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Juvenile hormone synthesis and signaling disruption triggering male offspring induction and population decline in cladocerans (water flea) : Review and adverse outcome pathway development

Kenji Toyota[†,‡,§,∞,*], Haruna Watanabe[∥,∞,*], Masashi Hirano[⊥], Ryoko Abe[∥], Hitoshi Miyakawa[#], You Song[▽], Tomomi Sato[○], Shinichi Miyagawa[§], Knut Erik Tollefsen[▽,◆], Hiroshi Yamamoto[∥], Norihisa Tatarazako[¶], Taisen Iguchi[○,*]

[†]Marine Biological Station, Sado Center for Ecological Sustainability, Niigata University, 87 Tassha, Sado, Niigata 952-2135, Japan.

[‡]Department of Biological Sciences, Faculty of Science, Kanagawa University, 2946 Tsuchiya, Hiratsuka, Kanagawa 259-1293, Japan.

[§]Department of Biological Science and Technology, Faculty of Advanced Engineering, Tokyo University of Science, 6-3-1 Niijuku, Katsushika-ku, Tokyo 125-8585, Japan.

[∥]Health and Environmental Risk Division, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan.

[⊥]Department of Bioscience, School of Agriculture, Tokai University, Kumamoto City, Kumamoto 862-8652, Japan

[#]Center for Bioscience Research and Education, Utsunomiya University, 350 Mine-machi, Utsunomiya, Tochigi 321-8505, Japan.

[▽]Norwegian Institute for Water Research (NIVA), Section of Ecotoxicology and Risk Assessment, Gaustadalléen, Oslo, Norway

23 ○Graduate School of Nanobioscience, Yokohama City University, 22-2 Seto, Kanazawa-ku,
24 Yokohama, Kanagawa 236-0027, Japan
25 ◆Norwegian University of Life Sciences (NMBU), Faculty of Environmental Science and
26 Technology, Department of Environmental Sciences (IMV), Ås, Norway
27 ¶Department of Science and Technology for Biological Resources and Environment, Graduate
28 School of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8566, Japan.
29 ∞These authors contributed equally to this work.
30
31 ***Corresponding authors:**
32 Kenji Toyota
33 Marine Biological Station, Sado Center for Ecological Sustainability, Niigata University, 87
34 Tassha, Sado, Niigata 952-2135, Japan.
35 Email: toyotak@cc.niigata-u.ac.jp
36
37 Haruna Watanabe
38 Health and Environmental Risk Division, National Institute for Environmental Studies, 16-2
39 Onogawa, Tsukuba, Ibaraki 305-8506, Japan.
40 Email: watanabe.haruna@nies.go.jp
41
42 Taisen Iguchi
43 Graduate School of Nanobioscience, Yokohama City University, Yokohama 236-0027, Japan
44 Email: taiseni@hotmail.co.jp

45 **Abstract**

46 Juvenile hormone (JH) are a family of multifunctional hormones regulating larval development,
47 molting, metamorphosis, reproduction, and phenotypic plasticity in arthropods. Based on its
48 importance in arthropod life histories, many insect growth regulators (IGRs) mimicking JH have
49 been designed to control harmful insects in agriculture and aquaculture. These JH analogs (JHAs)
50 may also pose hazards to nontarget species by causing unexpected endocrine-disrupting (ED)
51 effects such as molting and metamorphosis defects, larval lethality, and disruption of the sexual
52 identity. This critical review summarizes the current knowledge of the JH-mediated effects in the
53 freshwater cladoceran crustaceans such as *Daphnia* species on JHA-triggered endocrine
54 disruptive outputs to establish a systematic understanding of JHA effects. Based on the current
55 knowledge, adverse outcome pathways (AOPs) addressing the JHA-mediated ED effects in
56 cladoceran leading to male offspring production and subsequent population decline were
57 developed. The weight of evidence (WoE) of AOPs was assessed according to established
58 guidelines. The review and AOP development aim to present the current scientific understanding
59 of the JH pathway and provide a robust reference for the development of tiered testing strategies
60 and new risk assessment approaches for JHAs in future ecotoxicological research and regulatory
61 processes.

62

63 **Keywords**

64 AOP, *Daphnia*, endocrine disruption, juvenile hormone, environmental sex determination

65

66

67 ## 1. Introduction

68 Juvenile hormones (JHs) are a family of acyclic sesquiterpenoids that regulate a range
69 of physiological processes in insects. These substances regulate metamorphosis, ovarian
70 development, reproductive behavior, and various types of phenotypic plasticity, such as caste
71 determination in social insects and weapon traits development in beetles during their life cycles.[1,
72 2] In addition to insects, the JH system is believed to be conserved in the majority of arthropods,
73 including Malacostraca crustaceans (e.g., crabs and shrimps) and Cladocera (water flea)
74 (Branchiopoda, Figure S1).[3] Methyl farnesoate (MF), which is structurally related to the insect
75 JHs and identified in various Malacostracan species, is generally accepted as a significant innate
76 JH in Malacostracan crustaceans.[4-7] Moreover, MF likely acts as a natural JH molecule in
77 *Daphnia* species.[8]

78 A wide range of artificial JH analogs (JHAs) such as pyriproxyfen, fenoxycarb,
79 methoprene, and diofenolan, have been developed as insect growth regulators (IGRs) to control
80 harmful insects.[9] Since JHAs have been used worldwide in agriculture, aquaculture and
81 household applications (e.g., insect pest control on pet animals), environmental contamination by
82 JHAs and their adverse effects on nontarget arthropod species, such as molting and
83 metamorphosis defects, larval lethality, and disruption of sexual determination and reproduction,
84 are of high concern.[10–14] Water fleas reproduce by parthenogenesis and usually produce only
85 female offspring under appropriate environmental conditions. However, both JHs (e.g., JH III and
86 MF) and JHAs (methoprene, pyriproxyfen, fenoxycarb, hydroprene, kinoprene, epofenonane, and

87 diofenolan) induce dose-dependent increases in male offspring and decreases in reproduction
88 among many Cladocera genera (i.e., *Daphnia, Ceriodaphnia*, *Moina*, *Bosmina*, and *Oxyurella*).[15–
89 24] For example, pyriproxyfen was detected in the Júcar river, Spain, ranging from 83 to 100 ng/L[25]
90 and has been reported to reduce fecundity and induce male offspring in *Daphnia magna* at a
91 concentration 30 and 100 ng/L, respectively[16], suggesting potential JH disrupting risks of
92 pyriproxyfen to *Daphnia* in the Júcar river (maximum concentration 100 ng/L/LOEC for
93 reproduction 30 ng/L= 3.3).
94 Therefore, male offspring induction in *Daphnia* species has been applied as a new
95 endpoint for screening chemicals with JH activity in the OECD test guideline, *Daphnia magna*
96 Reproduction Test Annex 7 (OECD TG211)[26], and was cited as OECD non-mammalian test for
97 evaluating endocrine disrupting chemicals (EDCs).[27] However, this assay has not been applied
98 yet in regulations of EDCs such as the Biocidal Products Regulation (BPR, Regulation (EU)
99 528/2012) or the Plant Protection Products Regulation (PPPR, EC 1107/2009) in the EU due to
100 the scarce knowledge on the endocrinology for non-target invertebrate.[28] In particular, it should
101 also be considered that daphnids can also produce male offspring in response to natural
102 environmental factors (e.g., short photoperiod, temperature fluctuation, decreased food density,
103 and overpopulation).[27,29,30] Watanabe et al.[31] has demonstrated that the *D. magna* NIES strain does
104 not induce male offspring in response to these environmental changes, whereas the *D. magna*
105 LRV13.2 and LRV13.5-1 strains and the *D. pulex* WTN6 strain produce male offspring in
106 response to photoperiod differences.[8,30,32] It is urgently required to mechanistically understand
107 how chemicals and environmental stimuli perturb the JH signaling pathway in order to
108 discriminate effects due to chemical exposure or environmental stimuli. It is also crucial to

109 assemble existing knowledge and assess the weight of evidence (WoE) to better understand the
110 research status in this field and evaluate the suitability of test methods for detecting arthropod
111 JHAs.

112 Conceptual frameworks, such as adverse outcome pathways (AOPs), are increasingly
113 used to organize the existing knowledge and describe a sequential chain of causally linked events
114 at various levels of biological organization that lead to an adverse effect of regulatory concern.[33,34]
115 The OECD has published a guidance document for development, assessment and application of
116 AOPs for chemical safety evaluation[34]. An AOP describes a sequence of events commencing with
117 initial interaction of a stressor/chemical with a biomolecule within an organism (i.e., molecular
118 initiating event, MIE), which can progress through a dependent series of intermediate key events
119 (KEs) and culminate in an adverse outcome (AO) considered relevant to risk assessment or
120 regulatory decision-making. KEs are connected to one another via scientifically-based linkages
121 defined as KE relationships (KERs). The AOPs can better align information generated by *in vitro*
122 and *in silico* assays to conventional *in vivo* ecotoxicity testing results.

123 To assess the degree of confidence supporting an AOP, the Evolved Bradford–Hill
124 weight of evidence (WoE) considerations are recommended by the OECD.[34] Using these
125 harmonized WoE assessment criteria, one can efficiently capture the current knowledge status
126 and identify future research needs. At present (October 2021), as many as 375 AOPs have been
127 submitted to an AOP repository database, the AOPwiki (www.aopwiki.org), however, AOPs for
128 invertebrate species are currently limited. In the critical review, we summarize the current
129 knowledge on the JH system in Cladocera, propose and evaluate novel AOPs describing JH

130 synthesis and signaling disruption leading to male offspring induction, reproduction decrease, and
131 population decline.
132
133 ## 2. Methyl Farnesoate System in *Daphnia*
134 ### 2.1. Synthesis of MF
135 2.1.1. Genes responsible for MF biosynthesis pathway
136 In insect species, the concentration of innate JHs in the hemolymph, which regulate the
137 development, growth, and reproduction, is precisely controlled by various physiological
138 processes such as synthesis, degradation, sequestration, and secretion. The synthesis step has
139 generally been believed to be the most important for downstream effects of JHs.[35,36] The
140 endogenous MF is nearly identical in structure to insect JH, JH III, only differing in the presence
141 of an epoxide ring at the terminal end, indicating that the biosynthetic pathway of MF is very
142 similar to that of JHs (Figure S2). The biosynthetic pathway of JH consists of two main steps: the
143 mevalonate pathway where acetyl-CoA is converted to farnesyl diphosphate (FPP), and the JH-
144 specific pathway responsible for the conversion of FPP to MF and then to JH III.[37] The genes
145 involved in the mevalonate pathway and the putative farnesoic acid *O*-methylfransferase (FAMeT)
146 have been identified in *D. pulex*.[38] In addition to FAMeT, the *JH acid O-methylfransferase*
147 *(JHAMT)* gene was found.[39] Previous studies using recombinant JHAMT proteins of several
148 insect species such as *Bombyx mori*, *Drosophila melanogaster*, and *Aedes aegypti* demonstrated
149 that JHAMT has a vigorous methylation activity that not only converts JH III acid into JH III but
150 also converts FA into MF. However, a recent study has revealed that JHAMT of *D. pulex* only

151 converts FA to MF. In contrast, it does not generate JH III from JH III acid[8], suggesting that
152 JHAMT may catalyze the final step of MF synthesis in crustaceans, and MF is likely an innate
153 JH molecule in daphnids. The MF is finally converted to JH III by CYP15A1 in insects, except
154 for the Lepidoptera (Figure S2).[40] However, CYP15A1 orthologs have never been found in other
155 Arthropoda except for insects[8,41], indicating that the *CYP15A1* gene acquisition might have been
156 an important event enabling JH biosynthesis in insects.[40]
157
158 2.1.2. Regulation of MF synthesis
159 To date, there has been a large body of studies that have characterized and contributed to our
160 understanding of the regulatory mechanisms of JH synthesis (i.e., neuropeptides, and
161 neurotransmitters in insect species[35,37] and in Malacostracan decapod crustaceans[42]). However,
162 these factors are mostly unknown in daphnids due to a lack of baseline knowledge regarding
163 endogenous MF titers. Current instrumental analytical technologies, such as liquid or gas
164 chromatography-mass spectrometry (LC- or GC-MS), enable thedetection and quantification of
165 MFs and JHs in extracts and hemolymphs of several insect species.[43-45] In contrast, no studies
166 have yet successfully measured MF in daphnids due to the possibility that endogenous MF levels
167 are lower than those typically found in insect species, and thus, the analytical characterization of
168 MF titers in these small crustaceans is limited. The description of periodical fluctuation of
169 endogenous MF titers during the life cycle of *Daphnia* remains unknown. Until recently, such a
170 condition was a significant obstacle to characterizing the regulatory mechanism of MF synthesis.
171 A new approach taking advantage of the direct link between MF and male sex determination in
172 daphnids has recently overcome the obstacles of accurately controlling offspring sex using a

173 WTN6 strain of *D. pulex* through the alteration of culture photoperiod.[8,31] Female offspring is
174 predictability produced if a mother is reared under long-day conditions (14 h-light, 10 h-dark),
175 whereas male offspring production occurs when mothers are raised under short-day conditions
176 (10 h-light, 14 h-dark). This photoperiod-dependent sex determination system can be an excellent
177 tool for understanding the molecular basis for environmental sex determination and the role of
178 the MF system in daphnids.

179 Quantitative real-time PCR of *JHAMT* during the parthenogenetic reproductive cycle
180 of *Daphinia* demonstrated that the MF synthesis process is activated just before the male sex-
181 determining period during oocyte maturation *in ovo*.[8] Moreover, transcriptome and chemical
182 treatment assays with agonists and antagonists revealed that ionotropic glutamate receptors
183 (iGluRs), especially *N*-methyl-D-aspartic acid (NMDA) receptor subtypes, are an essential
184 element for male offspring induction as they act as an upstream regulator of MF signaling.[46]
185 Moreover, metabolome analysis supports the proposal that glutamate (known as one of the natural
186 ligands of NMDA receptor) accumulates dramatically in daphnid mothers at a sex-determining
187 period when reared under male-producing (short-day) conditions.[47] Similar to daphnids,
188 glutamate and its signaling pathway via NMDA receptor are also known to mediate the JH
189 synthesis in several insect species.[48,49] The NMDA signaling promotes the synthesis of JHs by
190 activating the transcription of *JHAMT* via the decapentaplegic-mediated transforming growth
191 factor β (TFG-β) signaling pathway in *D. melanogaster*.[48] Although NMDA receptor involvement
192 remains unknown, Ishimaru and colleagues[49] revealed that TFG-β signaling regulates the
193 synthesis of JH by upregulating *JHAMT* transcription in the cricket, *Gryllus bimaculatus*,

194 suggesting that the signaling pathway of TFG-β may be widely conserved to control the synthesis
195 of JH in insect species.
196 In addition to the NMDA pathway, involvement of the protein kinase C (PKC)
197 signaling pathway was identified in the male sex determination of *D. pulex*. This pathway acts as
198 an upstream regulator of MF signaling by cotreatment assay of inhibitor and MF with several
199 concentrations.[52] However, several pioneering kinds of research, using some insects and
200 crustaceans, revealed that PKC acts as a crucial element in the downstream of JH signaling
201 pathway. For instance, it associates with the membrane receptor of JH to mediate the JH signaling
202 in male accessory glands of *D. melanogaster* and ovarian follicle cells of *Locusta migratoria*.[5354]
203 In addition, its activation is induced by MF treatment to stimulate larval metamorphosis in the
204 barnacle *Balanus amphitrite*.[55] Although a previous study has demonstrated that PKC rapidly
205 recruits NMDA receptors to the surface of *Xenopus* oocyte cells and increases their channel-
206 opening rates[56], molecular relationships between NMDA and PKC signaling for MF synthesis in
207 daphnids remains unclear. More detailed analyses with various inhibitors and activators in
208 daphnids will inevitably help understand the diversified PKC signaling involved in the JH
209 pathway among arthropods.
210 Pantothenate (vitamin B5) was found to be accumulated in daphnid mothers at the
211 onset of the sex-determining period reared under male-producing conditions in the WTN6 strain
212 of *D. pulex*.[47] Pantothenate is a water-soluble vitamin ubiquitously present in living organisms,
213 also known as a precursor of co-enzyme A (CoA). Pantothenate administration to mother
214 individuals demonstrated that the male induction ratio is significantly increased, suggesting that
215 it might act as a male-sex determinant substance. The pantothenate' molecular mechanism during

216 the MF or JH synthesis pathway activation is mostly unknown, even in model insect species,

217 including *D. melanogaster*. One possible hypothesis is that it is supplied as a primary source for

218 the MF synthesis pathway because both MF and JHs are sesquiterpenoids that are initially

219 synthesized from acetyl-CoA through the mevalonate pathway. Previous studies using the

220 budding yeast *Saccharomyces cerevisiae* and engineered *Escherichia coli* demonstrated that

221 pantothenate is the rate-limiting precursor of CoA synthesis. When this substance is administrated

222 in the culture medium, it could increase the farnesoid yields.[57,58] More detailed analyses will be

223 necessary to elucidate the pantothenate involvement in MF biosynthesis in the insects and

224 daphnids.

225 Taking together the knowledge above based on the photoperiod-dependent sex

226 determination system of the *D. pulex* WTN6 strain, we propose the following possible hypothesis

227 underlying mechanisms of MF synthesis. When a mother detects the short-day cues: 1)

228 pantothenate accumulation occurs to activate the mevalonate and MF synthesis pathways; 2) PKC

229 pathway recruits the NMDA receptor and increases its channel-opening rates; and 3) the NMDA

230 signaling pathway mediates the MF synthesis via the activation of *JHAMT* expression (Table 1).

231 To reinforce those signaling pathways driving male sex-determination, it was recently found that

232 in some strains of *D. magna*, the proportion of female or male offspring varies depending on

233 photoperiod. The LRV13.2 strain produces female or male offspring, respectively, under the long-

234 day or short-day conditions (similar to *D. pulex* WTN6 strain), whereas the LRV13.5-1 strain

235 produces female or male offspring under the short-day or long-day conditions.[32] Furthermore,

236 both *D. magna* strains could alter female or male offspring production in response to photoperiod

237 differences as well as the *D. pulex* WTN6 strain by lifelong rearing experiments. These findings

238 suggest that MF signaling regulates the signaling pathways underlying sex determination

239 processes via iGluRs and PKC pathways in daphnids.

240 In contrast, iGluRs agonists and pantothenate did not show male inducibility in *D.*

241 *magna*, unlike the WTN6 strain (Table 1). This result indicates that molecular mechanisms

242 underlying male sex-determination may diverge between *D. magna* and *D. pulex*.[59] Additional

243 comparative analysis will become essential to verify whether other daphnid genotypes may

244 control offspring sexes in response to various environmental stimuli and assess whether this

245 process can be generalized to a larger domain of *Daphnia* species.

246

247 **2.2. MF receptor system**

248 The molecular structure of JH receptor in the daphnid has been a long-standing

249 mystery. In recent years, exploratory advances have been made to identify the Methoprene-

250 tolerant (Met) protein as a JH receptor in many insects.[60,61] The Met is a nuclear transcription

251 factor of the basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) family, which is generally known

252 to form homodimer or heterodimer complexes with other bHLH-PAS proteins to initiate DNA

253 binding and transcriptional regulation.[62] Recent studies showed that a bHLH-PAS protein, the

254 steroid receptor coactivator (SRC; also known as FISC and Taiman), forms a heterodimer with

255 Met in response to the presence of JHs. It activates the downstream JH-responsive genes (e.g.,

256 *early trypsin* in *A. aegypti*, and *Krüppel homolog 1* (*Kr-h1*) in *Tribolium castaneum* and *B. mori*

257 [560,61,63–65]), which suggests that Met-SRC heterodimeric complex plays a crucial role in the JH

258 signaling pathway in insect species. However, unlike in insect species, *Kr-h1* is not a JH-

259 responsive gene in *D. pulex*.[66] In addition to SRC, other bHLH-PAS proteins, such as Cycle

260 (CYC), were identified as distinct partner of Met in *A. aegypti*.[67] It was shown that the MET-CYC
261 heterodimer regulates the transcriptions of *Kr-h1* and *Hairy* in response to JH III in the context
262 of photoperiod-dependent circadian regulation in female *A. aegypti*. This implies that Met is an
263 obligatory component of JH receptor and can recruit different bHLH-PAS partners under sex-,
264 developmental stage-, tissue-, and gene-specific conditions.

265 The orthologs of Met and SRC were identified from two water fleas, *D. pulex* and *D.*
266 *magna*, and found that Met and SRC form a heterodimer in response to MF and other JH-like
267 chemicals[68,69], suggesting that the molecular mechanisms underlying JH reception and its
268 downstream transduction are conserved between insects and daphnids. Moreover, rhythmical
269 production and accumulation of Met as multimers were demonstrated in the absence of MF in *D.*
270 *pulex*.[670] In contrast, Met stimulates dissociation of its multimers to form a heterodimer with SRC
271 in the presence of MF.[70] Based on the finding of Met-SRC complex as a JH receptor of daphnids,
272 a highly-sensitive reporter system for detecting juvenoids was established to enable rapid and
273 cost-efficient *in vitro* screening of JH-active chemicals.[70] Up to now, Met has been identified in
274 many other species, including the shrimp *Neocaridina denticulata*[41], horseshoe crabs species
275 (*Carcinoscorpius rotundicauda*, *Limulus polyphemus*, and *Tachypleus tridentatus*), and the
276 chelicerate black-legged tick *Ixodes scapularis*[72], thereby suggesting the JH system
277 comprehensive conservation in Arthropoda.

278 A wide range of JH-active candidate chemicals makes it difficult to test the JH activity
279 of all chemicals using *in vivo* and *in vitro* assays using daphnids. Computational (*in silico*) models
280 using knowledge about ligand-binding interactions with endocrine receptors have been
281 increasingly implemented to characterize receptor-binding specificities and interspecies

282 differences in ligand binding. They also provide rapid (high-throughput) screening tools to
283 identify active receptor ligands in arthropods, including daphnids.[73,74] A set of homology docking
284 models of the PAS-B domain of Met in *D. pulex* and *D. magna* were recently developed based
285 on the crystal structure of HIF-2α to predict and simulate the binding activity between Met and
286 JH-active ligands in daphnids.[75] The three-dimensional structures of both models developed were
287 highly conserved, although there were eight mismatched amino acids located on the protein
288 surface (Figure 1A). Moreover, molecular docking simulations of JH and its analogs with Met in
289 daphnids provides a relatively better prediction for detecting JH-active ligands. This result shows
290 a positive correlation in the interaction energies with each of the experimental values of *in vitro*
291 Met-mediated transactivation potencies and *in vivo* JH activities based on male induction (Figure
292 1B).[75] *In silico* approaches using the *Daphnia* Met model may offer valuable screening tools for
293 detecting JH-active candidate chemicals and supporting development of an Integrated
294 Approaches to Testing and Assessment (IATA)
295 (https://ec.europa.eu/jrc/en/eurl/ecvam/alternative-methods-toxicity-testing/iata).
296

297 **3. Adverse Effect of Potential Antagonists and Inhibitors of MF Synthesis**

298 As aforementioned in section 2.1, MF synthesis is regulated by NMDA and PKC signalings in *D.*
299 *pulex*.[46,52] The NMDA receptor-mediated glutamate signaling is also crucial in the stimulatory
300 pathways of JH synthesis in cockroach *Diploptera punctata*.[49] Moreover, in the cricket *G.*
301 *bimaculatus*, MK-801 (a specific antagonist of NMDA receptor) inhibits *in vitro* JH synthesis in
302 the JH-synthesizing organs, corpora allata (CA), and reduces JH III titers in a dose-dependent

303 manner.[76] Likewise, in *D. pulex* WTN6 strain, MK-801 (20 μM) strongly suppresses the male
304 offspring production under the short-day condition that may induce an increase in endogenous
305 MF titers.[8] Although pharmacological assays with three agonists for iGluRs (i.e., NMDA, AMPA,
306 and kainate [100 μM each]) show stimulatory effects on male offspring production, treatment
307 with 2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline, NBQX (antagonist for AMPA and
308 kainate receptors; 100 and 200 μM) does not show significant suppressive effects.[46] As a PKC
309 inhibitor, Bisindolylmaleimide IV (BIM, 10 μM) was used for topical application to *D. pulex*
310 mature female daphnids and caused a substantial reduction of the male to female offspring ratio.[52]
311 Together, MK-801 and BIM act as inhibitors of MF synthesis in *D. pulex*. Topical application of
312 both chemicals does not change the offspring number associated with a male ratio decline.[46,52]
313

314 **4. Adverse Effects of MF Receptor Agonists**

315 After discovering the Met and SRC as JH receptors (JHR), a quantitative yeast two-hybrid assay
316 system was developed in *A. aegypti*.[63,67] Likewise, a two-hybrid luciferase assay with Met and
317 SRC was developed in two *Daphnia* species, *D. pulex* and *D. magna*, and three insects, such as
318 *D. melanogaster*, *T. castaneum*, and *A. aegypti*.[68,70,77] These systems enabled efficient screening
319 of JHAs by detecting heterodimerization of Met and SRC in response to a group of chemicals that
320 act as direct ligands for binding and activation of the JH receptor.

321 Numerous chemicals bearing JH activity have been artificially designed and developed
322 as novel insecticides.[9] Toxic effects of several JHAs were investigated followed by OECD
323 TG211[26] and found that four natural JHs (JH I, II, III, and MF) and seven JHAs (15ethoprene,

324 pyriproxyfen, fenoxycarb, hydroprene, kinoprene, epofenonane, and diofenolan) can induce a
325 dose (concentration)-dependent male offspring production in *D. magna*.[15–19,23,24,78] At present,
326 based upon the aforementioned two-hybrid assay, 16ethoprene, pyriproxyfen, fenoxycarb, and
327 diofenolan were confirmed as JH agonists in addition to MF and JH III.[24,68,77] Moreover, most of
328 these JHAs cause declines in the number of produced offspring.[16,18,24,78,79] The lowest observed
329 effect concentration (LOEC) for reproduction decrease (2–31,000 ng/l) is lower than or at least
330 equal to that of the LOECs for the induction of male offspring production (4–130,000 ng/l).[24,79]
331 Diofenolan shows the most substantial effects on induction of male offspring production and
332 reproduction suppression among them.[24] The increase of the proportion of male offspring and
333 reduction of offspring number triggered by JHAs exposure could have catastrophic effects on the
334 *Daphnia* populations in aquatic ecosystems. Indeed, some studies with other pesticides, such as
335 fenvalerate, demonstrated that severe reproduction perturbations might require 1–3 generations
336 before returning to normal.[80,81] Ginjupalli and Baldwin[82] show that the effects of pyriproxyfen on
337 *D. magna* population dynamics are time-dependent; longer exposures extend male production and
338 decrease reproduction. They also showed that juvenile daphnids exposed to pyriproxyfen for only
339 2–4 days needed approximately 10 days for recovery in reproduction.[82] These data suggest that
340 JHAs exhibit detrimental effects on daphnid population dynamics by increasing male population
341 ratios and that prolonged exposures cause are more severe effects. Although a direct experimental
342 study on population level was not available, a population model has separately predicted the
343 effects of male production and reproduction inhibition on the population growth rate.[83] Tanaka et
344 al.[83] established a population model based on the 21-day reproduction assay data using

345 pyriproxyfen[79], and they suggest a sex change decreased population growth rate comparable
346 magnitude with that posed by the reproduction inhibition.
347

348 5. AOP development and evaluation

349 5.1. Conceptual AOP assembly

350 Based on the comprehensive knowledge on the JH pathways in daphnids, we have assembled a
351 series of conceptual AOPs (Figure 2) describing perturbations to JH synthesis and downstream
352 signaling pathways, leading to population decline. The JH synthesis pathway induced by
353 environmental stimuli or chemicals presents an upstream of the JHR mediated pathway leading
354 to male offspring induction. The JHR mediated pathway is also initiated by JHAs independently
355 from the JH synthesis pathway and may lead to two parallel KEs, male offspring induction and
356 vitellogenin decrease, which finally cause reproduction impairment and population decline.
357

358 5.1.1. AOP for JH synthesis disruption

359 Based on the current experimental evidence described in section 2.1, the NMDA receptor (iGluR)
360 activation is considered as a MIE (MIE/KE1) of the AOP for JH synthesis disruption, triggered
361 by environmental stimuli (i.e., short-day conditions) or chemicals (iGluR agonist and antagonist).
362 This MIE promotes JH synthesis by activating *JHAMT* transcription (KE2), followed by a JH titer
363 increase (KE3) (Figure 2). Pantothenate accumulation (a possible precursor of acetyl-CoA) is
364 removed from the AOP at present due to inconsistent evidence being observed for *D. pulex* and
365 *D. magna*.[47,59] The PKC signaling activation is also involved in male offspring induction as an

366 upstream regulator of JH signaling. However, more work needs to be done to better elucidate the

367 relationship between the NMDA receptor and the PKC signaling pathway.

368

369 **5.1.2. AOPs for disruption of JHR signaling**

370 An increase in the JH titer (KE3) can lead to acvitation of the JHR (KE4). However, the JHR

371 mediated pathway is also promoted by many exogenous JHAs (e.g., pyriproxyfen, fenoxycarb,

372 hydroprene, kinoprene, and diofenolan).[18,24,78] Therefore, JHR activation is also considered as an

373 MIE for the AOPs (AOP2 and AOP3, Figure 2) that are directly triggered by JHAs. Activation of

374 the JHR can lead to male offspring induction and reproduction failure. Among several JHR target

375 genes reported in the literature,[48,84–86] *doublesex1* (*dsx1*) has been identified as a key regulator of

376 offspring sex differentiation in several daphnids.[22,89] As an activator and regulator of *dsx1*

377 expression, the basic-leucine zipper transcription factor Vrille and long noncoding RNAs (named

378 doublesex1 alpha promoter-associated long RNA) were also reported as the auxiliary events of

379 AOP.[90,91] The *dsx1* activation (KE5) has been considered the best suited marker due to its

380 essentiality in male trait formation, and thus it is proposed as a KE in the JHR AOPs (AOP1 and

381 AOP2; Figure 2).

382 An increase in the proportion of male offspring can normally lead to reproduction

383 decrease (AO1/KE6) and potential population decline.[83] Moreover, most of JHAs, except for

384 epofenonane, directly suppress reproduction at a lower concentration than where male offspring

385 is induced.[78] Therefore, both KEs/AOs simultaneously contribute to the final AO, population

386 decline. The biological cascades between JHR activation and reproduction decrease has not been

387 fully characterized, however, yolk protein vitellogenin (VTG) was found as the most abundant

388 polypeptidein in *D. magna* eggs [92] and several studies suggested that mRNA expression of *vtg*
389 was downregulated by JHAs.[84,85,88] Therefore, *vtg* decrease was selected as an upstream KE of
390 reproduction decline and proposed this as a separate linear AOP (AOP3, Figure 2). The AOP1
391 initiating from the JH synthesis pathway may also affect VTG production through JHR activation.
392 However, only the male offspring induction pathway is included for a linear pathway and a
393 branched pathway is independently evaluated as AOP3.
394

395 **5.2. Assessment of the AOPs.**

396 A WoE assessment has been performed to evaluate the strength of the proposed AOPs.[34]
397 Essentiality of the KEs (Table 2), biological plausibility, empirical support and quantitative
398 understanding of the KERs (Table 3) were used as the main assessment criteria[34]. The three AOPs
399 are assessed separately as shown below, and a detailed list of supporting evidence can be found
400 in the supporting information.
401

402 **5.2.1. AOP1: JH synthesis disruption pathway leading to induction of male offspring,**
403 **reproduction, and population decline**

404 **Essentiality of KEs**

405 The essentiality of iGluR activation (MIE/KE1) is considered high, based on direct experimental
406 evidence showing that the iGluR antagonist inhibited male offspring production under a short-
407 day condition for *D. pulex* WTN6 strain and *D. magna* LRV13.2 and LRV13.5-1 strains.[8,46,59]
408 The iGluR agonist treatment that induced male offspring in *D. pulex* WTN6 strain under a long-
409 day condition also provide additional support.[46,59] For essentiality of JHAMT activation (KE2),

410 enzymatic assays provide direct evidence showing that KE2 stimulated KE3 (JH titer increase).
411 In such assays, a recombinant JHAMT protein of *D. pulex* catalyzed MF from FA.[8] The MF is
412 now considered an innate JH in daphnids and other crustaceans, but a direct measurement method
413 for MF in the daphnids has still not been successfully established. Therefore, direct support for
414 essentiality of the JH titer increase (KE3) is not available at present. Nonetheless, apical treatment
415 of numerous JHs and JHAs in the JHR reporter gene assay and *in vivo* assay shows the *JHR*
416 activation,[68,71,77,69] *dsx1* activation,[22,89] and male offspring induction and reproduction
417 impairment.[18,24,78] These results support a moderate essentiality of KE3. The LC-MS/MS and GC-
418 MS methods have been established to quantify endogenous MF titers in insects and other small
419 crustaceans such as the amphipod *Gammarus locusta* and the branchiopod *Artemia franciscana*.[43-
420 45,94] We hypothesize it would also apply to the daphnid species in further studies.

421 The essentiality of JHR activation (KE4) and *dsx1* activation (KE5) leading to male
422 offspring induction (KE7) is highly supported by transcriptional knockdown studies resulting in
423 the aborted embryo and lack of male phenotype.[68,89] The essentiality of KE5 is also supported by
424 indirect evidence that *dsx1* expression was observed only in male offspring of several daphnid
425 species.[22] In a two-generation reproduction test using *D. magna*, pyriproxyfen (0.5 μg/L)
426 produced almost no female offspring in the first generation and no reproduction was observed in
427 the second generation, supporting the essentiality of KE7 and reproduction decrease
428 (KE8/AO2).[95] Tanaka et al.[83] has described a population model that provides indirect support for
429 the essentiality of KE7 and KE8, demonstrating that the effects of pyriproxyfen on both KE7 and
430 KE8 could lead to a decrease in population growth rate (AO3).

431

432 **WoE evaluation of KERs (KER1-KER7)**

433 The biological plausibility of the KERs (KER1, KER2) linking environmental stimuli (e.g., short-
434 day condition) to inhibition of JH synthesis is not considered strong, due to a lack of supportive
435 evidence. However, it may be biologically plausible, based on analogy to accepted biological
436 relationships, in other insect and crustaceans,[50,96,97] but the *NMDAR* and *JHAMT* genes have been
437 identified recently and their physiological function within the daphnids has not been completely
438 established.

439 For empirical support of the KERs, no experimental evidence is currently found to
440 directly support dose-response and incidence concordance. Temporal concordance is supported
441 by the upregulation of the JHR subunit genes (*Met* and *SRC*) after the induction of *NMDR-b* and
442 *JHAMT* at the early stage of reproductive cycle (Figure S3) in *D. pulex* WTN6 strain under short-
443 day conditions.[98] However, the regulatory mechanism between iGluR and JHAMT activation has
444 not been fully characterized. Some inconsistencies have also been reported for NMDA receptor
445 agonism between *D. pulex* and *D. magna*,[59] indicating that the molecular mechanism regulating
446 the NDMA signaling may differ between the two species. The JH titer should increase after the
447 *JHAMT* activation[8], but the temporal change of JH titer during the reproductive period was not
448 directly analyzed in daphnids. Therefore, more experimental evidence regarding KE3 is needed
449 after the appropriate analytical method for the determination of JH titer in daphnids is established.
450 Therefore, empirical support for KER1 and KER2 is considered low at present.

451 In contrast, the biological plausibility between the JH titer increase to male offspring
452 induction (KER3, KER4, KER5) is considered high based on the numerous JHR reporter gene
453 assay studies,[68–71,77,69] the function of *dsx1* geneduring sex differentiation (male trait development

454 in daphnids),[89–91,98] and male offspring observation in many JHs and JHAs exposure studies.[15–19,23,24,78,99,100] For empirical support, dose-response and temporal concordance between KE4 (JHR activation) and KE7 (male offspring induction) were demonstrated following exposure to several specific stressors (JHs and JHAs) using reporter assay and gene expression analysis *in vivo* studies,[68–71,77,69] whereas KE5 was only observed for one fenoxycarb concentration (1 μg/L).[89–91,98] The recently developed luciferase assay that detects the transcriptional activation of the modified JH response elements (JHRE) from *T. castaneum Krüppel homolog 1* (a major JH-responsive gene in insects) by the *Daphnia* JHR demonstrated that the *JHR* activation by fenoxycarb occurred at almost the comparable concentration (EC50 = 4.92×10^{-9} M)[71] as that of *dsx1* upregulation *in vivo* (LOEC ≤ 3.31×10^{-9} M).[89] On the basis of the limited number of supporting studies, no inconsistencies were found. Even though the three KEs have not been evaluated in the same study, the time-course gene expression analysis of the JHR subunits (*Met* and *SRC*) and *dsx1* indicated that KE4 occurred earlier (expression peak was observed ~36 h before ovulation[70,97]) than the KE5 (from 3 h post-ovulation[89,91,98]) and KE7 (Figure S3). At present, there is no inconsistency in empirical support across different *Daphnia* species. However, full-length sequences of *Met* and *SRC* genes were only identified in *D. pulex* and *D. magna.*[68] The *dsx1* genes' homologs were conserved in several cladoceran species, such as *D. pulex*, *D. magna* (NIES and Belgium strain), *Daphnia galeata*, *Moina macrocopa*, and *Ceriodaphnia dubia*.[22] In conclusion, the empirical support for KER3, KER4, and KRE5 is considered to be moderate. Further studies with simultaneous measurements (KE3 in particular) of all KEs in the same studies and following exposure to a wide range of stressors are still required.

475 It is considered that the biological plausibility level of downstream KERs linking male
476 production to reproduction impairment (KER6) and population decline (KER7) as moderate as
477 described in the essentiality support and the supporting information. The empirical support is
478 judged to be low, as only a two-generation reproduction test and population modeling using
479 pyriproxyfen in *D. magna* are currently available.[83,94] For the observation of population-level
480 effects in daphnids, microcosm studies are encouraged.

481

482 **5.2.2. AOP2: JHR mediated pathway leading to male offspring induction**

483 This AOP is the downstream part of AOP1. Hence, the assessment of KEs and KERs
484 has been described in 5.2.1. Dissimilar to AOP1, KE4 (JHR activation) is assigned as the MIE
485 for this AOP and a number of JHAs (e.g., pyriproxyfen, fenoxycarb, kinoprene, hydroprene, and
486 diofenolan) are known to directly mediate this MIE.[15-19,23,24,78,99,100] (Table S1).

487

488 **5.2.3. AOP3: JHR mediated pathway leading to reproduction decline.**

489 **Essentiality of KEs**

490 This AOP describes *JHR* activation leading to population decline via perturbation to *vtg*
491 expression. Essentiality of the *JHR* activation (MIE/KE4) leading to reductions in *vtg* expression
492 (KE6) is supported by direct evidence where JHAs (i.e., fenoxycarb and pyriproxyfen) treatment
493 caused downregulation of *vtg* expression.[84,85,87] In addition, Kato et al.[92] detected VTG as a
494 component of major yolk protein complexes in the eggs at early development stages. Tokishita et
495 al.[84] identified sequences resembling known JH-responsive and ecdysone-responsive elements in
496 the intergenic region of 2.6 kb between two *vtgs* (*Dmagvtg1, Dmagvtg2*), indicating that *vtg*

497 expression is involved in the JHR mediated pathway. However, there were a few contradictory

498 experimental studies where no downregulation of *vtg* expression was observed with the JHA

499 treatment.[24,86] Therefore, the essentiality of KE4 leading to KE6 is considered as moderate.

500 Transcription analysis of *vtg* was used in multiple studies as an early warning biomarker of

501 chronic reproductive effects in daphnids.[85,86,101–104] However, there are currently no experimental

502 studies supporting that decrease in VTG protein concentration can lead to impaired reproduction

503 in the daphnid study (Supporting information).[104] Therefore, the essentiality of KE6 (VTG

504 decrease) leading to KE8/AO2 (reproduction decrease) was determined as moderate. Therefore,

505 a knockdown study or further *in vivo* assays with the measurement of both *vtg* expression and

506 reproduction to demonstrate a statistically significant correlation between the two KEs will further

507 strengthen the data for supporting this KE.

508

509 **Assessment of the KERs (KER8, KER9)**

510 It could be biologically plausible that *vtg*-related genes are one of the JHR responsive genes.[84]

511 However, the regulation of VTG under different conditions has not been clarified yet. Many

512 JHs/JHAs down-regulate *vtg* expression,[84,85,87] whereas a few studies reported that the JHs/JHAs

513 exposure to adult females showed no effect on *vtg* mRNA levels.[24,86] A short-term exposure (<72

514 h) in the latter studies and time-dependent modulation of *vtg* expression during the reproduction

515 cycle, alternating with ecdysteroids, may explain this inconsistency. Inter-animal differences in

516 *vtg* expression levels at different molt/reproductive stage could confound the JHA effects.[86]

517 Generally, in insects, juvenoids and ecdysteroid hormones cooperatively control VTG synthesis;

518 juvenoids typically induce VTG and ecdysteroids have either a stimulatory or suppressive effect,

519 depending upon the species.[86,105,106] This JH and ecdysone cooperative regulation makes it
520 difficult to obtain clear experimental support for KER8. Therefore, the biological plausibility of
521 KER8 is considered moderate, and additional experiments with the efficient exposure period and
522 transcriptional analysis at appropriate timing are needed. KER9 (from *vtg* decrease to
523 reproduction decrease) may be biologically plausible based on analogy to accepted biological
524 relationships in the other species. However, biological linkage supported by evidence has not
525 been aquired yet in the daphnids.

526 Empirical support for dose-response concordance of KER8 is not sufficient at present.
527 Concerning temporality, *vtg2* mRNA level was upregulated between 12 and 24 h after the
528 previous molt and then downregulated between 24 and 48 h after the previous molt.[86] This
529 suggests that the peak expression of *vtg* occurs ~36 h after the egg development period in the
530 ovary, which is later than that of *JHR* activation (24 h of ovarian egg development).[70] For KER9,
531 direct experimental evidence assessing both KE6 and KE8 in the same study are not currently
532 available for JH/JHAs. Even in the studies evaluating the effects of several other chemicals (i.e.,
533 cyproterone acetate, acetone, triclosan, atrazine, and ecdysteroids 20-hydroxyecdysone and
534 ponasterone A, heavy metals, miconazole, perfluoroethylcyclohexane sulfonate, and bisphenol
535 A),[86,101–104] the evidence for a cause-effect relationship between the two KEs against these
536 chemicals is not conclusive (Supporting Information). Therefore, the empirical support for both
537 KER8 and KE9 is considered low, and further studies simultaneously measuring the change in
538 these events against JHs/JHAs exposure are required.

539

540 **5.2.4. Quantitative understanding**

541 The quantitative understanding of each adjacent KERs is low because most of the KEs,
542 particularly KEs in the JH synthesis pathway, were measured in a limited study with a few model
543 chemicals, concentrations, and species (Table 3). In KER4 (JHR activation to *dsx1* activation),
544 the *JHR* activation measured by reporter gene assays has dose-response data for several chemicals,
545 whereas *dsx1* activation was measured in male offspring induced in short-day condition and only
546 by fenoxycarb exposure (generally at 1 µg/L). The only non-adjacent KER: *JHR* activation (KE4)
547 leading to male offspring induction (KE7) can be quantitatively discussed. The KER's empirical
548 support were high, and KER's biological plausibility was moderate at present[108]. Considering
549 demand for a regulatory application in Japan, it is desirable to develop a quantitative prediction
550 of the proportion of male offspring (KE7) in reproduction based on chemical potency as JHR
551 agonists (JHR activation, KE4). At present, the response–response relationship can be described
552 only six chemicals between the two KEs; EC50 for JHR activation in reporter gene assays[24,68,71]
553 and EC50 for male induction *in vivo* assay[16] (Figure 3). The JH III, MF, fenoxycarb, and
554 hydroprene caused response at the same level (slightly lower in KE7 than KE4) on the two KEs.
555 However, pyriproxyfen and diofenolan are considerably more active in KE7 (two to five orders
556 of magnitude lower). It is not clear whether these outliers are due to their chemical specificity or
557 technical limit of the reporter assay where JHRE from *T. castaneum,* not from *Daphnia* species,
558 were used at present. Based on the docking simulation, both chemicals are expected to show
559 higher JHR activity than that showed in the reporter assay (Figure 1B). Therefore, further
560 improvement of the reporter assay and testing of more chemicals are needed. Thus, the
561 quantitative understanding of this KER is considered moderate.

562

563 **5.3. Domain of applicability**

564 The biological domain of AOP1 is currently limited to *D. pulex* WTN6 strain and *D. magna*
565 LRV13.2 and LRV13.5-1 strains because effect of iGluR antagonist/agonists in sex determination
566 have been only reported in these species of which sex determination is sensitive to change in the
567 photoperiod and response to iGluR agonist were different between *D. pulex* and *D. magna*.[8.46.59,97]
568 Therefore, stressors such as short-day condition and several iGluR antagonists/agonists, might be
569 valid only in the specific species or strain for AOP1. Moreover, further investigation of male
570 offspring induction by other environmental stimuli (e.g., low temperature, food shortage,
571 overpopulation)[31] will identify other KE and pathway in the near future.

572 For both AOP2 and AOP3, although the male offspring induction and reproduction
573 decrease by JH agonists are conserved across broad cladoceran genera, such as *Ceriodaphnia*,
574 *Moina*, *Bosmina*, and *Oxyurella*,[17,20–22] *in vitro* assay for detecting JHR activation is currently
575 limited to *D. magna* and *D. pulex*. *In silico* analysis of JHR, such as Sequence Alignment to
576 Predict Across Species Susceptibility (SeqAPASS; https://seqapass.epa.gov/seqapass/)[108] and
577 molecular docking simulations of JHAs with Met[75] will help to explore the taxonomic domain
578 and new stressors. The other chemicals reported to induce male offspring by *in vivo* assays are
579 summarized in Table S1, but the *JHR* activation potency of these chemicals has only minimally
580 been confirmed. Even in the same species, Oda et al.[19,109] demonstrated different strains of *D.*
581 *magna* had different sensitivity in the proportion of male offspring production by fenoxycarb.
582 Some strains (e.g. *D. magna* LRV13.5-1 [35]) constantly produce male offspring even in the
583 laboratory culturing condition, whereas the NIES strain that is used most of the KE4, KE5, KE7

584 studies, rarely produces males in response to environmental stress.[31] To avoid false positive

585 results in vivo assay detecting male offspring induction by chemicals, it is recommended to use a

586 strain which rarely produces males in control treatment or understand the basic male offspring

587 proportion of your strain in laboratory culturing condition and control treatment.

588

589 **5.4. Overall assessment of the AOP**

590 The KERs' biological plausibility, empirical support, and quantitative understanding and the

591 evidence supporting the KEs' essentiality in an AOP are assessed together for an AOP's overall

592 assessment.[34] For AOP1, even though all the KEs' essentiality are moderate/high, the WoE for

593 upstream KER1 and downstream KER6/KER7 are low, suggesting that further empirical support

594 for understanding the molecular relationship between short-day condition (or the other stressors)

595 to KE2 are expected to increase the level of WoE level for AOP1. For AOP2, the overall WoE

596 for upstream KERs (KER4/KER5) is moderate, whereas it is low for the downstream KERs

597 (KER6/KER7) as described forAOP1. The quantitative understanding of KER6 (KE7→AO1)

598 will be required to consider its potential regulatory application. For AOP3, both the evidence

599 supporting KEs and KERs are still moderate/low, and the further biological understanding of the

600 pathway is needed.

601

602 **5.5. Regulatory application of the AOPs**

603 Male offspring induction by chemical-mediated disruption of JH signaling is expected to be used

604 as a new endpoint in a regulatory context (such as BPR and PPPR[28]) to identify endocrine

605 disruptors in invertebrates (i.e., chemicals with JH activity), and the AOPs can help to develop

606 tiered testing strategies. The Ministry of the Environment of Japan developed a two-tier
607 framework for testing and assessing chemical ED effects on aquatic organisms.[110,111] The JH and
608 ecdysteroidal activities are also included in the framework (Figure S4).[110,111] Reporter gene assay
609 for detecting *JHR* activation is assigned as Tier 1 with *ecdysone receptor* activation assay[112] (to
610 assess actions to endocrine systems) *in vitro* assays.[68,71,77] The results of *in vitro* assays and
611 literature review will be used to prioritize chemicals for *in vivo* testing. As Tier 1 *in vivo* assay,
612 short-term JH Activity Screening Assay (JHASA)[23] was developed based on OECD TG 211
613 *Daphnia magna* Reproduction Test Annex 7,[25] which is assigned as Tier 2 (to characterize adverse
614 effects) *in vivo*. Based on the temporal concordance of KEs between KE4 to KE7 as described in
615 5.2.3, the JHASA starts exposure from matured adult and only observes offspring from the second
616 brood after exposure to shorten test duration and reduce laborious work for identification of all
617 offspring sex by microscope observation.[23] It is currently proposed as a new OECD test guideline
618 (a ring test report is in preparation). The JHASA and OECD TG211 Annex 7 are also suggested
619 as Level 3 and Level 4 *in vivo* assays, respectively, providing data about the selected endocrine
620 mechanism(s)/pathway(s) in the OECD Conceptual Framework for Testing and Assessment of
621 Endocrine Disrupting Chemicals.[27]

622 Because the JHASA still requires approximately 5–7 days, a suite of high-throughput
623 screening methods for MIEs and upstream KEs would be a utility to screen the high number of
624 chemicals. The quantitative understanding of the KERs is required to use an assay for upstream
625 KEs as an alternative to assays for downstream KEs. As discussed in 5.2.4, the quantitative
626 understanding between *JHR* activation and male offspring induction is moderate. More dose-
627 response data of target chemicals is required to develop a predictive model between the two KEs.

628 Besides, the *in silico* JHR binding model can facilitate the screening of JHR agonists, and more
629 training sets of chemicals are needed to improve the model's predictive power. Implementation
630 of these *in silico* and *in vitro* tools and knowledge reviewed herein will help to develop future
631 IATA initiatives.
632

633 **6. Future Directions**

634 Although *in silico*, *in vitro*, and *in vivo* assays to detect JHR agonists have been developed,
635 detection methods for chemicals disrupting the upstream JH synthesis pathway (KE1-KE3) have
636 not been established. Abe et al.[113,114] found that several macrolide pesticides (e.g., ivermectin) and
637 the secondary metabolites of plants (plant essential oil component) induced male offspring in
638 JHASA but did not show *JHR* activation in the reporter assay developed by Tanaka et al.[71]
639 (unpublished data). These compounds may stimulate an upstream JH synthesis pathway leading
640 to JH titer increase or involve in transcriptional regulation of *dsx* (KE5). To confirm the former
641 hypothesis, KE1–KE3 need to be investigated in the daphnids exposed to these candidate
642 compounds. Because the KE1 and the KE2 were only observed in male offspring producing *D.*
643 *pulex* stimulated by photoperiod condition, further investigation of various candidate chemicals
644 will probably identify other important upstream KEs in the JH synthesis disrupting pathway.
645 Moreover, new assessment approaches that can correctly detect the effect of anti-JH
646 using *in vivo*, *in vitro*, and *in silico* methods are required to complement the testing and assessment
647 framework. Most JH antagonists developed/discovered as IGRs (e.g. precocenes,
648 fluoromevalonate, compactin, and imidazoles) inhibit the JH synthesis pathway in insects by

649 either disrupting enzyme action or injuring the CA cells.[115] The JHR antagonists were also
650 identified from plant extract using the mosquito two-hybrid yeast assay.[116] As *in vivo* assay,
651 precocious metamorphosis-inducing activity using the silkworm (*B. mori*) larva is used to detect
652 anti-JH action in insects.[117] In the daphnids' case, repression of male offspring production
653 compared with that in an unexposed condition (e.g., *D. pulex* WTN6 strain) and has the potential
654 to be used as an endpoint of anti-JH *in vivo* assay.

655 This literature review found that several events in insects' JH system were not
656 observed in daphnids, suggesting that using only daphnids could not detect the insect-specific
657 disruptors in the JH systems. Understanding the evolutionary diversities and common principles
658 underlying the JH systems among arthropods is still needed for further development of a robust
659 testing approach. It would also contribute to expand the application domain of the current set of
660 AOPs.

661

662 LIST OF ABBREVIATIONS

663 AO adverse outcome

664 AOP adverse outcome pathway

665 AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

666 BIM bisindolylmaleimide IV

667 bHLH-PAS basic helix-loop-helix-Period-aryl hydrocarbon receptor nuclear
668 translocator-single-minded

669 bZIP basic-leucine zipper

670	CA	corpora allata
671	CoA	co-enzyme A
672	CYC	cycle
673	CYP15A1	cytochrome p450 15A1
674	DAPALR	doublesex1 alpha promoter-associated long RNA
675	dsx1	doublesex1
676	EAGMST	extended advisory group on molecular screening and toxicogenomics
677	EDC	endocrine disrupting chemical
678	FAMeT	farnesoic acid O-methylfransferase
679	FPP	farnesyl diphosphate
680	GC-MS	gas chromatography-mass spectrometry
681	IATA	integrated approaches to testing and assessment
682	iGluR	ionotropic glutamate receptor
683	IGR	insect growth regulator
684	JH	juvenile hormone
685	JHA	juvenile hormone analog
686	JHAMT	juvenile hormone acid O-methylfransferase
687	JHASA	juvenile hormone activity screening assay
688	JHR	juvenile hormone receptor
689	JHRE	juvenile hormone response element
690	KE	key event
691	KER	key event relationship

692 Kr-h1 krüppel-homolog 1

693 LC-MS liquid chromatography-mass spectrometry

694 LOEC lowest observed effect concentration

695 Met methoprene-tolerant

696 MF methyl farnesoate

697 MIE molecular initiating event

698 NMDA N-methyl-D-aspartic acid

699 NMDAR N-methyl-D-aspartic acid receptor

700 NBQX 2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline

701 OECD organization for economic co-operation and development

702 PKC protein kinase C

703 RT-PCR reverse transcription-polymerase chain reaction

704 SeqAPASS sequence alignment to predict across species susceptibility

705 SRC steroid receptor coactivator

706 TFG-β transforming growth factor β

707 vtg vitellogenin

708 WoE weight of evidence

709

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717

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1072 **Figure and Table Legends**

1073 Figure 1

1074 A. Homology models of Met PAS-B domain in *D. pulex* (dark cyan) and *D. magna* (orange).

1075 Nonconserved residues are shown as sticks.

1076 B. Relationships between U_dock values and EC_{50} values of *in vitro* assays for *D. pulex* and *D.*

1077 *magna*. Red and blue plots indicate the values for *D. pulex* and *D. magna*, respectively.

1078

1079 Figure 2

1080 Adverse outcome pathway for the JH synthesis and the JHR mediated disruption triggering male

1081 offspring induction and population decline in *Daphnia* species. MIE, molecular initiating event;

1082 KE, key event; AO, adverse outcome; iGluR, ionotropic glutamate receptor; PKC, protein kinase

1083 C; JHAMT, juvenile hormone acid O-methyltransferase; JH, juvenile hormone; JHR, juvenile

1084 hormone receptor; *dsx1*, double sex 1.

1085

1086 Figure 3

1087 The relationship of EC50 for the JHR activation (KE4) in reporter gene assays [24,66,71] and for male

1088 induction (KE7) in OECD TG211 Annex 7.[16,18]

1089

1090 Table 1

1091 Pharmacological assays summary using daphnids with photoperiod-dependent sex determination

1092 conditions.

1093

1094 Table 2

1095 Support for KEs essentiality and their detection methods.

1096 KE, key event; AO, adverse outcome; iGluR, ionotropic glutamate receptor; JHAMT, juvenile

1097 hormone acid O-methyltransferase, JH, juvenile hormone; JHR, juvenile hormone receptor; *dsx1*,

1098 *double sex 1*

1099

1100 Table 3

1101 The weight of evidence (WoE) assessment of Key Event Relationships' (KERs).

1102