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2

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4 signaling in the freshwater microcrustacean *Daphnia*

5

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25 **Abstract**

26

27 The two essential insect hormones, ecdysteroids and juvenile hormones, are  
28 possessed not only by insects, but also widely by arthropods, and regulate  
29 various developmental and physiological processes. In contrast to the  
30 abundant information about molecular endocrine mechanisms in insects, the  
31 knowledge of non-insect arthropod endocrinology is still limited. In this  
32 review, we summarize recent reports about the molecular basis of these two  
33 major insect hormones in the freshwater microcrustacean *Daphnia*, a  
34 keystone taxon in limnetic ecology and a bioindicator in environmental  
35 studies. Comprehensive comparisons of endocrine signaling pathways  
36 between insects and daphnids may shed light on the regulatory mechanisms  
37 of various biological phenomena and, moreover, evolutionary processes of  
38 arthropod species.

39

40

41 **Highlights:**

- 42 - Ecdysteroids and juvenile hormones synergistically regulate various  
43 phenomena in Arthropoda.
- 44 - Endocrine systems in non-insect arthropods are poorly understood.
- 45 - Endocrine systems differ in complexity among taxa, although general  
46 outline is conserved.

47

48 **Keywords:**

49 ecdysteroid, juvenile hormone, *Daphnia pulex*, *Daphnia magna*, hormonal

50 crosstalk

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68 1. Introduction

69

70 The freshwater microcrustacean or water flea, *Daphnia* (Branchiopoda:  
71 Cladocera), is a keystone taxon in limnetic ecology and is widespread around  
72 the world [1-3]. Generally, daphnids reproduce by a process referred to as  
73 cyclical parthenogenesis [4]. By employing clonal reproduction during spring  
74 and summer when conditions are warm and food is abundant, female  
75 daphnids lay eggs in the brood chamber situated in their dorsal region. The  
76 offspring are reared in the brood chamber until hatching and released into  
77 the water column immediately before molting. Under favorable conditions,  
78 adult daphnids have a reciprocally synchronized cycle of molting and  
79 reproduction throughout their lives. On the other hand, when environmental  
80 conditions become less favorable (e.g., a decrease in day length, temperature  
81 and food), they produce male offspring in a process referred to as  
82 environmental sex determination and employ sexual reproduction which  
83 increases genetic variation through recombination and the likelihood of  
84 survival under harsh environmental conditions [5]. Thus, these small  
85 crustaceans have very complicated life cycles and can alter their survival  
86 strategies drastically in response to the surrounding environment [6]. It has  
87 recently been demonstrated that these survival mechanisms are regulated by  
88 a variety of physiological and endocrine systems. For example, endocrine  
89 factors such as ecdysteroids and juvenile hormones (JHs), which are among

90 the most widely studied and well known insect hormones, are also considered  
91 to play important roles in daphnids [7, 8]. For instance, topical application  
92 of ecdysteroids or their agonists on *Daphnia magna* causes a delay and/or  
93 defect in molting, and consequently death [9-12]. Similarly, exposure to JHs  
94 or their analogs decreases the number of offspring in many daphnia species,  
95 and the sex ratio of offspring becomes male-biased, which is a typical  
96 example of the disruption of sex determination by chemicals [13-18]. In  
97 addition to the occurrence of clonal reproduction in daphnids, which is useful  
98 for producing large numbers of genetically identical organisms, their high  
99 sensitivity to insect hormones prompted the Organization for Economic  
100 Cooperation and Development (OECD) to establish a biological assay system  
101 for screening the hormonal activities of chemicals using daphnids as a model  
102 organism [19-20]. However, in contrast to insects, a lack of basic information  
103 about daphnid (and other non-insect arthropods) endocrine systems is an  
104 important limitation. Indeed, this has resulted in the OECD test guidelines  
105 being applied in the absence of developmental, physiological, and/or  
106 molecular information of the mode of action (MoA) and adverse effects of  
107 chemicals.

108 Under these circumstances, as the draft genome sequence of *D.*  
109 *pulex* was determined [21] and gene-function analytical methods, such as  
110 RNAi, TALEN and CRISPR/Cas9, were established in succession [22-27],  
111 striking findings and advances in the technical and genetic aspects of daphnid



112 genetics have emerged. In this review, we summarize recent reports on the  
113 molecular basis of biosynthesis; reception and signaling cascades of two  
114 major arthropod hormones, ecdysteroids and JHs, in *Daphnia*; and how these  
115 findings have increased the potential suitability of *Daphnia* as a model for  
116 environmental studies. Knowledge is not only important for removing the  
117 “black boxes” in biological assay systems, but it also helps to shed light on  
118 the regulatory mechanisms of various biological phenomena and to clarify the  
119 complicated life cycle observed in daphnids.

120

121 2. Insect hormones – biosynthesis and signaling

122

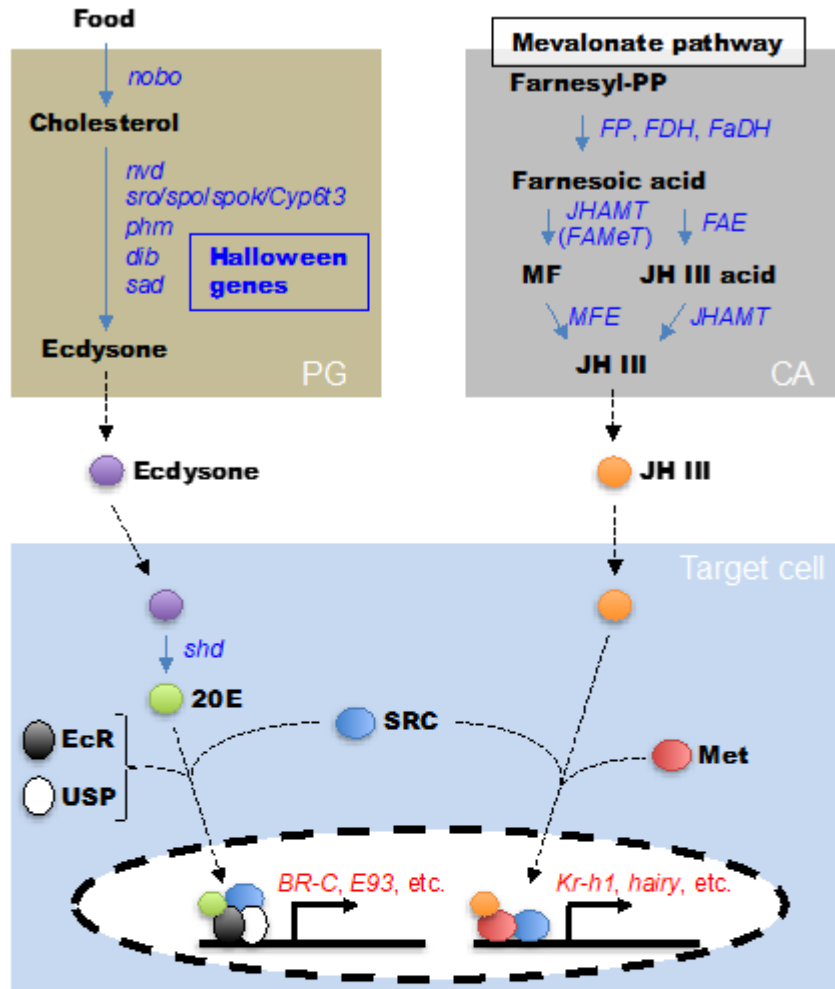
123 Before discussing the endocrinology of daphnids, it is important to understand  
124 the ecdysteroid- and JH-signaling pathways in insects. Ecdysteroids and JHs  
125 work in a synergistic manner to regulate the development and  
126 metamorphosis of most (perhaps all) insects studied to date [28, 29].  
127 Numerous studies on insect endocrinology have been undertaken using  
128 traditional model insects, such as the fruit fly (*Drosophila melanogaster*), red  
129 flour beetle (*Tribolium castaneum*) and moths (*Bombyx mori* and *Manduca*  
130 *sexta*) [7, 8, 29-33]. Although the endocrine systems and their underlying  
131 molecular mechanisms can differ markedly between divergent species, the  
132 endocrinology of more evolutionary related species such as insects are  
133 considered to be conserved.

134 Schematic pathways for both ecdysteroid and JH biosynthesis and  
135 their receptors can be seen in **Fig. 1A**. In insects, ecdysteroids are  
136 synthesized in a specific organ called the prothoracic gland (PG). Cholesterol  
137 contained in food is converted into the end product, ecdysone, by the action  
138 of a series of particular enzymes, and then ecdysone is released into  
139 hemolymph [33-43]. At the target peripheral cells, ecdysone is converted  
140 into 20-hydroxyecdysone (20E), an active form of an ecdysteroid, by *shade*  
141 (*shd*) [44]. Among these ecdysteroidogenic genes, *spook* (*spo*), *phantom*  
142 (*phm*), *disembodied* (*dib*), *shadow* (*sad*) and *shd* are referred to as

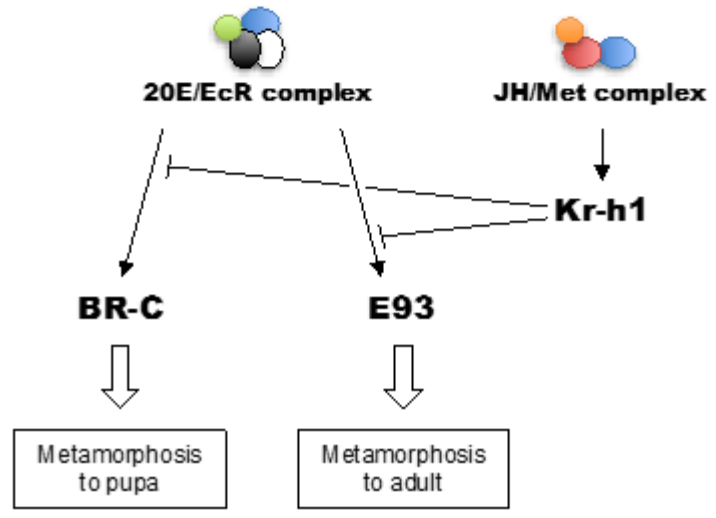
143 "Halloween genes", as knockout of these genes showed the naked cuticle  
144 phenotype in flies as a result of ecdysteroid synthesis deficiency **[45-46]**.  
145 Another major insect hormone, JH, is synthesized in the corpora allata (CA).  
146 JH biosynthesis starts with the mevalonate pathway, which is common in  
147 eukaryotes (and some prokaryotes) **[47]**. Farnesyl pyrophosphate (FPP), an  
148 end product of the mevalonate pathway, is then converted by a series of  
149 enzymes into active forms of JH, such as juvenile hormone III (JH III), which  
150 is most commonly found in insects. These active JHs are released into the  
151 hemolymph before exerting a variety of functions in the target peripheral cells  
152 **[48-55]**. This synthetic pathway differs slightly between Lepidoptera and  
153 other insect taxa. In the former, JHs are produced using juvenile hormone  
154 acid (JH acid) as an intermediate whereas the latter JHs are produced using  
155 methyl farnesoate (MF) as an intermediate. Although the cholesterol  
156 biosynthetic (mevalonate-squalene) pathway is considered highly conserved  
157 in many species, insects are found to be lacking the squalene synthase and  
158 genes involved in the squalene pathway, which means that insects are unable  
159 to synthesize cholesterol and therefore dependent on direct dietary sources  
160 **[56]**.  
161  
162



A



B



164

165

166 **Fig. 1.** A schematic view of the ecdysteroid and juvenile hormone (JH)  
167 signaling pathways in insects. (A) The pathways from biosynthesis in the  
168 specific organs to reception at peripheral target cells. (B) Hormonal crosstalk  
169 between ecdysteroid and JHs during regulation of metamorphosis.  
170 Abbreviations: PG, prothoracic gland; CA, corpora allata; Farnesyl-PP,  
171 farnesyl pyrophosphate; MF, methyl farnesoate; JH III, juvenile hormone III;  
172 20E, 20-hydroxyecdysone; EcR, ecdysone receptor; USP, ultraspiracle; Met,  
173 Methoprene-tolerant; SRC, steroid receptor coactivator; nobo, noppera-bo;  
174 nvd, neverland; sro, shroud; spo, spook; spok, spookier; phm, phantom; dib,  
175 disembodied; sad, shadow; shd, shade; FP, farnesyl phosphatase; FDH,  
176 farnesol dehydrogenase; FaDH, farnesal dehydrogenase; FAMeT, farnesoic  
177 acid *O*-methyltransferase; MFE, methyl farnesoate epoxidase; FAE, farnesoic  
178 acid epoxidase; JHAMT, juvenile hormone acid *O*-methyltransferase; BR-C,  
179 Broad-Complex; Kr-h1, Krüppel homolog 1.

180

181

182 In target peripheral cells, ecdysteroids and JHs bind to specific  
183 receptors (Fig. 1). The active ecdysteroid, 20E, transformed from ecdysone  
184 by *shd*, binds to a member of the nuclear receptor superfamily, ecdysone  
185 receptor (EcR) [57]. When EcR binds 20E, it forms a heterodimer with

186 another nuclear receptor superfamily protein, ultraspiracle (USP) **[58, 59]**.  
187 Following association with cofactors, such as steroid receptor coactivator  
188 (SRC), the EcR complex then binds to a specific sequence on the genome  
189 (ecdysone response element: EcRE) and acts as a transcription factor **[58-**  
190 **60]**. Unlike the ecdysteroids, the JH receptor was only discovered relatively  
191 recently. Juvenile hormone III and other JHs are bound by the basic helix-  
192 loop-helix-Per-Arnt-Sim (bHLH-PAS) family protein, Methoprene-tolerant  
193 (Met) **[61-63]**. After heterodimerization with SRC, which is another bHLH-  
194 PAS protein and a component of the EcR complex, in response to binding to  
195 JHs, the Met complex binds to the JH response element (JHRE) and acts as a  
196 transcription factor, similar to EcR **[64-66]**.

197 Ecdysone receptor complex transduces signaling to downstream  
198 factors by regulating the transcription of quite various genes **[67, 68]**. The  
199 transcription factors, *Broad-Complex (BR-C)* and *E93*, which are pupal- and  
200 adult-specifier genes, respectively, are representative targets that are  
201 directly regulated by EcR **[69-81]**. In contrast to EcR, the downstream  
202 signaling of the JH receptor is less understood, primarily because the Met/SRC  
203 complex has only recently been discovered. However, the transcription of the  
204 zinc finger transcription factor, *Krüppel homolog 1 (Kr-h1)*, which is directly  
205 regulated by the Met/SRC complex in response to binding to JHs, has been  
206 extensively studied **[82-85]**. Recent studies using *B. mori* demonstrated that  
207 *Kr-h1* prevents both larval-pupal and pupal-adult metamorphosis by binding

208 directly to the promoter region of *BR-C* and *E93*, repressing their expression,  
209 and in so doing, forming the molecular basis of a mutual inhibitory action  
210 (“status quo”) of JHs [**86, 87**] (**Fig. 1B**).  
211



212 3. Ecdysteroids in *Daphnia*

213

214 Like all members of the Ecdysozoa and Arthropoda, daphnids grow through  
215 molting. However, as mentioned previously, there are two major differences  
216 between daphnids and insects: 1) in contrast to insects which do not molt at  
217 the adult stage, daphnids continue to molt and increase in size over the  
218 course of their lives; 2) molting and reproduction are reciprocally  
219 synchronized in adult daphnids. Consequently, ecdysteroids are considered  
220 to be necessary for survival throughout their lifetime **[88, 89]**.

221

222

223 3.1. Ecdysteroid biosynthesis

224

225 Although the Y-organ in decapods is known to be responsible for ecdysteroid  
226 synthesis **[90, 91]**, little is known about where ecdysteroids are synthesized  
227 and released in other crustaceans, such as daphnids. Recent effort to identify  
228 key genes in ecdysteroidogenesis of *D. magna* by cloning led to the discovery  
229 of the genes *neverland* (*nvd*), which converts cholesterol into 7-dehydro  
230 cholesterol at the first step of the synthesis, and *shd*, which converts  
231 ecdysone to an active form of the ecdysteroid 20E in target cells **[12]**. In  
232 addition, *D. magna* has two *nvd* homologs (*nvd1* and *nvd2*), and these were  
233 expected to be paralogs acquired specifically in daphnids. At both embryonic

234 and adult stages, *nvd1* was strongly expressed in gut epithelial cells [12, 92].  
 235 Embryonic knockdown of *nvd1* by RNAi showed a striking reduction in the  
 236 internal ecdysteroid titer and a delay of development by inhibition of  
 237 embryonic molt [92]. A similar developmental delay was also observed in  
 238 *shd* RNAi embryos [92]. These results strongly suggest that both *nvd1* and  
 239 *shd* are involved in the ecdysteroidogenesis in *D. magna*, and that *nvd1*-  
 240 expressing gut epithelial cells may be responsible for ecdysteroid synthesis.  
 241 On the other hand, *nvd2* was expressed in germ cells of the ovary where *nvd1*  
 242 was absent [92]. Further studies are necessary to determine whether *nvd2*  
 243 is also involved in the ecdysteroidogenesis. In addition, several other genes  
 244 involved in ecdysteroid synthesis can be found in the daphnid genome (e.g.,  
 245 *phm* and *dib*), and expression and functional analyses of these genes could  
 246 be instrumental for elucidating where ecdysteroids are synthesized [93]  
 247 (**Table 1**).

248  
 249  
 250  
 251

Table 1. Accession numbers of *Daphnia* homologs of ecdysteroid and juvenile hormone signaling-related genes

Gene name	<i>Daphnia magna</i>	<i>Daphnia pulex</i>	Reference
Ecdysteroid biosynthesis			
nobo	?	?	

nvd	BAQ02388, BAQ02389	<i>EFX86361,</i> <i>EFX88304,</i> <i>EFX60729</i>	<b>[12, 92]</b>
sro	?	?	
spo/spok	<i>KZS09664</i>	<i>EFX88041</i>	
Cyp6t3	?	?	
phm	<i>KZS17835</i>	<i>EFX85499</i>	
dib	<i>KZS10280</i>	<i>EFX63066</i>	
sad	<i>KZS16986</i>	<i>EFX70970</i>	
shd	BAF35770	<i>EFX77008</i>	
Ecdysteroid receptor			
EcR	BAF49030	<i>EFX79409</i>	<b>[94]</b>
USP	BAF49028	<i>EFX88423</i>	<b>[94]</b>
Ecdysteroid-responsive gene			
E74	<i>KZS09932</i>	<i>EFX89297</i>	
E75	ABP48738	ADB79814	<b>[98, 99]</b>
HR3	ACY56690	ACY56691	<b>[98, 99]</b>
HR4	<i>KZS12701</i>	<i>EFX67867</i>	
betaFTZ-F1	BAU20372	<i>EFX77612</i>	
DDC	<i>KZS20105</i>	<i>EFX90074</i>	
BR-C	<i>KZS07124</i>	<i>EFX90040</i>	
E93	?	?	
Juvenile hormone biosynthesis			
FP	?	?	
FDH	<i>KZS13936</i>	<i>EFX87469</i>	
FaDH	<i>KZS05270</i>	<i>EFX71031</i>	
FAMeT/JHAMT	?	BAH86593	<b>[104]</b>
FAE/MFE	lost?	lost?	<b>[109]</b>
Juvenile hormone receptor			
Met	BAM83855	BAM83853	<b>[110]</b>
SRC	BAM83854	BAM83856	<b>[110]</b>
Juvenile hormone-responsive gene			

Kr-h1	<i>KZS09055</i>	LC270151	Miyakawa et al. unpublished
hairy	?	?	

---

Accession numbers in italic indicate computational gene models based on the genome sequences.

252

253

254 3.2. Ecdysteroid receptors

255

256 Homologs of both EcR and USP have been isolated from *D. magna*, and both  
257 have the characteristic structure of the nuclear receptor superfamily **[94]**.

258 The two-hybrid luciferase assay using mammalian cultured cells transfected  
259 with *D. magna EcR*, *USP* and *Drosophila Taiman* (the same as SRC)

260 demonstrated that *Daphnia EcR* and *USP* form a heterodimer specifically in  
261 response to ecdysteroids such as ecdysone and 20E **[94, 95]**. Homologs of

262 these ecdysteroid receptors are also found in the *D. pulex* genome **[96]**  
263 (**Table 1**), suggesting that daphnids bind to ecdysteroids by employing a

264 similar (or the same) receptor system to that of insects.

265

266

267 3.3. Downstream signaling of ecdysteroids

268

269 The heterodimer of EcR and USP acts as a transcription factor and activates  
270 downstream genes **[58-60]**. When *D. magna* embryos were injected with a

271 reporter plasmid containing insect EcRE fused to GFP and exposed to 20E,  
272 the fluorescent intensity increased dose-dependently with 20E exposure [97].  
273 These results suggest that ecdysteroid receptors have the same molecular  
274 function in insects and daphnids, although the direct targets of the receptors  
275 are not yet known in daphnids.

276           Among various ecdysteroid-responsive genes in insects [67, 68],  
277 *hormone receptor 3 (HR3)* and *E75* have already been isolated in both *D.*  
278 *pulex* and *D. magna* [98, 99]. In *D. magna*, *HR3* expression was upregulated  
279 in response to 20E, whereas *E75* expression remained unchanged [98].  
280 Although ecdysteroid-responsive genes such as *E74*, *HR4* and *βFTZ-F1* have  
281 been identified in the daphnid genome (Table 1), their involvement in the  
282 ecdysteroid signaling pathway in daphnids remains unknown. In order to  
283 clarify how daphnids regulate and synchronize their elaborate molting and  
284 reproduction cycles, and to perform a detailed functional analysis of each  
285 candidate gene, a comprehensive analysis of all of the molecular pathways  
286 (i.e., from hormone biosynthesis to the expression of biological phenomena)  
287 is considered necessary [100].

288

289 4. Juvenile hormones in *Daphnia*

290

291 In addition to ecdysteroids, JHs play very important roles in the reproduction  
292 and development of daphnids. In particular, their roles in sex determination  
293 is both unique and essential for their life history **[13-18, 101]**.

294

295

296 4.1. Juvenile hormone biosynthesis

297

298 The current progress status regarding studies on the JH-synthesizing organ  
299 in daphnids is similar to that for studies on ecdysteroids. Although the  
300 mandibular organ (MO) is known to be the site of synthesis in decapods **[102,**  
301 **103]**, homologous organs have not been found in daphnids and related taxa.

302 However, a considerable number of homologous genes involved in JH  
303 biosynthesis in the insect CA, have been identified in the genomes of daphnids,  
304 implying the existence of the same molecular synthesis pathway (**Table 1**).

305 Among these genes, especially *juvenile hormone acid O-methyltransferase*  
306 (*JHAMT*)/*farnesoic acid O-methyltransferase* (*FAMeT*) has the ability to  
307 convert farnesoic acid (FA) into MF, indicating that daphnids also appear to  
308 produce MF **[104]**. Decapods use MF as an active form of JH, without  
309 converting it to JH III **[105-107]**. Although unconfirmed, daphnids are  
310 considered to use MF as an active JH ligand, mainly because the male-

311 induction activity of MF is higher than it is for JH III [14, 108] and also  
312 because of the loss of *methyl farnesoate epoxidase* (*MFE*), a member of *cyp15*,  
313 that converts MF to JH III in insects [109]. However, given that several  
314 similar sequences could be found in a genome-wide survey for *MFE* genes  
315 (**Table 1**), further studies are awaited.

316

317

#### 318 4.2. Juvenile hormone receptors

319

320 We recently cloned and functionally analyzed the JH receptor *in daphnids* for  
321 the first time in crustaceans. Similarly to insects, the JH receptor was a JH-  
322 dependent heterodimer complex comprising Met and SRC, which responded  
323 to various JH analogs in addition to MF and JH III [110]. Ligand sensitivity  
324 profiles against these various JHs corresponded well with the male-induction  
325 activity of each chemical, suggesting that the JH receptor, Met, is a molecular  
326 target when sex determination is disrupted by exposure to exogenous JHs.  
327 Moreover, we found that two of the eight amino acids comprising the JH-  
328 binding pocket in Met differed between insects and daphnids, and that a single  
329 amino acid mutation of an insect-type residue into *Daphnia* Met caused a  
330 marked increase in response to JH III, just as in insect JH receptors [108,  
331 110]. It is possible that a change in the ligand selectivity of the JH receptor,  
332 Met, is related to the difference observed in the innate JH ligands between

333 insects and crustaceans. If so, then understanding the molecular evolution of  
334 JH receptors is one of the important topics in the evolution of the JH signaling  
335 pathway in Arthropoda.

336

337

338 4.3. Downstream signaling of juvenile hormones

339

340 Activation of the JH signaling pathway causes a lot of physiological changes  
341 in daphnids. One of the well-studied examples is environment-dependent  
342 male production [13, 14]. We recently used RNA-seq analysis to screen  
343 genes downstream of the JH signaling pathway that are activated when males  
344 are produced. The results showed that *hemoglobin*, *serine protease*, and  
345 numerous other genes (many of which are functionally unknown because of  
346 a lack of homology to known genes) were upregulated in male-producing  
347 mothers [111]. Both *hemoglobin* and *serine protease* genes have also been  
348 reported to be upregulated after topical application of JHs to *D. magna* at the  
349 juvenile stage (before starting reproduction), suggesting that these genes are  
350 typical downstream factors of the JH signaling pathway in daphnids [112].  
351 It is considered likely that these factors are involved in male determination  
352 in the ovary of parental daphnid, and after that, which fate-determined eggs  
353 spontaneously express the male specific gene, *doublesex*, in daphnids [113,  
354 114].



355           On the other hand, some *Daphnia* species are known to produce  
356 defensive morphs or structures in response to the presence of predators (i.e.,  
357 so called "inducible defense" mechanisms) [115-117], and JHs are also  
358 involved in regulation of the morphogenesis in these defensive morphs [118,  
359 119]. We previously proposed a signaling cascade model describing how, in  
360 individuals that have received chemical stimuli released by predators, the JH  
361 signaling pathway is activated and morphogenesis occurs in response to the  
362 involvement of several morphogenetic and cell-proliferating factors [96].

363           In this way, the molecular underpinnings of a variety of biological  
364 phenomena exhibited by daphnids, as well as the role of the components of  
365 the JH signaling pathway in these processes, are becoming increasingly clear.  
366 However, aspects related to the proximate signaling of JH, such as the direct  
367 target genes of the JH receptor, Met, are still unknown. In insects, one such  
368 direct target is *Kr-h1*, which is a well-studied and very important downstream  
369 component of JHs [82-87]. Although daphnids possess a *Kr-h1* ortholog, the  
370 findings of our recent study using a reporter assay to clarify the function of  
371 *Daphnia Kr-h1* strongly suggested that this ortholog is not involved in JH  
372 signaling (Miyakawa et al. unpublished). Consequently, identifying primary JH  
373 responsive genes is considered crucial for obtaining a comprehensive  
374 understanding of the various molecular characteristics of the JH signaling  
375 pathway in daphnids.

376

377 5. Summary and future directions

378

379 Ecdysteroids, JHs and their signaling pathways, are extremely important  
380 endocrine systems that elaborately regulate a vast array of physiological  
381 phenomena, not only in insects, but also in daphnids. While daphnid  
382 endocrinology is still less understood than that for the model insect species  
383 (e.g., *D. melanogaster* and *B. mori*), the overall nature of the hormonal  
384 pathways in daphnids has been gradually elucidated using a variety of  
385 molecular approaches since the draft genome of *D. pulex* was published.  
386 Findings published to date have shown that the endocrine systems of insects  
387 and daphnids, while generally conserved and similar in terms of their overall  
388 outline, differ in certain respects. It is possible that these slight differences in  
389 the molecular signaling pathways could serve as a basis for the novel traits  
390 or life histories acquired by specific taxa; for example, environmental sex  
391 determination and inducible defense in daphnids.

392           In many cases, ecdysteroids and JHs do not act independently of  
393 each other, with final developmental processes often being a sequel to  
394 complicated crosstalk between the two signaling pathways **[7, 8, 29]**. Recent  
395 studies have demonstrated that a cofactor of the EcR complex, SRC, is the  
396 heterodimeric partner of the JH receptor, Met **[64-66]**, and that Kr-h1, a  
397 direct target of the JH/Met/SRC complex, acts as a repressor of *BR-C* and *E93*  
398 expression by ecdysteroid signaling **[86, 87]**; the molecular basis of the

399 hormonal crosstalk has thus been partly elucidated. We have already  
400 successfully established several variations of reporter assay systems that can  
401 be used to analyze gene regulatory relationships within both ecdysteroid and  
402 JH signaling pathways in daphnids **[94, 108, 110]**. By comparing not only  
403 expression patterns and functions of individual genes, but also connections  
404 of each component of signaling pathway between insects and daphnids using  
405 these systems, we expect to understand how the alterations of endocrine  
406 systems have occurred in an ancestor of insects after diverging from  
407 branchiopod crustaceans.

408           To date, study of arthropod endocrinology has mainly relied on the  
409 specific taxa, holometabolous insects and decapod crustaceans. To  
410 understand the evolutionary processes of arthropod diversity from the aspect  
411 of physiological regulation, further studies should be performed using various  
412 arthropods broadly (i.e., hemimetabolous insects, non-decapod crustaceans,  
413 myriapods, arachnids, etc.).

414

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416

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424

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