonoic acid (20Eoic) in insects (Guittarda *et al*., 2011). The presence of 20Eoic has been observed during embryogenesis in the shrimp *Macrobrachium rosenbergii*, suggesting that maternal ecdysteroids are inactivated in the developing embryos in decapod crustaceans as in insects (Wilder *et al*., 1995). In chapter II, ELISA analysis did not show a detectable amount of ecdysteroids in early period of embryo development. However, a previous study demonstrated the presence of ecdysteroids in early developmental period of *D. magna* embryos by radioimmunoassay analysis (Mu and LeBlanc, 2004). The time-course expression pattern of *Cyp18a1* during the parthenogenetic reproductive cycle of *D. magna* counteracted the fluctuation of the innate ecdysteroids titre as shown in chapter I, and

might regulate the degradation of 20E to lower the ecdysteroids titre in the adult *D. magna* as well. Furthermore, the embryos injected with *Cyp18a1*-dsRNA failed the first moulting and died (Fig. 2-11). During this period, expression of the *Cyp18a1* gene tended to increase before *de novo* synthesis of ecdysteroids likely occurred. This suggests that the embryos need to break down maternal ecdysteroids before they start *de novo* synthesis with the maturation of their endocrine organ.

**Gut epithelial cells are the possible ecdysteroids synthetic tissue**

The synthesis of ecdysteroids occurs in a specialized organ or tissues (Lachaise *et al*., 1993; Gilbert, 2012). In insect larvae, the synthesis of ecdysone takes place in the PG. Thus, *nvd* and most of the Halloween genes are exclusively expressed in the PG (Ono *et al*., 2006; Yoshiyama *et al*., 2006, Iga and Smagghe, 2010; Marchal *et al*., 2011; Iga *et al*., 2013). However, it is unknown whether daphnids have a functional PG-like organ, Y-organ-like structure, or something very different. The present results showing that *D. magna nvd1* was prominently expressed in the gut epithelial cells of both embryo and adult suggest that 7dC might be synthesized (Fig. 2-5; Fig. 2-6). The 7-dC could be transported to another location for further conversions to ecdysone. Further study is required to specify the exact location of ecdysone and 20E production. Crustaceans including daphnids continue to moult throughout life even after sexual maturity. In contrast, most insect species stop moulting when they reach the adult stage, and PG degenerates during metamorphosis. However, ecdysteroids are required for stem cell niche formation and oogenesis, for instance (Carney and Bender, 2000;

König *et al*., 2011). Thus, ecdysteroidogenesis continues in adults. Indeed, some studies have reported that in some insects ecdysteroids are synthesized in follicle cells in the ovary (Gilbert, 2012). Although the synthesis of ecdysteroids in the ovary in other arthropods is poorly documented (Brown *et al*., 2009), ovarian expression of *disembodied* and *spook* were detected in chelicerates *V. destructor* and *O. moubata*, respectively (Cabrera *et al*., 2015; Ogihara *et al*., 2015).

Interestingly, *D. magna nvd2* was expressed in germ cells in the ovary (Fig. 2-7), but was not detected in embryos despite *vasa* being detected. Considering that *D. magna nvd1* and *nvd2* were generated by gene duplications in daphnids as demonstrated in chapter I, both paralogues might be capable of taking part in the synthesis of ecdysteroids since the characteristic domain and motif are highly conserved among insects and daphnids (chapter I). Although there is no clear evidence to date of ecdysteroid synthesis in the ovary of crustaceans, ecdysone is converted to 20E and some intermediates in ecdysteroidogenesis are found in the ovary of the crab *Cancer antennarius* (Spaziani *et al*., 1997; Brown *et al*., 2009). Alternatively, nvd2 could function differently other than ecdysteroidogenesis such as germ cell differentiation and maintenance. Further analysis is required to fully understand its role in *D. magna*. In terms of the possible synthesis of ecdysteroids in gut epithelial cells, other Halloween genes in ecdysteroidogenesis should also be studied.

## Conclusions

In chapter II, I show that *de novo* ecdysteroid synthesis is required for *D. magna* embryo development. The timing of moultings coincided with the decline of the ecdysteroids titre after each of the two peaks during embryo development. The first peak occurred at 41 HAO after the expression of *nvd1*, *nvd2* and *shd* began to rise, indicating likely start of *de novo* synthesis of ecdysteroids. The knockdown of *nvd1* or *shd* resulted in failure of embryonic moultings, which was accompanied with the decreased ecdysteroids titre. These results suggest that *nvd1* regulates the ecdysteroids titre to signal the embryonic moultings in *D. magna*.

**Table 2-1.** A summary of ecdysteroid synthesis and inactivation pathways. Known enzymes and receptor proteins in insects and their orthologues in daphnia species, *D. magna* and *D. pulex*, are shown.

| Enzymes and intermediates for ecdysteroids signaling pathway in insects (Gilbert, 2004; Niwa *et al*., 2010; Sin *et al*., 2015). | | Number of orthologues in *Daphnia magna* | Number of orthologues in *Daphnia pulex* |
|---|---|---|---|
| ***Synthesis*** | | | |
| Cholesterol | | | |
| ↓ | **Neverland** | 2 [+](BAQ02388.1, BAQ02389.1) | 3 **(187531, 341762, 311567) |
| 7-dehydrocholesterol | | | |
| "Black Box" | **Spook (CYP307A1)** | 1 *(Dapma7bEVm010925t3) | 1 **(42170) |
| (intermediates | **Spookier (CYP307A2)** | — | — |
| unknown) | **Shroud** | — | 1 **(330516) |
| Ketodiol | | | |
| ↓ | **Phantom (CYP306A1)** | 1 *(Dapma7bEVm001479t1) | 1 **(22404) |
| Ketotriol | | | |
| ↓ | **Disembodied (CYP302A1)** | 1 *(Dapma7bEVm001506t1) | 1 **(67413) |
| 2-deoxyecdysone | | | |
| ↓ | **Shadow (CYP315A1)** | 1 *(Dapma7bEVm004713t1) | 1 **(112233) |
| Ecdysone | | | |
| ↓ | **Shade (CYP314A1)** | 1 [+](BAF35770.1) | 1 **(321908) |
| 20-hydroxyecdysone | | | |
| ***Receptors*** | | | |
| | **Ecdysone receptor** | 3 proteins with 5 cDNAs [+]( BAF49029.1-BAF49033.1) | 2 **(245021, 218282) |
| | **Ultraspiracle** | 1 [+](BAF49028.1) | 1 **(219611) |
| ***Inactivation*** | | | |
| ↓ | **CYP18a1** | 1 [+](BAQ02390.1) | 1 **(314153) |
| ↓ | | | |
| 20-hydroxy-ecdysonoic acid | | | |

[+]Protein ID at GenBank (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/)

*wFlea Base (Genome Informatics Lab. Indiana University: http://arthropods.eugenes.org/Evidential Gene/ daphnia/ daphnia _magna/)

** Protein ID at the DOE Joint Genome Institute (The genome portal of the Department of Energy Joint Genome Institute: http://genome.jgi-psf.org/Dappu1/Dappu1.home.html)

## Table 2-2.

## Primer sequences used for quantitative real-time PCR

| genes | forward | reverse |
|---|---|---|
| *cyclophilin* | GACTTTCCACCAGTGCCATT | AACTTTCCATCGCATCATCC |
| *neverland1* | AGCACAAGGCGGGAAGAGT | GCTTCCCATTTCACCTTCCA |
| *neverland2* | CGTCGGTGACTGCATCGA | TGCCGTCGTTCCCATTG |
| *shade* | GACTGCTGAAGGCGTTGACA | CGGCTGCCACTAGGTCGATA |
| *Cyp18a1* | TACCCGATCGTCGGTTACCT | GAGCGCCGTCAGCTCTTC |
| *EcR* A1-α | TGGCTTTTTTCTGTGTTGAGTTG | CCGATCAAAAGTTACACGATGACA |
| *EcR* A1-*β* | CAGTCCGCCGGAGACAGA | CAACGAAAAATGAACCCAACTTG |
| *EcR* A2-α | CGGCCTCGCGTAATCG | TGAGTCTCTGCGCCACTTATCA |
| *EcR* A2-*β* | GACTGTGCGTGCCAGTGAAA | CGCCGAAAAACCAACGAA |
| *EcR B* | GCTGGTAGTGAGGCCACGAT | GCCCTCGCCACACACTCT |
| *USP* | GTTGGAGTCAAGGATGGTATCGT | AGCCGAGTTCCGGTGGAT |
| Analyses of dsRNA-injected embryos | | |
| **genes** | **forward** | **reverse** |
| *neverland1* | GGCAGATGGTCAATGCAC | CCGTTTACTTCGTCGCAA |
| *neverland2* (probe 1and 2) | GGGAAGTCGGATGGAAAGG | CGAAGAGACGCAAATCGTG |
| (probe 3) | CGGACAAGAGCCTCGAAC | CCAGATGGGCCACATCAG |

## Table 2-3.

## Primer sequences used for *in situ* hybridization and dsRNA generation

| ***in situ* hybridization** | | | |
|---|---|---|---|
| **genes** | | **forward** | **reverse** |
| *neverland1* | | GCGCGAGTTAAATCATGG | GGTGTAACCGTCTCGAAG |
| *neverland2* | | GGACGCGTATTGCCCTCATTTGGGA | TAATCCCGGCCCGATTTGTTTGGCT |
| *shade* | | ACTTGCTCTAGAGGTTTGTGGAAG | TAAATAGTGACGAGGCCACTGGA |
| *Cyp18a1* | | ATGATCGTGTGGAGTTTGGGA | TTTGGCGTTCTGGATCTCCAG |
| **dsRNA generation** | | | |
| **genes** | | **forward** | **reverse** |
| *neverland1* | | ATGGATGTCTTTTTCGTTTTCAAATCCG | TTTCGGTATTTTTCCCGATGTATAAGGA |
| *neverland2* | 1 | GGACGCGTATTGCCCTCATTTGGGA | AGGAGATCCCGGTTCGGGACCTT |
| | 2 | TAATACGACTCACTATAGGGGTTCATTTGAC | TAATACGACTCACTATAGGGTCACCAATCTA |
| | | TATGGG | TGGAGC |
| | 3 | TAATACGACTCACTATAGGGATCCGATT | TAATACGACTCACTATAGGGAGTTTAG |
| | | TGACGGCCATAA | CGTAAGGTGCAATGACGGTGG |
| *shade* | | ATGAGATTTTTAAAGAAATTAAAGCGTG | TGATGCCATTCCAATCCTTGGACGTTAA |
| *Cyp18a1* | | ATGATCGTGTGGAGTTTGGGACGGCTGG | ACACTTCGATCATAATTCGGTTCTCCAT |

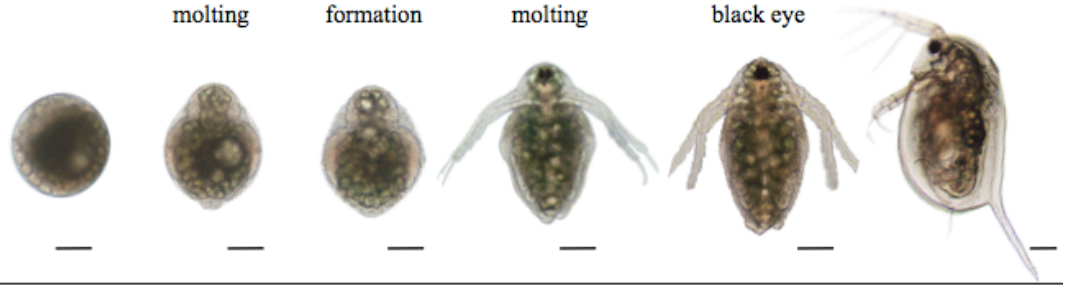
| Stage: | 1 | 2 | 3 | 4 | 5 | 1st instar neonate |
|---|---|---|---|---|---|---|
| | segmentation | 1st molting | eye formation | 2nd molting | single black eye | 3rd molting |
| |  | | | | | |
| Development at 18 C°, HAO | | | | | | |
| | 25.7 | 32.1 | 38.1 | 54.6 | 60.6 | 72.6 |
| Development at 20 C°, HAO | | | | | | |
| | 23.0 | 34.1 | 37.2 | 51.6 | 53.6 | 68.4 |

**Figure 2-1.** Characteristic events during development of *D. magna* embryo. Hours after oviposition (HAO) refers to the time after the eggs were oviposited into a brood pouch. The embryos were dissected out from the brood pouch and kept individually at 18 C˚. The staging and time at 20 ˚C are as described elsewhere (Hiruta *et al*., 2014; Toyota *et al*., in press). Scale bars: 100 µm.

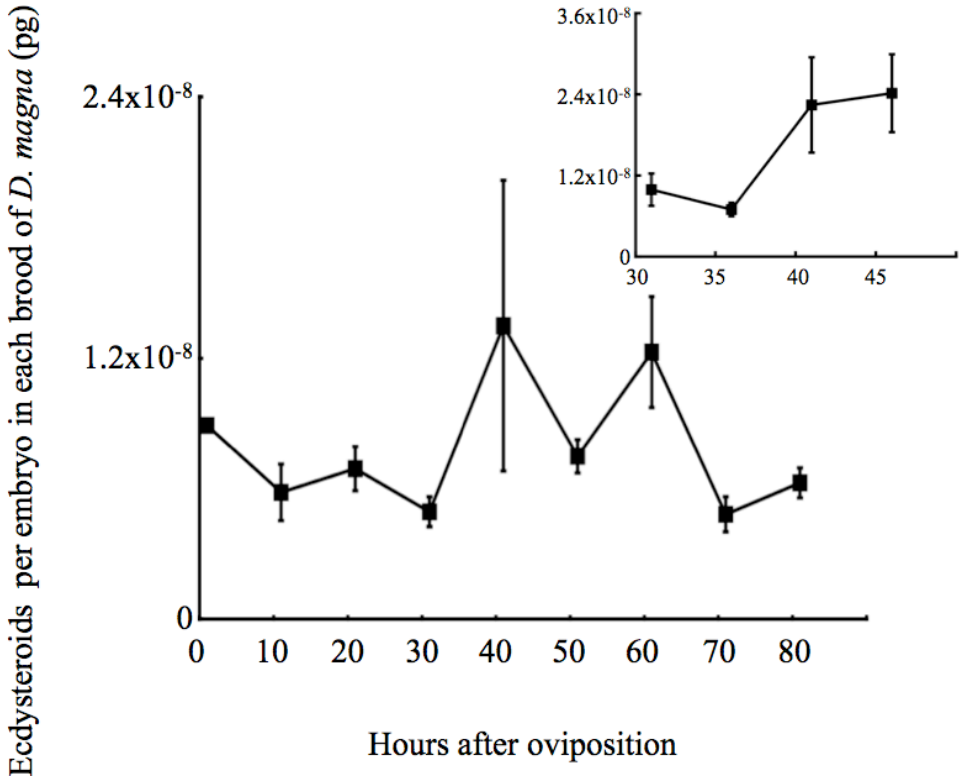



**Figure 2-2.** Fluctuation of ecdysteroids titre in *D. magna* embryos during development after oviposited. The titre was determined by ELISA. Each point represents average ecdysteroids titre per embryo in each of five broods at each time point. Any values of broods below the lowest standard point ($9.6x10^{-6}$ pg) were analyzed with this value. Bars represent standard errors.

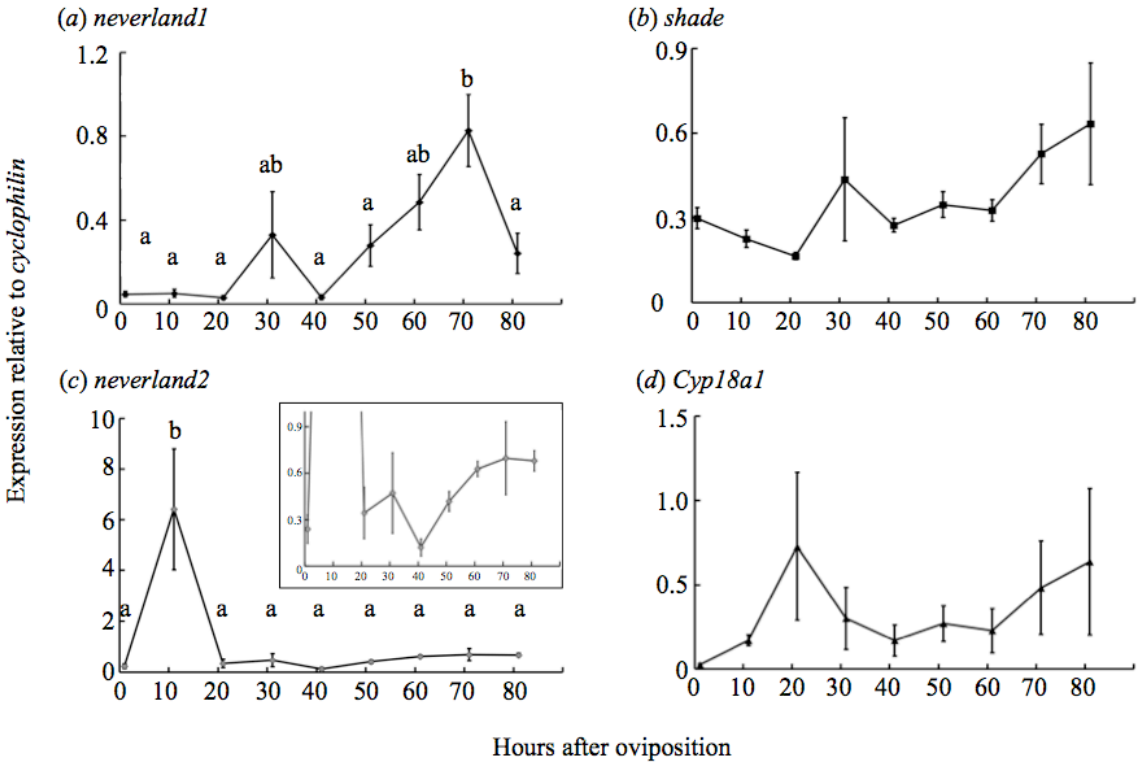



**Figure 2-3.** Gene expression profiles during embryonic development of *D. magna*. (*a*) *nvd1*, (*b*) *shd*, (*c*) *nvd2* and (*d*) *Cyp18a1*. Hours after oviposition refers to the time after the eggs were oviposited into a brood pouch. Three replicates were used for each time point. *D. magna cyclophilin* was used as the reference gene. Bars represent standard errors. Different letters indicate significant differences among the time points. $P < 0.05$ (ANOVA followed by the Tukey-Kramer test).

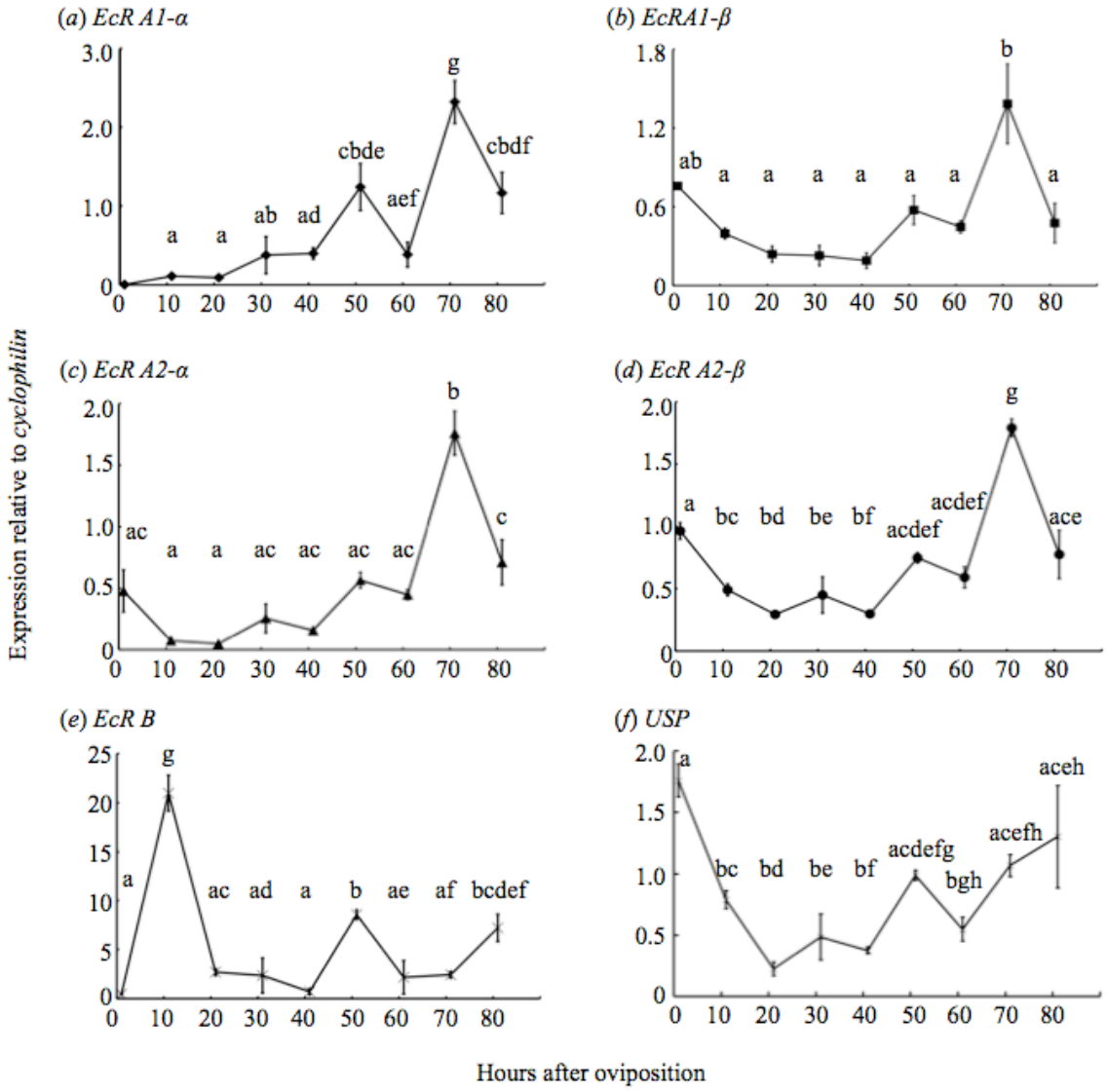



**Figure 2-4.** Gene expression profiles of *EcR* and *USP* during embryonic development of *D. magna*. Three subtypes of *EcR*, *A1*and *A2* each with two UTRs, and *B* were analyzed. (*a*) *EcR A1-α* (the level at 1 HAO was below the detectable limit), (*b*) *EcR A1-β*, (*c*) *EcR A2-α*, (*d*) *EcR A2-β*, (*e*) *EcR B* and (*f*) *USP*. Hours after oviposition refers to the time after the eggs were oviposited into a brood pouch. Three replicates were used for each time point. *D. magna cyclophilin* was used as the reference gene. Bars

represent standard errors. Different letters indicate significant differences among the time points. $P < 0.05$ (ANOVA followed by the Tukey-Kramer test).

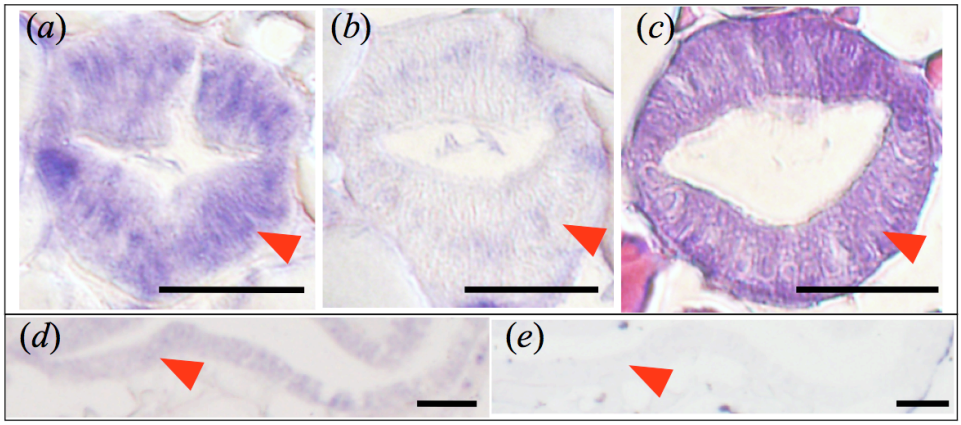



**Figure 2-5.** *Nvd1* expression in a serially sectioned gut of *D. magna* embryo. Tissue-specific expression of *nvd1* was observed by sectioned *in situ* hybridization. Cross sections with (*a*) an antisense probe, (*b*) a sense probe, and (*c*) HE staining. Lateral sections with (*d*) an antisense probe, (*e*) a sense probe. Arrowheads show the gut. Scale bars: (*a*) and (*b*): 25 µm; (*c*) and (*d*): 100 µm.

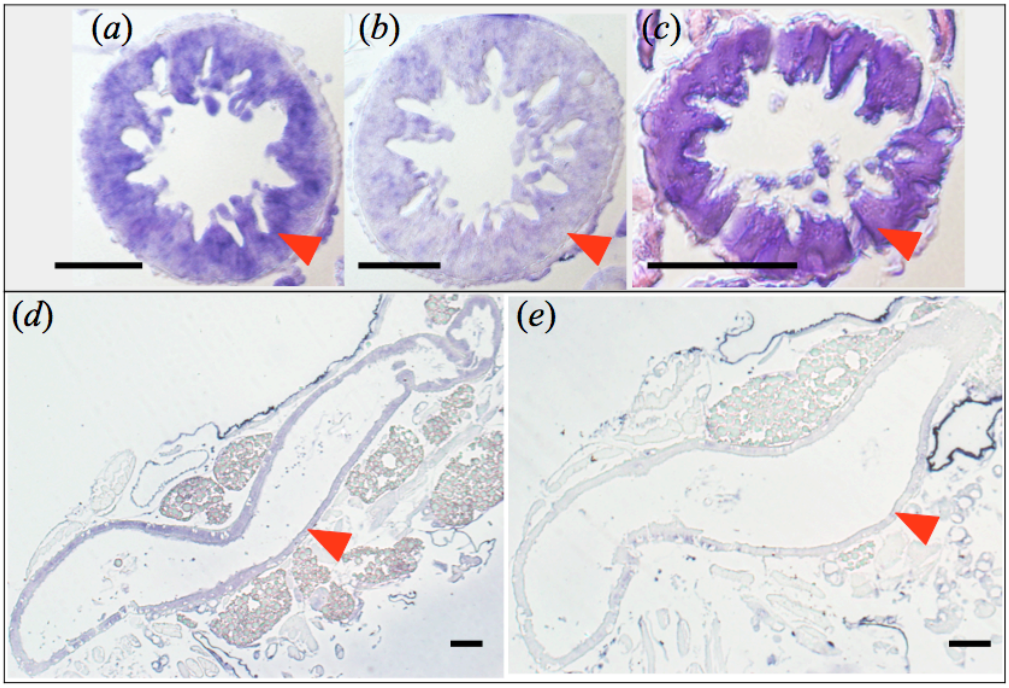



**Figure 2-6.** *Nvd1* expression in a serially sectioned gut of adult *D. magna*. Tissue-specific expression of *nvd1* was observed by sectioned *in situ* hybridization. Cross sections with (*a*) an antisense probe, (*b*) a sense probe, and (*c*) HE staining. Lateral sections with (*d*) an antisense probe and (*e*) a sense probe. Arrowheads show the gut. Scale bars: (*a*), (*b*) and (*c*): 50 µm; (*d*) and (*e*): 200 µm.

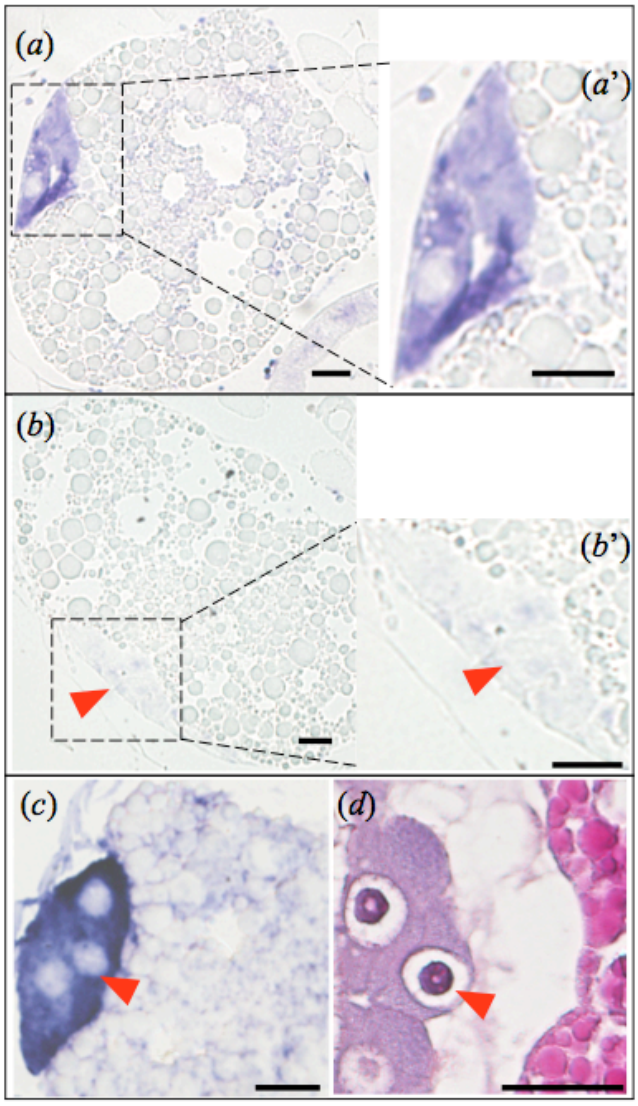



**Figure 2-7.** *Nvd2* expression in a serially sectioned ovary of adult *D. magna*. Tissue-specific expression of *nvd2* was analyzed by sectioned *in situ* hybridization. Results with (*a*) the *nvd2* antisense probe, (*b*) the *nvd2* sense probe, or (*c*) the *vasa* antisense probe as a positive control. (*d*) HE-stained ovary. (*a*') and (*b*') represent magnified views of (*a*) and (*b*), respectively showing germ cells. Arrowheads indicate undifferentiated germ cells. Scale bars: 50μm.

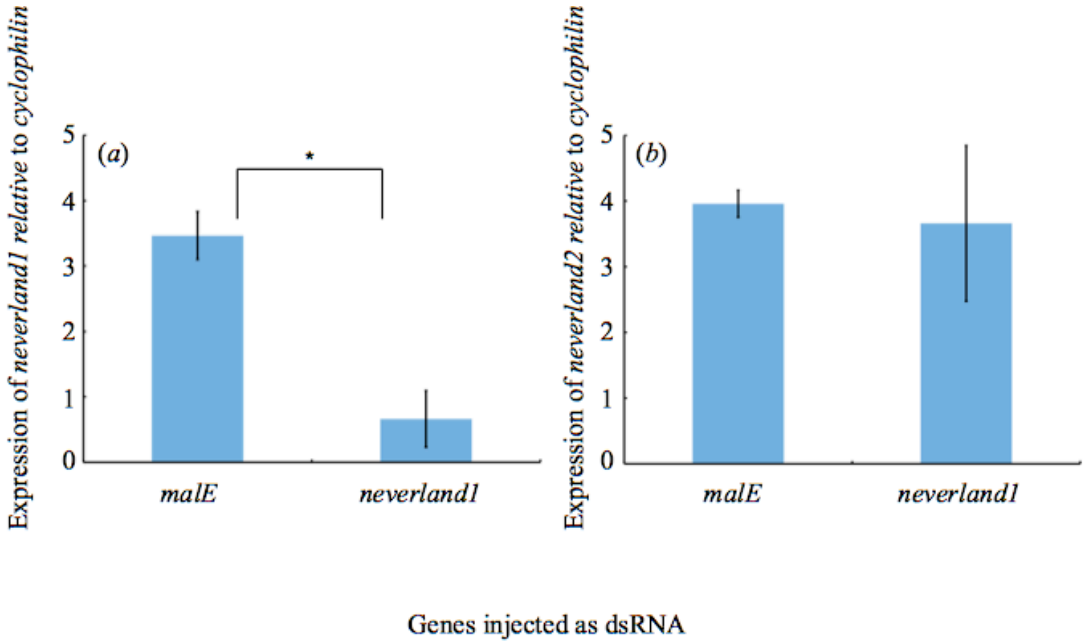



**Figure 2-8.** Relative expression of *nvd1* contrary to *nvd2* in *D. magna* embryos injected with *nvd1*- or *malE*-dsRNA. (*a*) *nvd1* and (*b*) *nvd2*. Ten embryos for each gene were used in triplicate. *D. magna* cyclophilin was used as the reference gene. Bars represent standard errors. * values differ significantly between *mal*E-dsRNA and *nvd1*-dsRNA ($P < 0.01$; Student's *t*-test).

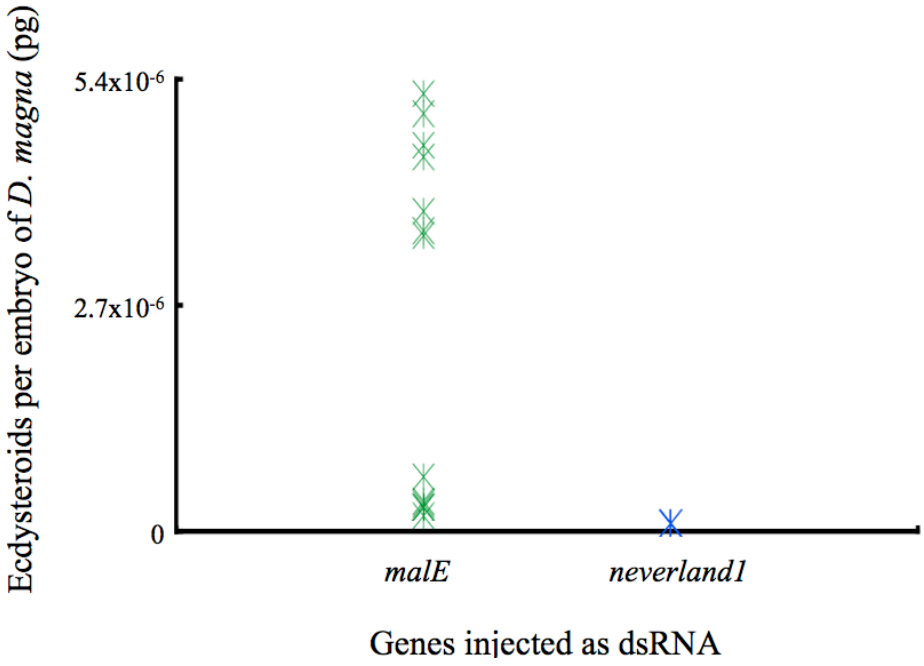



**Figure 2-9.** Ecdysteroids titre in *D. magna* embryos after RNAi knockdown of *nvd1* or *malE*. The titre was determined by ELISA. Each point represents the ecdysteroid titre per embryo. 28 and 30 embryos, each consisting of three broods, were used for *nvd1* and *malE*, respectively.

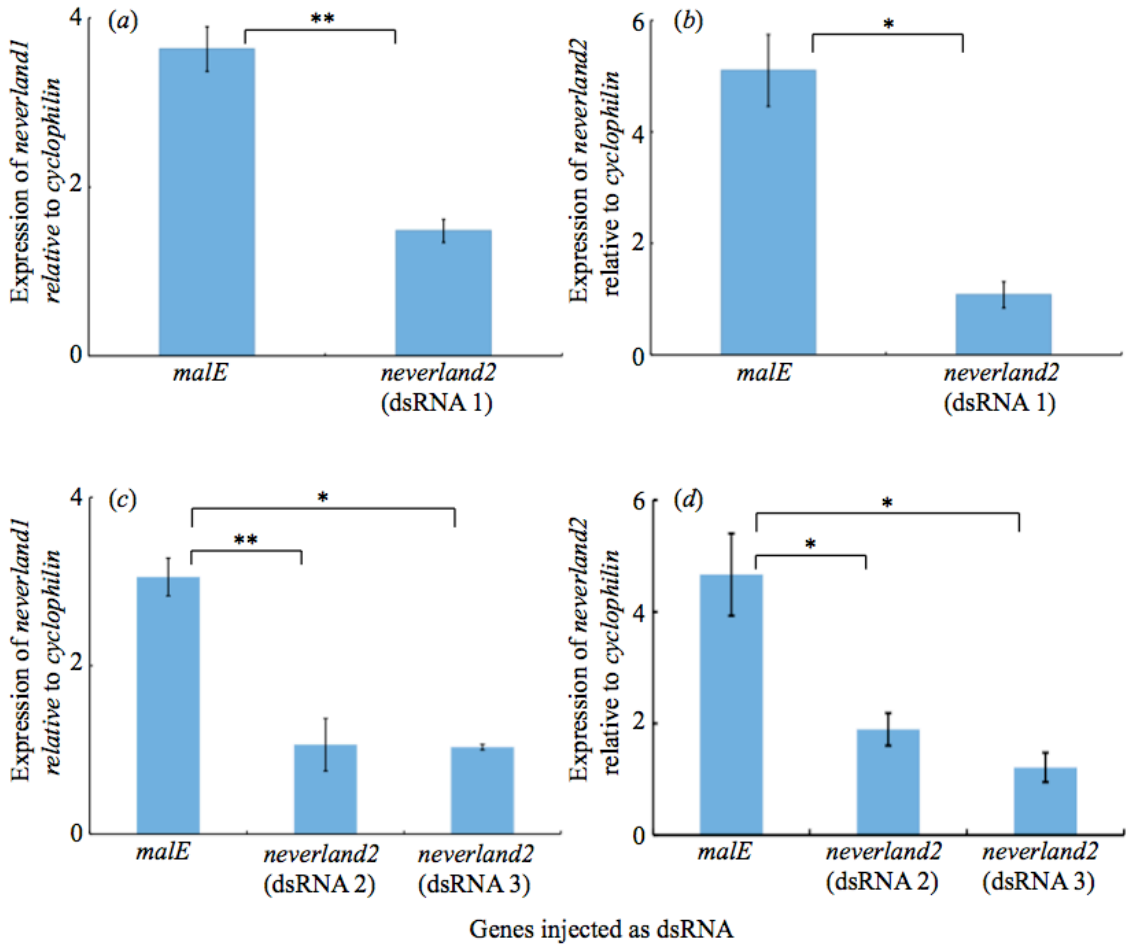



**Fig. 2-10.** Relative expression of *nvd1* or *nvd2* in *D. magna* embryos injected with *nvd2*- or *malE*-dsRNA. Relative expression of *nvd1* (*a*, *c*) and *nvd2* (*b*, *d*). Three different dsRNA were used for *nvd2*-dsRNA-injected embryos. dsRNA numbers in parentheses represent probe used for dsRNA generation as listed in Table 2-3. Ten embryos for each gene were used in triplicate. *D. magna cyclophilin* was used as the reference gene. Bars represent standard errors. Asterisks represent values differ significantly between *mal*E-dsRNA and *nvd1*-dsRNA (**$P < 0.01$, *$P < 0.05$; Student's *t*-test).

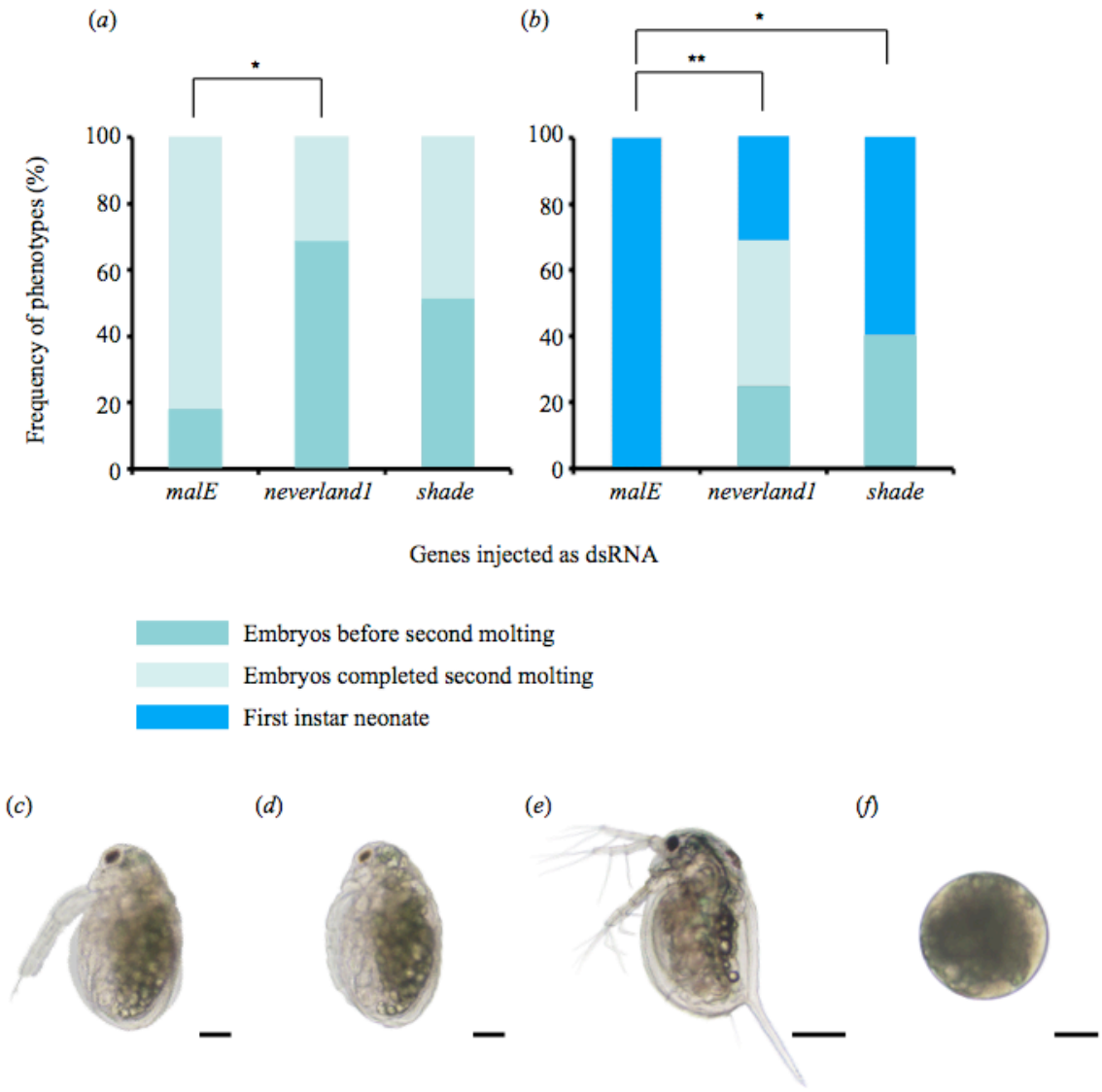



**Figure 2-11.** Defects in embryonic moultings of *D. magna* after *nvd1*-, *shd*-, or *cyp18a1*-dsRNA injection. Embryo development can be traced by embryonic moultings and eye development (Hiruta *et al*., 2014; Toyota *et al*., in press). *Daphnia* embryos shed their membranes twice during early and mid embryogenesis (stages 1 and 4; Fig. 2-1), and the final embryonic moulting that is to become first instar neonate occurs just after leaving the brood pouch. The second embryonic moulting uncovers second

antennae, and the final moulting uncovers a tail spine. (*a*) Embryos at 72 HAI. (*b*) Embryos at 96 HAI. *$P < 0.05$, **$P < 0.01$ (Steel test). Photos of the embryos at different developmental stages. (*c*) Control embryo after the second moulting. (*d*) *Nvd1*-dsRNA-injected embryo that failed the second moulting. (*e*) Control first instar neonate after the third moulting. (*f*) *Cyp18a1*-dsRNA-injected embryo. Scale bars: 100 μm.

## General Discussion

In this thesis, the roles of an arthropod steroid hormone, ecdysteroids, in moulting, the reproductive cycle and embryonic development in *D. magna* have been presented. Genetic and molecular analyses were performed to understand how ecdysteroids contribute to coordinated progression of the *D. magna* lifecycle, a parthenogenetic reproductive cycle linked to a moulting cycle, and embryogenesis.

In chapter I, I identified genes in the synthesis of ecdysteroids or in the inactivation pathway in *D. magna*. Cloning and phylogenetic analyses showed that nvd, shd as well as Cyp18a1 are evolutionary conserved in *D. magna*, suggesting that these genes appeared in arthropods before the radiation of insects. I demonstrated, using a 20E exposure experiment, that a reduction of the ecdysteroids titre was required for the progression of the reproductive cycle of adult *D. magna* and meiosis in the eggs. In addition, Cyp18a1, an enzyme known to inactivate 20E in insects, might regulate the decrease of ecdysteroids titre in *D. magna* as well. These reports are the first in a crustacean species to show an integrated study of molecular analyses and physiological events associated with the activities of ecdysteroids.

In chapter II, I demonstrated how ecdysteroids play crucial roles in developing embryos. ELISA analyses revealed that the ecdysteroids titre fluctuated during embryo development, with two apparent peaks. A decline of the ecdysteroids titre after these peaks signaled the timings of moults. These embryonic moults ensure normal embryogenesis, and a knockdown of *nvd1* resulted in a failure to activate the embryonic moults. Furthermore, *nvd1* likely regulates the ecdysteroids titre through

ecdysteroids synthesis. Therefore, it can be concluded that *de novo* ecdysteroids synthesis stimulated by *nvd1* is indispensable for embryonic moults. Tissue-specific gene expression analyses were also performed to show where ecdysteroids are synthesized in the embryo. The prominent expression of *nvd1* in the gut cells suggests that ecdysone is synthesized in the gut cells. In support of this, *nvd1* was also exclusively detected in the gut cells of the adult. Such analyses provide a concrete basis for understanding the endocrine system of *D. magna*.

For arthropods, moulting is essential for embryo and larval growth, and for adult growth as well in crustaceans. In this thesis, I showed that a controlled level of ecdysteroids is necessary for moults during embryogenesis and for the reproductive cycle in *D. magna*. Reports in insect species show how the ecdysteroids titre fluctuates with developmental events such as hatching, moulting, pupation and metamorphosis (Thummel 2001; Bond *et al.*, 2011). Consequently, ecdysteroids should be controlled in two dimensions: the correct timing and the right amount. The present studies showed that *D. magna* moulted after the ecdysteroids titre declined from the peak to the basal level. The embryos moult three times and have two peaks whereas the adults moult once in the moulting/reproductive cycle and have a single peak. A prolonged peak of the ecdysteroids titre after treatment with 20E resulted in a failure of not only moulting, but also oviposition in the adult. The inhibition of *de novo* ecdysteroids synthesis by *nvd1* knockdown produced moulting defects in the embryos. These results suggest that precise control of the quantity and timing of the ecdysteroids titre is required for normal reproduction and embryo development. The peak of the ecdysteroids titre in adult *D.*

*magna* may represent a high demand of ecdysteroids for cuticle and yolk depositions as these are synchronous events in daphnids. In a prawn *Macrobrachium rosenbergii*, which also exhibits synchronized moulting and reproduction, the ovarian ecdysteroids titre increased by 20-fold in the premoult stage (Subramoniam, 2000). Similarly, in *D. magna* embryos, the two peaks are likely responsible for the deposition of new embryonic membranes. The fluctuating ecdysteroids titre indicates that ecdysteroid inactivation also appears to be critical. Cyp18a1 inactivates 20E through hydroxylation into 20-hydroxyecdysonoic acid (20Eoic) in insects (Guittarda *et al.*, 2011). The time-course expression of *Cyp18a1* in adult *D. magna* counteracted the fluctuation of the ecdysteroids titre and might regulate the inactivation of 20E to lower the ecdysteroids titre in the adult *D. magna* as well. Furthermore, the embryos injected with *Cyp18a1*-dsRNA failed the first moult and died at stage 1 (Hiruta *et al.*, 2014 for stages). During this period, expression of the *Cyp18a1* gene tended to increase before *de novo* synthesis likely occurred. This suggests that the embryos need to break down maternal ecdysteroids before they start *de novo* synthesis with the formation of the synthetic organ. The presence of 20Eoic was observed during embryogenesis of *M. rosenbergii*, suggesting that maternal ecdysteroids are inactivated in the developing embryos (Wilder *et al.*, 1995).

In the adult *D. magna* treated with exogenous 20E in the middle of the reproductive cycle, which is a day before moulting and oviposition occur, meiosis was inhibited in the eggs inside the ovary. Meiotic reinitiation from Prophase I arrest by ecdysteroids has been reported in the spider crab *Acanthonyx lunulatus* and the prawn

*Palaemon serratus* (Chaix and De Reggi, 1982; Lanot and Clédon; 1989; Subramoniam, 2000). Although these reinitiations occurred under a high titre of ecdysteroids, the present study indicates that 20E treatment inhibited meiotic reinitiation in *D. magna*. Parthenogenetic *D. magna* exhibits abortive meiosis where the first meiosis ceases at anaphase, and then the second one starts over (Hiruta *et al.*, 2010). The decline of the ecdysteroids titre at the end of the reproductive cycle might regulate the reinitiation of the second meiosis in *D. magna*, assuring viable eggs. Moreover, expression analysis of *nvd2* implies that germ cells may synthesize ecdysteroids in adult *D. magna*. These cells might possibly be the source of ecdysteroids in the eggs. Insects synthesize ecdysteroids in follicle cells (Gilbert, 2012), which also produce yolk proteins to which ecdysteroids can tightly bind (Bownes *et al.*, 1988). When the yolk proteins are stored in oocytes, ecdysteroids are also carried with them. After the oocytes develop into embryos, the yolk proteins are broken down and the ecdysteroids serve as developmental regulators (Bownes *et al.*, 1988). The present results of *nvd2* expression in germ cells and the presence of maternal ecdysteroids in early embryos suggest that ecdysteroids may have been synthesized and allocated to the ovary of the adult *D. magna*. To further test this hypothesis, strategies for the functional analysis of specific genes are needed. Gene knockout techniques, a clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) system and transcription activator-like effector nuclease (TALEN), are now available for *D. magna* (Nakanishi *et al.*, 2014; Naitou *et al.*, 2015). Knocking out *nvd1* and/or *nvd2* would show whether the enzymes of these genes regulate ecdysteroids synthesis in the adult, and if having two paralogous

*nvd* genes play a unique role in *D. magna*. To further clarify whether the gut cells synthesize ecdysteroids, other genes of the ecdysteroid biosynthesis pathway identified in insects should be identified and analyzed for expression in the gut, or an organ culture method could be used by analyzing the level of ecdysteroids after incubation with a precursor.

In conclusion, *D. magna* possess evolutionary conserved CYP450 genes and two *nvd* paralogues. In the adult *D. magna*, results of 20E treatment suggest that the ecdysteroids titre was controlled for following moults and oviposition. The ecdysteroids titre seemed to be regulated by Cyp18a1. In the embryos, the ecdysteroids titre also fluctuated and initiated the embryonic moults. The successful moults were regulated by *nvd1* and/or *shd* suggesting the conserved roles of these genes among arthropods. The specific expression of *nvd1* in the gut cells of the embryo and adult suggest that the gut cells synthesize ecdysteroids in *D. magna*. The other paralogue, *nvd2*, showed germ cell-specific expression implying ecdysteroidogenesis or a different role unique to the adult. The ecdysteroids act on the eggs inside the ovary, oviposited eggs, developing embryos and adult. Clearly, the precise regulation of ecdysteroids is a central aspect of the successful parthenogenetic life cycle in *D. magna*.

**References**


Ables ET, Drummond-Barbosa D. (2010). The steroid hormone ecdysone functions with intrinsic chromatin remodeling factors to control female germline stem cells in *Drosophila*. *Cell Stem Cell*, **7**: 581-592.

Agawa Y, Sarhan M, Kageyama Y, Akagi K, Takai M, Hashiyama K, Wada T, Handa H, Iwamatsu A, Hirose S, Ueda H. (2007). *Drosophila* Blimp-1 is a transient transcriptional repressor that controls timing of the ecdysone-induced developmental pathway. *Mol Cell Biol*, **27**: 8739-8747.

Akagi K, Ueda H. (2011). Regulatory mechanisms of ecdysone-inducible *Blimp-1* encoding a transcriptional repressor that is important for the prepupal development in *Drosophila*. *Devel Growth Differ*, **53**: 697-703.

Andersen CL, Jensen JL, Ørntoft TF. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res*, **64**: 5245-5250.

Asada M, Kato Y, Matsuura T, Watanabe H. (2014). Early embryonic expression of a putative ecdysteroid-phosphate phosphatase in the water flea, *Daphnia magna* (Cladocera: Daphniidae). *J Insect Sci*, **14**: 181.

Asazuma H, Nagata S, Nagasawa H. (2009). Inhibitory effect of molt-inhibiting hormone on phantom expression in the Y-organ of the kuruma prawn, *Marsupenaeus japonicus*. *Arch Insect Biochem Physiol*, **70**: 220-233.

Bliss DE. (1983). Ecological aspects of reproduction. In *The Biology of Crustacea.* Edited by Vernberg FJ, Vernberg WB. New York: Academic Press, Inc.: 179-191.

Block DS, Bejarano AC, Chandler GT. (2003). Ecdysteroid concentrations through various life-stages of the meiobenthic harpacticoid copepod, *Amphiascus tenuiremis* and the benthic estuarine amphipod, *Leptocheirus plumulosus*. *Gen Comp Endocrinol*, **132**: 151-160.

Bond ND, Nelliot A, Bernardo MK, Ayerh MA, Gorski KA, Hoshizaki DK, Woodard CT. (2011). ßFTZ-F1 and Matrix metalloproteinase 2 are required for fat-body remodeling in *Drosophila*. *Dev Biol*, **360**: 286-296.

Bownes M, Shirras A, Blair M, Collins J, Coulson A. (1988). Evidence that insect embryogenesis is regulated by ecdysteroids released from yolk proteins. *PNAS*, **85**: 1554-1557.

Brown MR, Sieglaff DH, Rees HH. (2009). Gonadal ecdysteroidogenesis in Arthropoda: occurrence and regulation. *Annu Rev Entomol*, **54**: 105-125.

Cabrera AR, Shirk PD, Evans JD, Hung K, Sims J, Alborn H, Teal PEA. (2015). Three Halloween genes from the Varroa mite, *Varroa destructor* (Anderson & Trueman) and their expression during reproduction. *Insect Mol Biol*, **24**: 277-292.

Carney GE, Bender M. (2000). The Drosophila *ecdysone receptor* (*EcR*) gene is required maternally for normal oogenesis. *Genetics*, **154**: 1203-1211.

Chaix JC, De Reggi M. (1982). Ecdysteroid levels during ovarian development and embryogenesis in the spider crab *Acanthonyx lunulatus*. *Gen Comp Endocrinol*, **47**: 7-14.

Charmantier G, Charmantier-Daures M. (1998). Endocrine and neuroendocrine regulations in embryos and larvae of crustaceans. *Invert Reprod Dev*, **33**: 273-287.

Chávez VM, Marqués G, Delbecque JP, Kobayashi K, Hollingsworth M, Burr J, Natzle JE, O'Connor MB. (2000). The *Drosophila disembodied* gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development*, **127**: 4115-4126.

Colbourne JK, Pfrender ME, Gilbert D, Thomas WK, Tucker A, Oakley TH, Tokishita S, Aerts A, Arnold GJ, Basu MK, Bauer DJ, Cáceres CE, Carmel L, Casola C, Choi J, Detter JC, Dong Q, Dusheyko S, Eads BD, Fröhlich T, Geiler-Samerotte KA, Gerlach D, Hatcher P, Jogdeo S, Krijgsveld J, Kriventseva EV, Kültz D, Laforsch C, Lindquist E, Lopez J, *et al*.. (2011). The ecoresponsive genome of *Daphnia pulex*. *Science*, **331**: 555-561.

Elendt BP, Bias WR. (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.

Gilbert LI. (2004). Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*. *Mol Cell Endocrinol*, **215**: 1-10.

Gilbert LI. (2012). *Insect Endocrinology*, 1st edition. London: Academic Press.

Gilbert LI, Bollenbacher WE, Granger NA. (1980). Insect endocrinology: regulation of endocrine glands, hormone titer, and hormone metabolism. *Ann Rev Physiol*, **42**: 493-510.

Guittarda E, Blais C, Maria A, Parvy J, Pasricha S, Lumb C, Lafont R, Daborn PJ, Dauphin-Villemant C. (2011). CYP18A1, a key enzyme of *Drosophila* steroid hormone inactivation, is essential for metamorphosis. *Dev Biol*, **349**: 35-45.

Gunamalai V, Kirubagaran R, Subramoniam T. (2004). Hormonal coordination of molting and female reproduction by ecdysteroids in the mole crab *Emerita asiatica* (Milne Edwards). *Gen Comp Endocrinol*, **138**: 128-138.

Hannas BR, Wang YH, Thomson S, Kwon G, Li H, LeBlanc JA. (2011). Regulation and dysregulation of vitellogenin mRNA accumulation in daphnids (*Daphnia magna*). *Aquat Toxicol*, **101**: 351-357.

Hiruta C, Nishida C, Tochinai S. (2010). Abortive meiosis in the oogenesis of parthenogenetic *Daphnia pulex*. *Chromosome Res*, **18**: 833-840.

Hiruta C, Toyota K, Miyakawa H, Sumiya E, Iguchi T. (2014). Sexual reproduction is a key element in the life history strategy of water fleas, *Daphnia magna* and *Daphnia pulex*: casting a spotlight on male induction and its morphology. In *Daphnia*: *biology and mathematics perspectives*. Edited by El-Doma M. New York: Nova Science Publishers, Inc: 261-278.

Hopkins PM. (2009). Crustacean ecdysteroids and their receptors. In *Ecdysone: structures and functions.* Edited by Smagghe G. Netherlands: Springer: 73-97.

Huberman A. (2000). A shrimp endocrinology. A review. *Aquaculture*, **191**: 191-208.

Iga M, Smagghe G. (2010). Identification and expression profile of Halloween genes involved in ecdysteroid biosynthesis in *Spodoptera littoralis*. *Peptides*, **31**: 456-467.

Iga M, Blais C, Smagghe G. (2013). Study on ecdysteroid levels and gene expression of enzymes related to ecdysteroid biosynthesis in the larval testis of *Spodoptera littoralis*. *Arch Insect Biochem Physiol*, **82**: 14-28.

Kast-Hutcheson K, Rider CV, LeBlanc GA. (2001). The fungicide propiconazole interferes with embryonic development of the crustacean *Daphnia magna*. *Environ Toxicol Chem*, **20**: 502-509.

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**: 83-194.

Kato Y, Kobayashi K, Watanabe H, Iguchi T. (2011). Environmental sex determination in the branchiopod crustacean *Daphnia magna*: deep conservation of a *Doublesex* gene in the sex-determining pathway. *PLoS Genetics*, **7**: e1001345.

Kato Y, Shiga Y, Kobayashi K, Tokishita SI, 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.

Kenny NJ, Quah S, Holland PW, Tobe SS, Hui JH. (2013). How are comparative genomics and the study of microRNAs changing our views on arthropod endocrinology and adaptations to the environment? *Gen Comp Endocrinol*, **188**: 16-22.

König A, Yatsenko AS, Weiss M, Shcherbata HR. (2011). Ecdysteroids affect *Drosophila* ovarian stem cell niche formation and early germline differentiation. *EMBO J*, **30**: 1549-1562.

Kozlova T, Thummel CS. (2003). Essential roles for ecdysone signaling during *Drosophila* mid-embryonic development. *Science*, **301**: 1911-1914.

Lachaise F, Le Roux A, Hubert M, Lafont R. (1993). The molting gland of crustaceans: localization, activity, and endocrine control (a review). *J Crustacean Biol*, **13**: 198-234.

Lafont R. (2000). The endocrinology of invertebrates. *Ecotoxicology*, **9**: 42–57.

Lafont R, Mathieu M. (2007). Steroids in aquatic invertebrates. *Ecotoxicology*, **16**: 109-130.

Lanot R, Clédon P. (1989). Ecdysteroids and meiotic reinitiation in *Palaemon serratus* (Crustacean Decapoda natantia) and in *Locusta migratoria* (Insecta orthoptera). A comparative study. *Invert Reprod Dev*, **16**: 169-175.

LeBlanc GA. (2007). Crustacean endocrine toxicology: a review. *Ecotoxicology*, **16**: 61-81.

Lee HG, Seong CS, Kim YC, Davis RL, Han KA. (2003). Octopamine receptor OAMB is required for ovulation in *Drosophila melanogaster*. *Dev Biol*, **264**: 179- 190.

Li Z, Ge X, Ling L, Zeng B, Xu J, Aslam AFM, You L, Palli SR, Huang Y, Tan A. (2014). *CYP18A1* regulates tissue-specific steroid hormone inactivation in *Bombyx mori*. *Insect Biochem Mol Biol*, **54**: 33-41.

Marchal E, Badisco L, Verlinden H, Vandersmissen T, Soest SV, Wielendaele PV, Broeck JV. (2011). Role of the Halloween genes, *Spook* and *Phantom* in ecdysteroidogenesis in the desert locust, *Schistocerca gregaria*. *J Insect Physiol*, **57**: 1240-1248.

Martin-Creuzburg D, Westerlund SA, Hoffmann KH. (2007). Ecdysteroid levels in *Daphnia magna* during a molt cycle: Determination by radioimmunoassay (RIA) and liquid chromatography-mass spectrometry (LC-MS). *Gen Comp Endocrinol*, **151**: 66-71.

Mu X, LeBlanc GA. (2002). Environmental antiecdysteroids alter embryo development in the crustacean *Daphnia magna*. *J Exp Zool*, **292**: 287-292.

Mu X, LeBlanc GA. (2004). Synergistic interaction of endocrine-disrupting chemicals: Model development using an ecdysone receptor antagonist and a hormone synthesis inhibitor. *Environ Toxicol Chem*, **23**: 1085-1091.

Nagaraju GPC. (2010). Reproductive regulators in decapod crustaceans: an overview. *J Exp Biol*, **214**: 3-16.

Naitou A, Kato Y, Nakanishi T, Matsuura T, Watanabe H. (2015). Heterodimeric TALENs induce targeted heritable mutations in the crustacean *Daphnia magna*. *Biology Open*, **4**: 364-369.

Nakanishi T, Kato Y, Matsuura T, Watanabe H. (2014). CRISPR/Cas-mediated targeted mutagenesis in *Daphnia magna*. *PLoS ONE*, **9**: e98363

Namiki T, Niwa R, Sakudoh T, Shirai KI, Takeuchi H, Kataoka H. (2005). Cytochrome P450 CYP307A1/Spook: a regulator for ecdysone synthesis in insects. *Biochem Bioph Res Co*, **337**: 367-374.

Nijhout HF. (1994). *Insect Hormones*. Princeton: Princeton University Press.

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-moulting glossy*/*shroud* encodes a short-chain dehydrogenase/reductase that functions in the 'Black Box' of the ecdysteroid biosynthesis pathway. *Development*, **137**: 1991-1999.

Ogihara MH, Hikiba J, Suzuki Y, Taylor D, Kataoka H. (2015). Ovarian ecdysteroidogenesis in both immature and mature stages of an acari, *Ornithodoros moubata*. *PLoS ONE*, **10**: e0124953.2015.

Okumura T. (2004). Perspectives on hormonal manipulation of shrimp reproduction. *Japan agricultural research quarterly*, **38**: 49-54.

Ono H, Rewitz KF, Shinoda T, Itoyama K, Petryk A, Rybczynski R, Jarcho M, Warren JT, Marqués G, Shimell MJ, Gilbert LI, O'Connor MB. (2006). *Spook* and *Spookier* code for stage-specific components of the ecdysone biosynthetic pathway in Diptera. *Dev Biol*, **298**: 555-570.

Pankotai T, Popescu C, Martin D, Grau B, Zsindely N, Bodai L, Tora L, Ferrús A, Boros I. (2010). Genes of the ecdysone biosynthesis pathway are regulated by the dATAC histone acetyltransferase complex in *Drosophila*. *Mol Cell Biol*, **30**: 4254-4266.

Payne AH, Hales DB. (2004). Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr Rev*, **25**: 947-970.

Pechenik JA. (2005). *Biology of the Invertebrates*. 5th edition. McGraw-Hill Higher Education.

Petryk A, Warren JT, Marques G, Jarco MP, Gilbert LI, Kahler J, Parvy J, Li Y, Dauphin-Villemant C, O'Connor, MB. (2003). Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *PNAS*, **100**: 3773-13778.

Rewitz KF, Gilbert LI. (2008). *Daphnia* Halloween genes that encode cytochrome P450s mediating the synthesis of the arthropod molting hormone: Evolutionary implications. *BMC Evol Biol*, **8**: 60.

Rewitz KF, Rybczynski R, Warren JT, Gilbert LI. (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**: 188-99.

Revelli A, Massobrio M, Tesarik J. (1998). Nongenomic actions of steroid hormones in reproductive tissues 1. *Endocr Rev*, **19**: 3-17.

Rodríguez-Valentín R, López-González I, Jorquera R, Labarca P, Zurita M, Reynaud E. (2006). Oviduct contraction in *Drosophila* is modulated by a neural network that is both, octopaminergic and glutamatergic. *J Cell Physiol*, **209**: 183-198.

Riddiford LM, Truman JW. (1993). Hormone receptors and the regulation of insect metamorphosis. *AM Zool*, **33**: 340-347.

Sagawa K, Yamagata Shiga Y. (2005). Exploring embryonic germ line development in the water flea, *Daphnia magna*, by zinc-finger-containing VASA as a marker. *Gene Expr Patterns*, **5**: 669-678.

Shimada-Niwa Y, Niwa R. (2014). Serotonergic neurons respond to nutrients and regulate the timing of steroid hormone biosynthesis in *Drosophila*. *Nat Commun*, 5.

Shyama SK. (1987). Studies on moulting and reproduction in the prawn *Macrobrachium idella* (hellar). *Mahasagar-Bulletin Natl Inst Oceanog*, **20**: 15-21.

Sin YW, Kenny NJ, Qu Z, Chan KW, Chan KW, Cheong SP et al.. (2015). Identification of putative ecdysteroid and juvenile hormone pathway genes in the shrimp *Neocaridina denticulata*. *Gen Comp Endocrinol*, **214**: 167-176.

Soumoff C, Skinner DM. (1983). Ecdysteroid titers during the molt cycle of the blue crab resemble those of other Crustacea. *Biol Bull*, **165**: 321-329.

Spaziani E, DeSantis K, O'Rourke BD, Wang WL, Weld JD. (1997). The clearance in vivo and metabolism of ecdysone and 3- dehydroxyecdysone in tissues of the crab *Cancer antennarius*. *J Exp Zool*, **278**: 609–619.

Subramoniam T. (2000). Crustacean ecdysteroids in reproduction and embryogenesis. *Comp Biochem Phys C*, **125**: 135-156.

Swetha CH, Sainath SB, Reddy PR, Reddy PS. (2011). Reproductive endocrinology of female crustaceans: perspective and prospective. *J Marine Sci Res Devel*, **S3**: 2.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*, **28**: 2731-2739.

Techa S, Alvarez JV, Chung JS. (2014). Changes in ecdysteroid levels and expression patterns of ecdysteroid-responsive factors and neuropeptide hormones during the embryogenesis of the blue crab, *Callinectes sapidus*. *Gen Comp Endocrinol*, **214**: 157-166.

Thummel CS. (2001). Molecular mechanisms of developmental timing in *C. elegans* and *Drosophila*. *Dev Cell,* **1**: 453-465.

Tom M, Manfrin C, Giulianini PG, Pallavicini A. (2013). Crustacean oxi-reductases protein sequences derived from a functional genomic project potentially involved in ecdysteroid hormones metabolism-A starting point for function examination. *Gen Comp Endocrinol*, **194**: 71-80.

Toyota K, Hiruta C, Ogino Y, Miyagawa S, Okumura T, Onishi Y, Tatarazako N, Iguchi T. Comparative developmental staging of the female and male water fleas *Daphnia pulex* and *Daphnia magna* during embryogenesis. In press.

Truman JW, Riddiford LM. (2002). Endocrine insights into the evolution of metamorphosis in insects. *Annu Rev Entomol*, **47**: 467-500.

Vandesompele J, de Preter K, Pattyn F, Poppe B, van Roy N, de Paepe A, Speleman F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*, **3**: research0034.

Warren JT, Petryk A, Marqués G, Parvy JP, Shinoda T, Itoyama K, Kobayashi J, Jarcho M, Li Y, O'Connor MB, Dauphin-Villemant C, Gilbert LI. (2004). Phantom encodes the 25-hydroxylase of *Drosophila melanogaster* and *Bombyx mori*: a P450 enzyme critical in ecdysone biosynthesis. *Insect Biochem Mol Biol*, **34**: 991-1010.

Wilder MN, Fusetani N, Aida K. (1995). The presence of 20-hydroxyecdysonoic acid and ecdysonoic acid in eggs of the giant freshwater prawn *Macrobrachium rosenbergii*. *Fisheries Sci*, **61**: 101-106.

Yamada R, Yamahama Y, Sonobe H. (2005). Release of ecdysteroid-phosphates from egg yolk granules and their dephosphorylation during early embryonic development in silkworm, *Bombyx mori*. *Zool Sci*, **22**: 187-198.

Yamanaka N, Honda N, Osato N, Niwa R, Mizoguchi A, Kataoka H. (2007). Differential regulation of ecdysteroidogenic P450 gene expression in the silk moth, *Bombyx mori*. *Biosci Biotechnol Biochem*, **71:** 2808-2814.

Yanai H. (2004). *Statcel, the Useful Add-in Software Forms on Excel*. 2nd edn. Tokyo: OMS.

Yanai H. (2011). *Statcel, the Useful Add-in Software Forms on Excel*. 3rd edn. Tokyo: OMS.

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**: 2565-2575.

Zhu XX, Oliver JH, Dotson EM. (1991). Epidermis as the source of ecdysone in an argasid tick. *PNAS*, **88**: 3744-3747.

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