Comparative ovarian microarray analysis of juvenile hormone-responsive genes in water flea Daphnia magna:

Toyota, Kenji; Williams, Timothy; Sato, Tomomi; Tatarazako, Norihisa; Iguchi, Taisen

DOI:
10.1002/jat.3368

Short Abstract

A rapid screening method for detection of chemicals with juvenile hormone (JH)-activity was developed using adult *Daphnia magna* based on the phenomenon of induction of male offspring. However, JH-responsive genes in the ovary are still largely undescribed. Here, we conducted comparative microarray analyses using ovaries treated with fenoxycarb (artificial JH agonist) or methyl farnesoate (a putative innate JH in daphnids) to elucidate responses to JH agonists in ovary, including developing oocytes, at a JH-sensitive period for male sex determination.

Comparative ovarian microarray analysis of juvenile hormone-responsive genes in water flea *Daphnia magna*: potential targets for toxicity

Kenji Toyota[1,2], Timothy D. Williams[1], Tomomi Sato[3], Norihisa Tatarazako[4], Taisen Iguchi[2,3*]

[1]School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.

[2]Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, Department of Basic Biology, Faculty of Life Science, SOKENDAI (Graduate University for Advanced Studies), 5-1 Higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan.

[3]Graduate School of Nanobioscience, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan.

[4]Environmental Quality Measurement Section, Research Center for Environmental Risk, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan.

*Correspondence: Taisen Iguchi, Graduate School of Nanobioscience, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan.

E-mail: taisen@nibb.ac.jp

Short title: Ovarian transcriptome of *Daphnia magna*

Abstract

The freshwater zooplankton *Daphnia magna* has been extensively employed in chemical toxicity tests such as OECD Test Guidelines 202 and 211. Previously, it has been demonstrated that treatment of juvenile hormones (JHs) or their analogs to female daphnids can induce male offspring production. Based on this finding, a rapid screening method for detection of chemicals with JH-activity was recently developed using adult *D. magna*. This screening system determines whether a chemical has JH-activity by investigating the male offspring inducibility. Although this is an efficient high-throughput short-term screening system, much remains to be discovered about JH-responsive pathways in the ovary, and whether different JH-activators act via the same mechanism. JH-responsive genes in the ovary including developing oocytes are still largely undescribed. Here, we conducted comparative microarray analyses using ovaries from *Daphnia magna* treated with fenoxycarb (Fx; artificial JH agonist) or methyl farnesoate (MF; a putative innate JH in daphnids) to elucidate responses to JH agonists in ovary, including developing oocytes, at a JH-sensitive period for male sex determination. We demonstrate that induction of hemoglobin genes is a well-conserved response to JH even in the ovary, and a potential adverse effect of JH agonist is suppression of vitellogenin gene expression, that might cause reduction of offspring number. This is the first report demonstrating different transcriptomics profiles from MF and an artificial JH agonist in *D. magna* ovary, improving understanding the tissue-specific mode-of-action of JH.

Key words

Daphnia magna, juvenile hormone, juvenile hormone-responsive gene, microarray, ovarian transcriptome

Introduction

The cladoceran crustacean genus *Daphnia* is a model freshwater zooplankton forming the basis of a fundamental food-chain network in aquatic ecosystems. They have been used for ecological, developmental, evolutionary, and ecotoxicological studies, since they have unique and suitable features including; highly-diverged species around the world; ease of manipulation in the laboratory; production of genetically identical offspring by parthenogenesis; and, high-sensitivity to chemicals released into the natural environment. For ecotoxicological research, *Daphnia magna* has been used as a model species by the Organization for Economic Co-operation and Development Test Guidelines OECD TG202 (acute toxicity test; OECD, 1998) and TG211 (chronic toxicity test; OECD, 2004). Based on these toxicological tests, extensive data on effects of various chemicals have been accumulated for *D. magna* (Leiss *et al*., 2005), although as yet there is limited information on the modes-of-action of each chemical in *Daphnia*.

Describing the molecular impact of chemicals upon an organism is required to

elucidate the chemical-specific mode-of-action of the toxic effects. For this reason, transcriptomic technologies have recently been applied for ecotoxicology to facilitate understanding the causal relationship between chemical exposure and its molecular adverse effects (Waters and Fostel, 2004; Poynton et al., 2007). Application of microarray and high-throughput sequencing technology to ecotoxicology has been termed “ecotoxicogenomics” (Snape *et al*., 2004; Iguchi *et al*., 2006). Focusing on *Daphnia* studies, transcriptomic approaches (e.g., microarray and RNA-seq) have contributed to accumulating fundamental knowledge about molecular impacts of chemicals. For example, *D. magna* microarray analysis has been conducted by several research groups (Watanabe et al., 2008; Poynton et al., 2007; Connon et al., 2008; Toyota et al., 2014; Abe et al., 2015a). Additionally, RNA-seq analyses enable us to more comprehensively and easily identify candidate transcripts and signaling pathways responding to chemical exposure (Toyota et al., 2015b).

Daphnia species are known to switch reproductive strategy between parthenogenesis and sexual reproduction in response to changing environmental conditions within their habitat. They produce, in general, female offspring under favorable environmental conditions. However, they begin to produce exclusively male offspring in response to unsuitable environmental conditions such as shortened day-length, low temperature, lack of nutrients, and crowding (referred to as environmental sex determination). Sexual reproduction then occurs to produce resting eggs, which remain viable for long periods, in excess of a century in some cases, in adverse environments such as dry and freezing conditions (Hobæk and Larsson, 1990;

Kleiven et al., 1992; Smith, 1915; Toyota et al., 2015b).

Previously, it has been demonstrated that treatment of juvenile hormones (JHs) or their analogs to female daphnids could induce male offspring production even under female-producing conditions (Olmsted and LeBlanc, 2002; Tatarazako et al., 2003). Additionally, we have demonstrated that endogenous methyl farnesoate (MF; a putative innate JH molecule in daphnids) is likely increased when mother produces male offspring (Toyota et al., 2015a).

The induction of males following JH agonist exposure has become a useful endpoint for screening of chemicals with JH activity. This approach has been adopted for the OECD Validation Management Group for Ecotoxicity testing (OECD VMGeco), and added as a new endpoint in the OECD TG211 ANNEX 7 to detect JH-like activity (OECD, 2012). Furthermore, the JH-sensitive period for male sex determination in daphnids has been clarified as an oocyte maturation stage within the ovary (Kato et al., 2011; Toyota et al., 2015a). Based on this finding, a rapid screening method for detection of chemicals with JH-activity was recently developed by using adult *D. magna*. This screening system can validate whether an arbitrary chemical has JH-activity by investigating the male offspring inducibility (Abe et al., 2015b). Although this short-term screening system will be able to dramatically improve testing throughput, JH-responsive genes regulating the physiological response and male induction in the ovary, including developing oocytes, at a JH-sensitive period are still largely unknown. Therefore, in this study, in order to detect JH-responsive genes, we conducted comparative microarray analyses using two kinds of JH chemical;

fenoxycarb (an artificial JH agonist) and MF in *D. magna* ovaries.

Materials and methods

Daphnia strain and rearing conditions

The *Daphnia magna* strain (NIES clone) was obtained from the National Institute for Environmental Studies (NIES; Tsukuba, Japan) (Tatarazako et al., 2003). The strain originated from the Environmental Protection Agency (USA) and was maintained for more than 15 years at NIES. The synthetic M4 growth medium was used (Elendt and Bias, 1990). Cultures of 20 individuals per liter were incubated at 20±1°C under a 14-h light/10-h dark photoperiod. A 0.01-ml suspension of 4.3×10^8 cells ml^{-1} Chlorella (*Chlorella vulgaris*) was added daily to each culture.

Chemicals and concentrations

Fenoxycarb (Fx) and methyl farnesoate (MF) were used as JH agonists in this study. Their chemical structures are shown in **Figure 1A**. Fx and dimethyl formamide (DMF) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). MF was obtained from Echelon Bioscience (Salt Lake City, UT, USA). Experiments were conducted at the nominal concentrations of 2 ppb and 200 ppb for Fx and MF, respectively, dissolved in DMF. Those concentrations were decided according to previous studies (Tatarazako et al., 2003; Oda et al., 2005). Solvent DMF concentration in all test solutions was less than 0.01% v/v.

***Daphnia magna* oligonucleotide microarray**

A custom 4×44k oligonucleotide microarray was developed (Agilent Technologies, Earray Design ID: 020586). This microarray was designed from our developed expressed sequence tag (EST) database containing ~11k transcripts (Watanabe *et al*., 2005; 2007; 2008). Four probes were generally designed to each transcript sequence and the oligonucleotides (60 mers) were selected using the Agilent web design application (http://earray.chem.agilent.com/earray). Details of the platform design of the *D. magna* microarray and raw intensity values for each microarray are available at Gene Expression Omnibus with accession number GPL17297, series GSE81083.

RNA extraction and hybridization

20 individuals were cultured in 1 L of rearing medium with Fx or MF treatment. They were sacrificed at 2-3 weeks age, during the MF-sensitive period for male sex determination of the developing oocytes (Kato et al., 2011). Ovary samples consisted of three individuals per replicate, and quadruplicates were prepared for both Fx and MF treatments, respectively. Harvested ovaries were homogenized using a physcotron NS-310E (Nichion, Tokyo, Japan). Total RNA was extracted by TRIZOL reagent (Invitrogen, Tokyo, Japan), and purified using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols and including RNase-free DNase (Qiagen) treatment. The quality and concentration of

total RNA was determined by NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

500 ng of total RNA were prepared for hybridization using the Quick Amp Labeling Kit and One-color RNA Spike-in kit (Agilent Technologies), and purified using the RNeasy Micro Kit (Qiagen) following the manufacturer's protocol. The quality of Cyanine3-labeled complementary RNA (cRNA) was analyzed using the 2100 Bioanalyzer. 165 μg of Cyanine3-labeled cRNA were hybridized to the custom 4x44k *D. magna* microarray according to the manufacture's protocol. After 17 h incubation at 65^{o}C with rotation, the microarrays were washed with Gene Expression Wash Buffer Kit (Agilent Technologies). DNA microarrays were scanned using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) at 5 μm resolution. The signal intensity of the spots was digitized using the microarray imager software (Combimatrix Molecular Diagnostics, Irvine, CA, USA).

Differential gene expression analysis

Data were input to Genespring v7.3 (Agilent), filtered to remove low-intensity features and quantile-normalised with geWorkbench (Floratos et al., 2010). SAM differential gene expression analysis (Tusher et al, 2001) was conducted within MeV (Saeed et al., 2003). Comparisons were made between

control and treated groups, with a fold change of 1.5 or greater and FDR<0.1 considered significant.

Gene ontology enrichment analysis

GO annotations were assigned to this microarray previously (Toyota et al., 2014). GO enrichment analysis was carried out using the gene score resampling method in ErmineJ (v3.0.2) with full resampling of average of fold change used as gene scores (Lee et al., 2005). From 5,201 contigs or clones (total 10,135) bearing at least one GO term, GO subsets containing between 5 and 150 genes were considered in this analysis, and GO terms with a Benjamini-Hochberg FDR < 0.1 were defined as significant.

Results

Ovarian gene expression profile in response to fenoxycarb treatment

Microarray analyses were conducted using two kinds of JH agonist (Fx and MF), independently. Fx concentration was 2 ppb, which is able to induce male offspring exclusively (Tatarazako et al., 2003). Based on quality check of signal intensity of spots (detail in Materials and methods), we removed one replicate each from Fx and DMF (solvent control) treatments, resulting in analysis of this microarray data as triplicate samples **(Fig. 1B)**.

The number of differentially expressed ovarian genes (DEGs; fold change < 1.5 and FDR < 0.1) was 41 transcripts (77 probes) in response to 2 ppb Fx treatment; 26

(52 probes) up-regulated and 15 (25 probes) down-regulated **(Fig. 2; Table S1)**. Furthermore, cuticle- and cytochrome-related genes were up-regulated, whereas hemoglobin-, collagen-related and vitellogenin fused with superoxide dismutase genes were down-regulated **(Table 1; Table S1)**. Additionally, gene ontology (GO) enrichment analysis showed that GO terms related to hemoglobin (e.g., oxygen transport, heme binding, and hemoglobin complex) and vitellogenin (e.g., superoxide metabolic process, and lipid transporter activity) were varied statistically significantly **(Table 3)**.

Ovarian gene expression profile in response to methyl farnesoate treatment

Ovarian microarray analysis was performed following 200 ppb MF treatment, which is a sufficient concentration for 100% male offspring induction (Tatarazako et al., 2003). The number of DEGs in response to MF treatment was 41 transcripts (82 probes); 32 (60 probes) up-regulated and 9 (22 probes) down-regulated **(Fig. 2; Table S2)**. Although several already-known JH-responsive genes encoding cytochrome b5, trehalase and hemoglobin were differentially expressed in response to MF treatment as well as Fx, vitellogenin-related genes were not changed **(Tables 2 and 3)**. Additionally, several novel JH-responsive candidates responded only to MF treatment; for example, genes encoding chk1 checkpoint-like protein, aquaporin, and sterol desaturase **(Tables 2 and S2)**. Moreover, 8 transcripts (cytochrome b5, thioredoxin domain-containing protein 17, trehalase, and 5 unidentified genes) were up-regulated by both Fx and MF treatments **(Fig. 2; Table 3)**.

In order to functionally overview the ovarian MF-responsive genes, GO enrichment analysis was conducted. Changes in hemoglobin-related GO terms were more statistically significant with MF treatment than Fx treatment (e.g., heme binding and tetrapyrrole binding). Moreover, cuticle- and sugar transport-related terms were identified as statistically significant following MF treatment **(Table 4)**.

Discussion

We demonstrated that differentially expressed gene profiles in response to Fx and MF treatments were different in *D. magna* ovary, consistent with our previous microarray study using whole-bodies of *D. magna* neonates treated by three JH agonists (Toyota et al., 2014). In order to more comprehensively overview the trends of gene expression profiles in response to Fx and MF treatments, we conducted GO enrichment analysis, successfully identifying the hemoglobin gene family as varying in expression in response to both Fx and MF treatments. Interestingly, hemoglobin genes are highly-duplicated, forming a tandemly duplicated gene cluster in the *Daphnia* genome (Colbourne et al., 2011). Additionally, several previous studies demonstrated that hemoglobin genes of daphnids show high JH-responsiveness based on alteration of gene expression level (Eads et al., 2008; Gorr et al., 2006; Hannas et al., 2011). Although, based on those data, it has been considered that hemoglobin genes are up-regulated by JH agonist treatment, our recent microarray and RNA-seq analyses demonstrated that hemoglobin genes were often down-regulated in the whole-body of neonate treated by

other JH agonists; methoprene and epofenonane (Toyota et al., 2014), and in the whole-body or ovary of adult *D. pulex* treated by MF (Toyota et al., 2015b) **(Table S3)**. Additionally, the current study showed that ovarian hemoglobin genes were down-regulated by both Fx and MF treatments. Surprisingly, expression patterns showed the opposite when whole adult *D. magna* and *D. pulex* were treated with 200 ppb MF (Hannas et al., 2011; Toyota et al., 2015b) **(Table S3)**. These data imply that, although hemoglobin genes are undoubtedly JH-responsive genes in daphnids, the regulation of their expression pattern is tissue-specific and might also be affected by life-stage, species, chemical sensitivity and selectivity.

According to the OECD TG211 ANNEX 7 (OECD, 2004), toxic impacts of Fx and MF to fertility and male-inducibility on *D. magna* have been investigated as well as that of other JH agonists. Those studies revealed that Fx has high repro-toxic effects such as decreasing the number of offspring and increasing the male sex ratio of offspring even at less than 1.0 ppb. Although, likewise, repro-toxic effects of MF have been clarified, it was more than 1000 times less potent than Fx (Tatarazako et al., 2003; Oda et al., 2005). It has been reported that binding abilities of ligand (JH molecule) to the JH receptor complex is quite different between JH agonists, and that concentrations triggering the conjugation of JH agonist to JH receptor complex were consistent with each male-inducible concentration estimated by OECD TG211 test data (Miyakawa et al., 2013). In other words, the difference of male-inducibility among JH agonists could be explained by ligand selectivity of JH receptor complex. Despite these findings, the cause of differential toxic effects on fertility among JH agonists is still largely unknown.

In this study, DEG analysis revealed that vitellogenin-related genes (e.g., vitellogenin fused with superoxide dismutase) were apparently down-regulated in response to Fx treatment, but not to MF treatment. This tendency was more clearly shown by GO enrichment analysis. Moreover, similar to Fx exposure, it has been reported that expression levels of vitellogenin genes were decreased by other JH agonists in whole-body of juvenile individuals (Tokishita et al., 2006; Kim et al., 2011) and adult ovaries (Toyota et al., 2015b); however, interestingly, one report showed that expression level of vitellogenin gene was not affected by Fx treatment in adult female whole-body (Hannas et al., 2011). These data indicate that responsiveness of vitellogenin gene to JH is different depending on sample features such as age, tissue, and JH agonists treated. The vitellogenin of daphnids contains the superoxide dismutase (SOD)-like domain and might play a crucial role in the protection of oocytes against oxidative stress (Kato et al., 2004). Additionally, in general, vitellogenin is known as a precursor of yolk protein which is an essential factor for oocyte/egg maturation and embryo development. Therefore, it could be suggested that the reduction of offspring number in response to JH agonists might be due to suppression of vitellogenin-related expression in ovary. Although, to date, underlying mechanisms connecting JH agonist exposure and suppression of vitellogenin gene expression are still unclear, elucidation of those regulatory mechanisms will provide us important knowledge about the mode-of-action of JH agonist toxicity.

Although only eight transcripts could be identified as common up-regulated DEGs, expression profiles of two genes encoding cytochrome b5 and contig854

(without annotation) were consistent with our previous transcriptome analysis using adult whole-body and ovary of *D. pulex* (Toyota et al., 2015b), suggesting that these are strong candidates for well-conserved JH-responsive genes in daphnids. The expression level of contig1856 was up-regulated in this study; however, our previous transcriptome data showed that it was down-regulated (Toyota et al., 2015b). Trehalase is a glycoside hydrolase enzyme and catalyzes the conversion of trehalose to glucose. A recent study using red flour beetle *Tribolium castaneum* revealed that knock-down of JH acid methyltransferase (JHAMT) involved in JH synthesis represses trehalase gene expression, suggesting that JH regulates trehalose homeostasis (Xu et al., 2013). Therefore, in the daphnids, trehalase and trehalose homeostasis might also be downstream targets of JH signaling. Thioredoxin domain-containing protein 17 (TXNDC17) has peroxidase activity and could contribute to the removal of hydrogen peroxide generated by redox reactions (Jeong et al., 2004). Up-regulation of TXNDC17 might play important role in protection of ovary and/or oocyte from oxidative stress, since the amount of oxidant is increased by up-regulation of hemoglobin genes in whole-body responsive to JH treatment **(Table S3)**. Furthermore, three other transcripts (contig538, contig2690, and contig3811) are novel candidates for JH-responsive transcripts, although their functions are still unknown.

Finally, several MF-specific responsive candidates were identified, including genes encoding chk1 checkpoint-like protein, aquaporin, and sterol desaturase. Chk1 checkpoint protein acts for monitoring the DNA quality and can control delay in, or arrest of, the cell cycle at multiple points during the cycle (Purdy et al., 2005).

Although, to date, its function in oogenesis of daphnids responding to JH remains unknown, a recent study revealed that JH activates other checkpoint gene, cell-division-cycle 6 (cdc6), in vitellogenesis and oogenesis of migratory locust (Wu et al., 2016). These results suggest that regulation of the cell cycle can be altered by MF exposure in daphnids.

Taken together, in the current study, we discovered genes responsive to Fx or MF treatments by microarray analysis. Furthermore, we demonstrated that the hemoglobin genes are well-conserved JH-responsive elements even in the ovary, and a potential repro-toxic mechanism of JH agonists is suppression of vitellogenin gene expression, potentially leading to reduction of offspring number. Despite these findings, no candidate gene clearly involved in control of male induction was identified. The possible reason is that function and annotation of genes in *D. magna* are still largely undescribed, although recent progress in high-throughput sequencing technology paves the way for the ecotoxicogenomics research using non model organisms including daphnid species. Indeed, several unknown-function genes were contained in our current candidate DEGs. In order to overcome this limitation of gene annotation, large-scale *D. magna* transcriptome data has rapidly been accumulated (Orsini et al., 2016), making more efficient screening of genes involved with male induction and JH-response possible. Our findings provide fundamental information for understanding the alteration of tissue- and chemical-specific transcriptome in response to JH agonist treatment accompanied by male offspring production.

Conflict of Interests

The authors declare that they have no conflict of interests.

Author's Contributions

KT, TS, NT and TI conceived and designed the study. KT performed experiments. KT and TW analyzed the data. KT, TW and TI wrote a first draft. All authors participated in the modification of draft, and approved the final manuscript.

Acknowledgements

We would like to thank Ms. Kaoru Kobayashi, National Institute for Basic Biology (NIBB), for her technical assistance. Computational resources were provided by the Data Integration and Analysis Facility, and supported by Drs. Hiroyo Nishide and Ikuo Uchiyama, NIBB. This work was partly supported by the Japan Society for the Promotion of Science (JSPS) Research Fellowship for Young Scientists (KT), the Sasakawa Scientific Research Grant from The Japan Science Society (KT), the Saito Ho-on Kai Scientific Research Grant from The Saito Gratitude Foundation (KT), and grants from Ministry of the Environment of Japan (TI).

References

Abe R, Toyota K, Miyakawa H, Watanabe H, Oka T, Miyagawa S, Nishide H, Uchiyama I, Tollefsen KE, Iguchi T. 2015a. Diofenolan induces male offspring production through binding to the juvenile hormone receptor in *Daphnia magna*. *Aquat. Toxicol.* 159: 44-51. DOI: 10.1016/j.aquatox.2014.11.015.

Abe R, Watanabe H, Yamamuro M, Iguchi T, Tatarazako N. 2015b. Application of a short-term screening method for detecting chemicals having juvenile hormone activity using *Daphnia magna*. *J. Appl. Toxicol.* 35: 75-82. DOI: 10.1002/jat.2989.

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 JH, 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, Manak JR, Muller J, Pangilinan J, Patwardhan RP, Pitluck S, Pritham EJ, Rechtsteiner A, Rho M, Rogozin IB, Sakarya O, Salamov A, Schaack S, Shapiro H, Shiga Y, Skalitzky C, Smith Z, Souvorov A, Sung W, Tang Z, Tsuchiya D, Tu H, Vos H, Wang M, Wolf YI, Yamagata H, Yamada T, Ye Y, Shaw JR, Andrews J, Crease TJ, Tang H, Lucas SM, Robertson HM, Bork P, Koonin EV, Zdobnov EM, Grigoriev IV, Lynch M, Boore JL. 2011. The ecoresponsive genome of *Daphnia pulex*. *Science* 331: 555-561. DOI: 10.1126/science.1197761.

Connon R, Hooper HL, Sibly RM, Lim FL, Heckmann LH, Moore DJ, Watanabe H, Soetaert A, Cook K, Maund SJ, Hutchinson TH, Moggs J, De Coen W, Iguchi T, Callaghan A. 2008. Linking molecular and population stress responses in *Daphnia magna* exposed to cadmium. *Environ Sci Technol* 42: 2181-2188. DOI: 10.1021/es702469b.

Eads BD, Andrews J, Colbourne JH. 2008. Ecological genomics in *Daphnia*: stress responses and environmental sex determination. *Heredity* 100: 184-190. DOI: 10.1038/sj.hdy.6800999.

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 *D. magna*. *Water Res.* 24: 1157-1167. DOI:10.1016/0043-1354(90)90180-E.

Floratos A, Smith K, Ji Z, Watkinson J, Califano A. 2010. geWorkbench: an open source platform for integrative genomics. *Bioinformatics* 26(14): 1779-1780. DOI: 10.1093/bioinformatics/btq282.

Gorr TA, Rider CV, Wang HY, Olmstead AW, LeBlanc GA. 2006. A candidate juvenoid hormone receptor *cis*-element in the *Daphnia magna* hb2 hemoglobin gene promoter. *Mol. Cell. Endocrinol.* 247: 91-102. DOI: 10.1016/j.mce.2005.11.022.

Hannas BR, Wang YH, Thomson S, Kwon G, Li H, LeBlanc GA. 2011. Regulation and dysregulation of vitellogenin mRNA accumulation in daphnids (*Daphnia magna*). *Aquatic Toxicol* 101: 351-357. DOI: 10.1016/j.aquatox.2010.11.006.

Hobæk A, Larsson P. 1990. Sex determination in *Daphnia magna. Ecology* 71: 2255–2268. DOI: 10.2307/1938637.

Iguchi T, Watanabe H, Katsu Y. 2006. Application of ecotoxicogenomics for studying endocrine disruption in vertebrates and invertebrates. *Environ. Health Perspect.* 114: 101-105. DOI: 10.1289/ehp.8061.

Jeong W, Yoon HW, Lee SR, Rhee SG. 2004. Identification and characterization of TRP14, a thioredoxin-related protein of 14 kDa. *J. Biol. Chem.* 279(5): 3142-3150. DOI: 10.1074/jbc.M307932200.

Kato Y, Tokishita S, Ohta T, Yamagata H. 2004. A vitellogenin chain containing a superoxide dismutase-like domain is the major component of yolk proteins in cladoceran crustacean *Daphnia magna*. *Gene* 334: 157-165. DOI: 10.1016/j.gene.2004.03.030.

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 Genet.* 7(3): e1001345. DOI: http://dx.doi.org/10.1371/journal.pgen.1001345.

Kim J, Kim Y, Lee S, Kwak K, Chung WJ, Choi K. 2011. Determination of mRNA expression of DMRT93B, vitellogenin, and cuticle 12 in *Daphnia magna* and their biomarker potential for endocrine disruption. *Ecotoxicology* 20: 1741-1748. DOI: 10.1007/s10646-011-0707-0.

Kleiven OT, Larsson P, Hobæk A. 1992. Sexual reproduction in *Daphnia magna* requires three stimuli. *Oikos* 65: 197–206. DOI: 10.2307/3545010.

Lee HK, Braynen W, Keshav K, Pavlidis P. 2005. ErmineJ: tool for functional analysis of gene expression data sets. *BMC Bioinformatics* 6: 269. DOI: 10.1186/1471-2105-6-269.

Liess M, Von Der Ohe PC, 2005. Analyzing effects of pesticides on invertebrate communities in streams. *Environ. Toxicol. Chem.* 4: 954-965. DOI: 10.1897/03-652.1.

Miyakawa H, Toyota K, Hirakawa I, Ogino Y, Miyagawa S, Oda S, Tatarazako N, Miura T, Colbourne JK, Iguchi T. 2013. A mutation in the receptor Methoprene-tolerant alters juvenile hormone response in insects and crustaceans. *Nat. Commun.* 4:1856.

DOI: 10.1038/ncomms2868.

Oda S, Tatarazako N, Watanabe H, Morita M, Iguchi T. 2005. Production of male neonates in *Daphnia magna* (Cladocera, Crustacea) exposed to juvenile hormones and their analogs. *Chemosphere* 61: 1168-1174. DOI:10.1016/j.chemosphere.2005.02.075.

Olmstead AW, LeBlanc GA. 2002. Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia manga*. *J. Exp. Zool.* 293: 736-739. DOI: 10.1002/jez.10162.

Orsini L, Gilbert D, Podicheti R, Jansen M, Brown JB, Solaari OS, Spanier KI, Colbourne JK, Rush D, Decaestecker E, Asselman J, Schamphelaere KACD, Ebert D, Haag CR, Kvist J, Laforsch C, Petrusek A, Beckerman AP, Little TL, Chaturvedi A, Pfrender ME, Meester LD, Frilander MJ. 2016. Data Descriptor: *Daphnia magna* transcriptome by RNA-Seq across 12 environmental stressors. *Sci. Data* 3:160030. DOI: 10.1038/sdata.2016.30.

Poynton HC, Varshavsky JR, Chang B, Cavigiolio G, Chan S, Holman PS, Loguinov AV, Bauer DJ, Komachi K, Theil EC, Perkins EJ, Hughes O, Vulpe CD. 2007. *Daphnia magna* ecotoxicogenomics provides mechanistic insights into metal toxicity. *Environ. Sci. Technol.* 41: 1044–1050. DOI: 10.1021/es0615573.

Purdy A, Uyetake L, Cordeiro MG, Su TT. 2005. Regulation of mitosis in response to damaged or incompletely replicated DNA require different levels of Grapes (*Drosophila Chk1*). *J. Cell Sci.* 118: 3305-3315. DOI: 10.1242/jcs.02454.

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A, Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V, Quackenbush J. 2003. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 34(2): 374-378.

Smith G. 1915. The life-cycle of Cladocera, with remarks on the physiology of growth and reproduction in crustacea. *Proc. R. Soc. Lond. B Biol. Sci.* 88: 418-435.

Snape JR, Maund SJ, Pickford DB, Hutchinson TH. 2004. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *Aquat. Toxicol.* 67: 143-154. DOI:10.1016/j.aquatox.2003.11.011.

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

Tokishita S, Kato Y, Kobayashi T, Nakamura S, Ohta T, Yamagata H. 2006.

Organization and repression by juvenile hormone of a vitellogenin gene cluster in the crustacean, *Daphnia magna*. *Biochem. Biophys. Res. Commun.* 345: 362-370. DOI: 10.1016/j.bbrc.2006.04.102.

Toyota K, Kato Y, Miyakawa H, Yatsu R, Mizutani T, Ogino Y, Miyagawa S, Watanabe H, Nishide H, Uchiyama I, Tatarazako N, Iguchi T. 2014. Molecular impact of juvenile hormone agonists on neonatal *Daphnia magna*. *J. Appl. Toxicol.* 34:537-544. DOI: 10.1002/jat.2922.

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

Toyota K, Miyakawa H, Yamaguchi K, Shigenobu S, Ogino Y, Tatarazako N, Miyagawa S, Iguchi T. 2015b. NMDA receptor activation upstream of methyl farnesoate signaling for short day-induced male offspring production in the water flea, *Daphnia pulex*. *BMC Genomics* 16:186. DOI: 10.1186/s12864-015-1392-9.

Tusher VG, Tibshirani R, Chu G. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. USA* 98(9): 5116-5121. DOI: 10.1073/pnas.091062498.

Watanabe H, Tatarazako N, Oda S, Nishide H, Uchiyama I, Morita M, Iguchi T. 2005. Analysis of expressed sequence tags of the water flea *Daphnia magna*. *Genome* 48: 606-609. DOI: 10.1139/g05-038.

Xu J, Sheng Z, Palli SR. 2013. Juvenile hormone and insulin regulate trehalose homeostasis in the red flour beetle, *Tribolium castaneum*. *PLoS Genet.* 9(6): e1003535. DOI: 10.1371/journal.pgen.1003535.

Watanabe H, Takahashi E, Nakamura Y, Oda S, Tatarazako N, Iguchi T. 2007. Development of a *Daphnia magna* DNA microarray for evaluating the toxicity of environmental chemicals. *Environ. Toxicol. Chem.* 26: 669-676. DOI: 10.1897/06-075R.1.

Watanabe H, Kobayashi K, Kato Y, Oda S, Abe R, Tatarazako N, Iguchi T. 2008. Transcriptome profiling in crustaceans as a tool for ecotoxicogenomics. *Cell Biol. Toxicol.* 24: 641-647. DOI: 10.1007/s10565-008-9108-4.

Waters MD, Fostel J.M., 2004. Toxicogenomics and systems toxicology: aims and prospects. *Nature Rev. Genet.* 5: 936-948. DOI:10.1038/nrg1493.

Tables

Table 1.

Representative up- or down-regulated transcripts in response to fenoxycarb treatment (FC > 1.5, $q < 0.1$). Refer to Supplemental Table S1 for complete listing.

Probe ID	Hit Contig	Description (BlastX)	Fold Change (unlogged)	*q* value
Up-regulation				
DM08957_1	IGU001_0007_B05.r	beta-ig-h3 fasciclin	1.77	<0.001
DMAG0001S00004120	Contig4120	cub and sushi domain-containing protein 3	1.53	0.041
DMAG0001S00001893	Contig1893	cub and sushi domain-containing protein 3-like	1.55	0.067
DM09619_2	WTH001_0003_O02.f	cuticular protein analogous to peritrophins 3-a1	1.90	<0.001
DM02762_3	Contig5168	cysteine-rich secretory protein 2	2.20	<0.001
DM05678_1	Contig4172	cytochrome b5	1.59	0.056
DMAG0001S00006990	dm005p22.r	minichromosome maintenance deficient 8 (cerevisiae)	1.50	0.088
DMAG0001S00004449	Contig4449	papilin	3.14	<0.001
DM12229_2	Contig3209	phospholipase a2	1.63	0.024
DM06173_2	Contig2484	ras suppressor protein 1	1.52	0.054
DM11265_2	dm027b16.f	retinaldehyde-binding protein 1-like protein 1	1.79	0.059
DMAG0001S00001937	Contig1937	stress protein ddr48 (dna damage-responsive protein 48)	1.75	0.099
DMAG0001S00004737	Contig4737	thioredoxin domain-containing protein 17	1.63	<0.001

DM05149_2	dm037g17.f	xylose isomerase	2.11	<0.001
Down-regulation				
DMAG0001S00004100	Contig4417	2-domain hemoglobin protein subunit	0.13	0.086
DMAG0001S00005307	IGU001_0006_H04.f	2-domain hemoglobin protein subunit	0.14	0.086
DMAG0001S00005655	IGU001_0049_F06.f	2-domain hemoglobin protein subunit	0.15	0.086
DMAG0001S00001119	Contig1119	aplp_locmi ame: full=apolipophorins contains	0.14	0.086
DM06048_1	Contig4848	collagen alpha-1 chain	0.37	0.023
DMAG0001S00001196	Contig1196	collagen alpha-2	0.46	0.086
DM06590_1	Contig2473	steroid dehydrogenase	0.43	0.086
DM14629_2	Contig373	transducin -like 1 x-linked receptor 1-like	0.64	0.086
DMAG0001S00007057	dm006p15.f	vitellogenin fused with superoxide dismutase	0.29	0.086
DMAG0001S00008423	dm026o19.f	vitellogenin fused with superoxide dismutase	0.17	0.086
DM01857_2	dm043j10.r	vitellogenin fused with superoxide dismutase	0.16	0.086
DMAG0001S00009929	dm058b09.f	vitellogenin fused with superoxide dismutase	0.16	0.086

Table 2.

Representative up- or down-regulated transcripts in response to methyl farnesoate treatment (FC > 1.5, $q < 0.1$). Refer to Supplemental Table S2 for complete listing.

Probe ID	Hit Contig	Description (BlastX)	Fold Change (unlogged)	*q* value
Up-regulation				
DM04056_2	Contig1848	alpha-mannosidase 2	2.87	0.086
DM01384_3	Contig1921	angiotensin converting enzyme	2.16	0.030
DM02550_3	Contig5000	aquaporin 3-like	3.58	<0.001
DM05943_2	dm051j21.f	aquaporin isoform cra_b	3.67	0.020
DM08718_2	Contig4485	ccaat enhancer binding protein	2.61	<0.001
DMAG0001S00009113	dm040h12.f	chk1 checkpoint-like protein	3.00	<0.001
DM04958_1	Contig4172	cytochrome b5	1.86	0.055
DM10603_1	Contig1639	lim domain-binding protein	1.85	<0.001
DM09817_2	Contig817	microsomal dipeptidase	1.59	0.086
DM05459_1	Contig5232	midline fasciclin	1.78	0.018
DM02339_3	Contig2588	phosphatidylserine decarboxylase	1.53	0.085
DMAG0001S00004737	Contig4737	thioredoxin domain-containing protein 17	1.74	<0.001
DM00993_1	dm009m07.r	trehalase	1.78	0.091
Down-regulation				

DM11523_1	Contig4100	2-domain hemoglobin	0.10	0.079
DM08378_2	Contig3556	camp-dependent protein kinase r2	0.49	0.064
DM05703_1	dm005p22.r	minichromosome maintenance deficient 8 (cerevisiae)	0.37	0.054
DM03522_2	Contig1132	sterol desaturase	0.25	0.045

Table 3.

Common up-regulated transcripts in response to both treatments (FC > 1.5, $q < 0.1$).

Probe ID	Hit Contig	Description (BlastX)	Fx		MF	
			Fold Change	*q* value	Fold Change	*q* value
DM05678_1	Contig4172	cytochrome b5	1.59	0.056	-	-
DMAG0001S00004172			1.59	0.056	-	-
DM04958_1			-	-	1.86	0.055
DM10334_1			-	-	2.10	0.086
DMAG0001S00004737	Contig4737	thioredoxin domain-containing protein 17	1.63	<0.001	1.74	<0.001
DM11877_1			1.65	0.024	1.68	<0.001
DM00993_1	dm009m07.r	trehalase	-	-	1.78	0.091
DMAG0001S00001856	Contig1856	hypothetical protein DAPPUDRAFT_309304 [Daphnia pulex]	9.62	<0.001	6.08	<0.001
DM07245_2			17.95	<0.001	7.51	<0.001
DM07245_1			-	-	4.65	<0.001
DMAG0001S00000854	Contig854	hypothetical protein DAPPUDRAFT_308669 [Daphnia pulex]	1.76	<0.001	-	-
DM08418_1			2.70	<0.001	2.45	<0.001
DM08418_2			-	-	2.21	<0.001
DM06798_1	Contig538	hypothetical protein DAPPUDRAFT_303931	1.80	<0.001	1.65	0.091

		[Daphnia pulex]				
DM06100_2	Contig2690	Unknown	3.77	<0.001	6.10	0.086
DM06100_1			8.99	<0.001	22.84	0.053
DMAG0001S00003811	Contig3811	Unknown	-	-	1.52	0.031

Table 4.

List of GO terms varied in response to both treatments.

GO Name	GO_ID	Same As	Corrected *p* value		MF
			Fx		
Biological Process					
oxygen transport	GO:0015671	GO:0005344, GO:0019825	**0.0918**	**<0.0001**	
gas transport	GO:0015669		0.1102	**<0.0001**	
superoxide metabolic process	GO:0006801		**0.0128**	0.3504	
reactive oxygen species metabolic process	GO:0072593		**0.0608**	0.4771	
lipid transport	GO:0006869		**0.0875**	0.1070	
Molecular Function					
oxygen transporter activity	GO:0005344	GO:0015671, GO:0019825	**0.0810**	**<0.0001**	
oxygen binding	GO:0019825	GO:0015671, GO:0005344	**0.0810**	**<0.0001**	
heme binding	GO:0020037		0.5139	**<0.0001**	
tetrapyrrole binding	GO:0046906		0.4405	**<0.0001**	
oxidoreductase activity, acting on single donors with	GO:0016702		1.0000	**0.0887**	

incorporation of molecular oxygen, incorporation of two atoms of oxygen				
oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	GO:0016701		1.0000	**0.0985**
lipid transporter activity	GO:0005319		**0.0634**	**0.0853**
structural constituent of cuticle	GO:0042302		0.0622	**0.0114**
chitin binding	GO:0008061		0.5857	**0.0288**
carbohydrate transmembrane transporter activity	GO:0015144	GO:1901476	1.0000	**0.0783**
carbohydrate transporter activity	GO:1901476	GO:0015144	1.0000	**0.0783**
transferase activity, transferring pentosyl groups	GO:0016763		1.0000	**0.0950**
Cellular Function				
extracellular region	GO:0005576		**0.0056**	**<0.0001**
hemoglobin complex	GO:0005833		**0.0718**	**<0.0001**
cytosolic part	GO:0044445		1.0000	**<0.0001**

Supplemental table S1.

Up- or down-regulated transcripts in response to fenoxycarb treatment (FC > 1.5, $q < 0.1$).

Probe ID	Hit Contig	Description (BlastX)	Fold Change (unlogged)	*q* value
Up-regulation				
DM01917_2	Contig1411	hypothetical protein DAPPUDRAFT_224365 [Daphnia pulex]	1.72	0.024
DM04940_1	Contig1818	Unknown	1.76	0.024
DM04940_2	Contig1818	Unknown	1.76	0.041
DM07245_2	Contig1856	hypothetical protein DAPPUDRAFT_309304 [Daphnia pulex]	17.95	<0.001
DMAG0001S00001856	Contig1856	hypothetical protein DAPPUDRAFT_309304 [Daphnia pulex]	9.62	<0.001
DMAG0001S00001893	Contig1893	cub and sushi domain-containing protein 3-like	1.55	0.067
DMAG0001S00001937	Contig1937	stress protein ddr48 (dna damage-responsive protein 48)	1.75	0.099
DM06173_2	Contig2484	ras suppressor protein 1	1.52	0.054
DM13504_2	Contig2536	hypothetical protein DAPPUDRAFT_302150 [Daphnia pulex]	2.15	0.024
DM11193_1	Contig2536	hypothetical protein DAPPUDRAFT_302150 [Daphnia pulex]	3.13	<0.001
DMAG0001S00002536	Contig2536	hypothetical protein DAPPUDRAFT_302150 [Daphnia pulex]	3.12	<0.001
DM13504_1	Contig2536	hypothetical protein DAPPUDRAFT_302150 [Daphnia pulex]	2.53	<0.001
DM06100_1	Contig2690	Unknown	8.99	<0.001
DM06100_2	Contig2690	Unknown	3.77	<0.001
DM12229_2	Contig3209	phospholipase a2	1.63	0.024

DM03175_2	Contig3209	phospholipase a2	1.66	0.054
DMAG0001S00003209	Contig3209	phospholipase a2	1.69	0.088
DM07000_2	Contig3690	Unknown	2.61	0.099
DMAG0001S00003864	Contig3864	hypothetical protein DAPPUDRAFT_49070 [Daphnia pulex]	1.59	0.024
DM04523_1	Contig3864	hypothetical protein DAPPUDRAFT_49070 [Daphnia pulex]	1.63	<0.001
DMAG0001S00004120	Contig4120	cub and sushi domain-containing protein 3	1.53	0.041
DMAG0001S00004172	Contig4172	cytochrome b5	1.59	0.056
DM05678_1	Contig4172	cytochrome b5	1.59	0.056
DMAG0001S00004449	Contig4449	papilin	3.14	<0.001
DM11877_1	Contig4737	thioredoxin domain-containing protein 17	1.65	0.024
DMAG0001S00004737	Contig4737	thioredoxin domain-containing protein 17	1.63	<0.001
DMAG0001S00004753	Contig4753	hypothetical protein DAPPUDRAFT_224638 [Daphnia pulex]	2.16	<0.001
DM08270_1	Contig4753	hypothetical protein DAPPUDRAFT_224638 [Daphnia pulex]	2.06	<0.001
DM02762_3	Contig5168	cysteine-rich secretory protein 2	2.20	<0.001
DM02762_2	Contig5168	cysteine-rich secretory protein 2	2.18	<0.001
DM02437_2	Contig5168	cysteine-rich secretory protein 2	2.18	<0.001
DMAG0001S00005168	Contig5168	cysteine-rich secretory protein 2	2.18	<0.001
DM02437_1	Contig5168	cysteine-rich secretory protein 2	2.14	<0.001
DM02437_3	Contig5168	cysteine-rich secretory protein 2	2.09	<0.001
DM06798_1	Contig538	hypothetical protein DAPPUDRAFT_303931 [Daphnia pulex]	1.80	<0.001
DM08418_1	Contig854	hypothetical protein DAPPUDRAFT_308669 [Daphnia pulex]	2.70	<0.001

DMAG0001S00000854	Contig854	hypothetical protein DAPPUDRAFT_308669 [Daphnia pulex]	1.76	<0.001
DMAG0001S00006990	dm005p22.r	minichromosome maintenance deficient 8 (cerevisiae)	1.50	0.088
DMAG0001S00008166	dm022l12.f	Unknown	2.12	<0.001
DM11265_2	dm027b16.f	retinaldehyde-binding protein 1-like protein 1	1.79	0.059
DM11265_1	dm027b16.f	retinaldehyde-binding protein 1-like protein 1	1.62	0.078
DMAG0001S00008430	dm027b16.f	retinaldehyde-binding protein 1-like protein 1	1.75	0.088
DM05149_2	dm037g17.f	xylose isomerase	2.11	<0.001
DMAG0001S00008977	dm037g17.f	xylose isomerase	2.08	<0.001
DM05149_1	dm037g17.f	xylose isomerase	2.03	<0.001
DMAG0001S00009463	dm047g18.f	Unknown	2.18	0.041
DM11275_1	dm047g18.f	Unknown	2.04	<0.001
DM08957_1	IGU001_0007_B05.r	beta-ig-h3 fasciclin	1.77	<0.001
DM08957_2	IGU001_0007_B05.r	beta-ig-h3 fasciclin	1.68	<0.001
DM09619_2	WTH001_0003_O02.f	cuticular protein analogous to peritrophins 3-a1	1.90	<0.001
DMAG0001S00005944	WTH001_0003_O02.f	cuticular protein analogous to peritrophins 3-a1	1.72	<0.001
DM09619_1	WTH001_0003_O02.f	cuticular protein analogous to peritrophins 3-a1	1.66	<0.001

Down-regulation

DMAG0001S00001119	Contig1119	aplp_locmi ame: full=apolipophorins contains	0.14	0.086
DMAG0001S00001196	Contig1196	collagen alpha-2	0.46	0.086
DM12094_2	Contig220	Unknown	0.48	0.086

DM06590_1	Contig2473	steroid dehydrogenase	0.43	0.086
DM06590_2	Contig2473	steroid dehydrogenase	0.40	0.086
DM14629_2	Contig373	transducin -like 1 x-linked receptor 1-like	0.64	0.086
DM12258_2	Contig4365	Unknown	0.65	0.086
DMAG0001S00004365	Contig4365	Unknown	0.63	0.086
DMAG0001S00004100	Contig4417	2-domain hemoglobin protein subunit	0.13	0.086
DM06048_1	Contig4848	collagen alpha-1 chain	0.37	0.023
DMAG0001S00007057	dm006p15.f	vitellogenin fused with superoxide dismutase	0.29	0.086
DMAG0001S00008423	dm026o19.f	vitellogenin fused with superoxide dismutase	0.17	0.086
DM03613_3	dm026o19.f	vitellogenin fused with superoxide dismutase	0.14	0.086
DM03613_1	dm026o19.f	vitellogenin fused with superoxide dismutase	0.14	0.086
DM01857_2	dm043j10.r	vitellogenin fused with superoxide dismutase	0.16	0.086
DMAG0001S00001655	dm043j10.r	vitellogenin fused with superoxide dismutase	0.14	0.086
DM11621_2	dm043j10.r	vitellogenin fused with superoxide dismutase	0.14	0.086
DM01857_1	dm043j10.r	vitellogenin fused with superoxide dismutase	0.14	0.086
DM11621_1	dm043j10.r	vitellogenin fused with superoxide dismutase	0.14	0.086
DM01857_3	dm043j10.r	vitellogenin fused with superoxide dismutase	0.13	0.086
DMAG0001S00009929	dm058b09.f	vitellogenin fused with superoxide dismutase	0.16	0.086
DMAG0001S00005307	IGU001_0006_H04.f	2-domain hemoglobin protein subunit	0.14	0.086
DM08998_2	IGU001_0012_D09.f	Unknown	0.19	0.086
DM08998_1	IGU001_0012_D09.f	Unknown	0.19	0.086

DMAG0001S00005655	IGU001_0049_F06.f	2-domain hemoglobin protein subunit	0.15	0.086

Supplemental table S2.

Up- or down-regulated transcripts in response to methyl farnesoate treatment (FC > 1.5, $q < 0.1$).

Probe ID	Hit Contig	Description (BlastX)	Fold Change (unlogged)	*q* value
Up-regulation				
DM08098_2	Contig1409	hypothetical protein DAPPUDRAFT_311096 [Daphnia pulex]	3.22	<0.001
DM01338_2	Contig1639	lim domain-binding protein	1.67	0.018
DM10603_1	Contig1639	lim domain-binding protein	1.85	<0.001
DM10603_2	Contig1639	lim domain-binding protein	1.75	<0.001

DM01338_1	Contig1639	lim domain-binding protein	1.74	<0.001
DMAG0001S00001639	Contig1639	lim domain-binding protein	1.56	<0.001
DM04056_2	Contig1848	alpha-mannosidase 2	2.87	0.086
DM07245_2	Contig1856	hypothetical protein DAPPUDRAFT_309304 [Daphnia pulex]	7.51	<0.001
DMAG0001S00001856	Contig1856	hypothetical protein DAPPUDRAFT_309304 [Daphnia pulex]	6.08	<0.001
DM07245_1	Contig1856	hypothetical protein DAPPUDRAFT_309304 [Daphnia pulex]	4.65	<0.001
DM01384_3	Contig1921	angiotensin converting enzyme	2.16	0.030
DM01384_1	Contig1921	angiotensin converting enzyme	1.99	0.031
DM02339_3	Contig2588	phosphatidylserine decarboxylase	1.53	0.085
DM06100_1	Contig2690	Unknown	22.84	0.053
DM06100_2	Contig2690	Unknown	6.10	0.086
DMAG0001S00002984	Contig2984	Unknown	3.25	0.012
DM08692_1	Contig2984	Unknown	2.77	<0.001
DM08692_2	Contig2984	Unknown	2.62	<0.001
DMAG0001S00003104	Contig3104	hypothetical protein DAPPUDRAFT_303742 [Daphnia pulex]	1.52	0.090
DM06987_2	Contig3285	hypothetical protein DAPPUDRAFT_303367 [Daphnia pulex]	1.55	0.012

DM07199_1	Contig3481	Unknown	3.95	0.018
DM07418_1	Contig3481	Unknown	6.12	0.020
DM07199_2	Contig3481	Unknown	4.31	0.020
DMAG0001S00003481	Contig3481	Unknown	3.95	0.031
DM07418_2	Contig3481	Unknown	6.42	<0.001
DM05204_1	Contig3580	hypothetical protein DAPPUDRAFT_331983 [Daphnia pulex]	16.84	0.093
DMAG0001S00003632	Contig3632	hypothetical protein DAPPUDRAFT_231816 [Daphnia pulex]	2.93	0.053
DM06179_2	Contig3632	hypothetical protein DAPPUDRAFT_231816 [Daphnia pulex]	5.01	0.054
DM07000_1	Contig3697	Unknown	6.89	0.074
DMAG0001S00003811	Contig3811	Unknown	1.52	0.031
DM04958_1	Contig4172	cytochrome b5	1.86	0.055
DM10334_1	Contig4172	cytochrome b5	2.10	0.086
DMAG0001S00004485	Contig4485	ccaat enhancer binding protein	1.57	0.027
DM08718_1	Contig4485	ccaat enhancer binding protein	2.21	0.031
DM08718_2	Contig4485	ccaat enhancer binding protein	2.61	<0.001
DMAG0001S00004737	Contig4737	thioredoxin domain-containing protein 17	1.74	<0.001
DM11877_1	Contig4737	thioredoxin domain-containing protein 17	1.68	<0.001
DM14953_1	Contig4790	Unknown	1.90	0.086

DM02550_2	Contig5000	aquaporin 3-like	3.67	0.012
DM02550_3	Contig5000	aquaporin 3-like	3.58	<0.001
DM02550_1	Contig5000	aquaporin 3-like	3.39	<0.001
DMAG0001S00005000	Contig5000	aquaporin 3-like	3.25	<0.001
DM05459_1	Contig5232	midline fasciclin	1.78	0.018
DM06798_1	Contig538	hypothetical protein DAPPUDRAFT_303931 [Daphnia pulex]	1.65	0.091
DM09817_2	Contig817	microsomal dipeptidase	1.59	0.086
DM08418_1	Contig854	hypothetical protein DAPPUDRAFT_308669 [Daphnia pulex]	2.45	<0.001
DM08418_2	Contig854	hypothetical protein DAPPUDRAFT_308669 [Daphnia pulex]	2.21	<0.001
DM00993_1	dm009m07.r	trehalase	1.78	0.091
DMAG0001S00008858	dm034j11.f	Unknown	1.91	<0.001
DMAG0001S00009113	dm040h12.f	chk1 checkpoint-like protein	3.00	<0.001
DM04846_2	dm046e14.f	Unknown	1.57	<0.001
DM05943_2	dm051j21.f	aquaporin isoform cra_b	3.67	0.020
DMAG0001S00009682	dm051j21.f	aquaporin isoform cra_b	3.45	0.031
DM01160_2	dm051j21.f	aquaporin isoform cra_b	4.15	0.055
DM05943_1	dm051j21.f	aquaporin isoform cra_b	4.01	0.086
DM09287_1	IGU001_0045_A04.f	Unknown	1.53	0.027

DM09591_1	WTH001_0003_F20.f	Unknown	3.07	0.018
DM09591_2	WTH001_0003_F20.f	Unknown	3.60	0.031
DMAG0001S00005915	WTH001_0003_F20.f	Unknown	3.11	<0.001
DMAG0001S00006450	WTH001_0012_C01.r	hypothetical protein DAPPUDRAFT_45609 [Daphnia pulex]	1.67	0.027
Down-regulation				
DM03522_1	Contig1132	sterol desaturase	0.30	0.045
DM03522_3	Contig1132	sterol desaturase	0.29	0.084
DM03522_2	Contig1132	sterol desaturase	0.25	0.045
DM02956_2	Contig1132	sterol desaturase	0.25	0.084
DM02956_3	Contig1132	sterol desaturase	0.24	0.075
DMAG0001S00001132	Contig1132	sterol desaturase	0.22	0.084
DM02956_1	Contig1132	sterol desaturase	0.19	0.064
DM05290_1	Contig2478	hypothetical protein DAPPUDRAFT_101535 [Daphnia pulex]	0.52	0.064
DM11240_1	Contig2647	Unknown	0.22	0.084
DMAG0001S00003556	Contig3556	camp-dependent protein kinase r2	0.52	0.064
DM08378_2	Contig3556	camp-dependent protein kinase r2	0.49	0.064
DM02646_2	Contig3556	camp-dependent protein kinase r2	0.44	0.097
DM02845_1	Contig4100	2-domain hemoglobin	0.24	0.084

DM11523_2	Contig4100	2-domain hemoglobin	0.12	0.079
DM11523_1	Contig4100	2-domain hemoglobin	0.10	0.079
DM04574_1	Contig4811	hypothetical protein DAPPUDRAFT_305103 [Daphnia pulex]	0.06	0.064
DMAG0001S00006990	dm005p22.r	minichromosome maintenance deficient 8 (cerevisiae)	0.38	0.064
DM05703_1	dm005p22.r	minichromosome maintenance deficient 8 (cerevisiae)	0.37	0.054
DMAG0001S00010206	dm065a02.f	hypothetical protein DAPPUDRAFT_330570 [Daphnia pulex]	0.15	0.064
DM12494_1	dm065a02.f	hypothetical protein DAPPUDRAFT_330570 [Daphnia pulex]	0.14	0.084
DM12494_2	dm065a02.f	hypothetical protein DAPPUDRAFT_330570 [Daphnia pulex]	0.10	0.090
DMAG0001S00005739	WTH001_0001_B21.f	Unknown	0.50	0.076

Supplemental table S3.

Summary of expression patterns of hemoglobin-related gene in response to JH agonists.

Figure legends

Figure 1.

Chemical structure of fenoxycarb and methyl farnesoate (A) and schematic diagram of experimental procedure (B).

Figure 2.

Venn diagram representing the number of up-regulated (A) and down-regulated (B) contigs (probes) with significant expression change (FC > 1.5, $q < 0.1$) between treated and control *Daphnia* for fenoxycarb and methyl farnesoate treatments.