Differentially Expressed Genes in *Daphnia magna* Exposed to Methyl Farnesoate

Shu Shang

Abstract Endocrine disrupting chemicals (EDCs) are chemicals with the potential to interfere with the function of endocrine systems. When accumulated in the environment, they pose a great risk to the healthy functioning of most ecological food chains. Methyl farnesoate, the major terpenoid hormone in insects, could stimulate production of male offsprings in normally asexually produced *Daphnia magna*. Commonly known as water fleas, *D. magna* is a standard Environmental Protection Agency (EPA) species used in water toxicity tests. The methyl farnesoate signaling pathway is especially susceptible to the disruption of external signals. Thus, gene expression changes of *D. magna* under acute exposure of methyl farnesoate were studied to better understand the character of methyl farnesoate signaling pathway. Gene expression profiles for methyl farnesoate exposures were obtained by using a *D. magna* cDNA microarray following each acute exposure at an established effective concentration (EC_{50}). Differentially expressed genes were determined using a statistical method developed in Vulpe lab, UC Berkeley, which employs an outlier approach. Those differentially expressed genes by methyl farnesoate were analyzed in order to determine which genes were similarly affected in different exposures. These genes are likely candidate genes that initiate male production in *D. magna*. The results suggested that this approach could enable more understanding of what genes were involved in methyl farnesoate signaling pathway, as well as further substantiating the uses of toxicogenomic methods in the study of toxicants' effects on environmental organisms.

Introduction

It has been widely established that a wide range of chemicals are capable of disrupting and/or interfering with the endocrine system of animals (Tyler 1998). Environmental contaminants, including organochlorine pesticides (DDT and its metabolites), polychlorinated biphenyls (PCBs) and dioxins (PCDDs and PCDFs), could act as hormone mimics through endocrine systems, which regulate important biological processes in bodies of all mammals, birds, fish, and many other types of living organisms (Kavlock 1999). Collectively, these chemicals with the potential to interfere with the function of endocrine systems are called Endocrine Disruptive Chemicals (EDCs) (Kavlock 1999).

Much attention has been given to the study of EDCs' effects because of the apparent increase in adverse physiological effects which may be indicative of exposure to EDCs. Consequently, the volume of published literature about endocrine disruption in a variety of wildlife species has also significantly increased (Rodriguez *et al*. 2007). However, despite that many endocrine disruption cases having been reported and examined in great detail (Kendall *et al.* 1998), studies on the specific mechanisms by which the endocrine system can be disrupted by external signals are very limited (Rodriguez *et al*. 2007). Therefore, to fully understand the risk of known EDCs, and predict its potentially deleterious effects, more research on the mechanisms of EDCs is needed (LeBlanc 2007).

The ideal organisms for examining possible pollutant-species interactions are Crustaceans. They are one of the most ubiquitous groups of invertebrates constituting 95% of all known animal species in nature (LeBlanc 2007), and have previously been studied in EDC tests (Kendall *et al*. 1998). Major signal transducers in Crustacean endocrine systems are peptide hormones (LeBlanc 2007), which may function as the terminal hormone or as intermediates in a signaling pathway. Methyl farnesoate is the major terminal hormone of Crustaceans. Methyl farnesoate signaling pathway is proposed to mediate many regulatory functions associated with juvenile hormone in Crustaceans (LeBlanc 1999). These functions include larval development, metamorphosis, male reproductive organ development or maturation, and sexual differentiation. As an endogenous hormone, methyl farnesoate is also very susceptible to disruption by environmental chemicals such as its synthetic analogue Pyriproxyfen, which is commonly used as a pesticide in agricultural practices (LeBlanc 2007).

Among all regulatory functions that methyl farnesoate has on Crustaceans, its effects on the physical translation of the genetic sex in *Daphnia magna* have been repeatedly demonstrated (LeBlanc 2002). *Daphnia magna* is the EPA model organism used in water toxicity tests. It commonly goes through cyclic parthenogenesis in which the *Daphnia* population employs both asexual and sexual reproduction (Hebert 1978). Under favorable environmental conditions, the population exists as genetically uniformed female clones. Under specific environmental cues that forecast the unsuitability of the habitat (less light, scarce food resources or over-crowded population size), the females will produce male offsprings which are followed by the entry of the population into a sexual reproductive phase (Hebert 1978). In particular, high levels of methyl farnesoate exposure in the maternal *Daphnia* could also result in the production of male offsprings (Olmstead and LeBlanc 2003). However, the methyl farnesoate signaling pathway is rather poorly understood and only a small number of studies confirm that the disruption of this endocrine signaling pathway exists (LeBlanc 2007).

Therefore, recognizing the effects of methyl farnesoate on *D.magna* at the molecular level enables one to understand how EDCs interact with this methyl farnesoate signaling pathway. This can be achieved by toxicogenomics, the emerging sub-discipline of toxicological approaches (Nuwayris *et al.* 1999). Toxicogenomic methods normally combine the classical toxicity testing and one genetic approach which assesses genetic response changes either at the transcriptional or the translational level (Poynton 2007). As the genomic information for *D .magna* has become available, the application of toxicogenomics to *D. magna* could become a powerful research tool for evaluating the effects of hormonal chemicals on Crustaceans.

Based on my research goals, three research questions were developed:

1. Does methyl farnesoate initiate male reproduction in sexually matured *Daphnia magna* under standard laboratory conditions?

2. What are some most responsive genes in *Daphnia magna* to methyl farnesoate exposure?

3. Can toxicogenomics be used to help evaluating methyl farnesoate signaling pathway in a representative Crustacean species?

Through these research questions, I hope to understand the molecular basis of the one Crustacean endogenous hormone pathway that can be disrupted by the presence of EDCs in the surrounding. I hypothesized that differentially expressed *D. magna* genes under methyl farnesoate exposure were involved in *Daphnia* sex determination mechanism.

To find genes responsive to external methyl farnesoate signals, I compared gene expression profiles before and after a 24-hour acute methyl farnesoate exposure. cDNA microarray techniques, which provide us with a diagnostic tool to screen the many variables required to examine gene-expression patterns (Iguchi *et al.* 2006), were employed as the major method to compare the relative mRNA transcript levels between different biological samples. When looking for differentially expressed genes in methyl farnesoate exposed and untreated *Daphnia*, up-regulated (over-expressed) and down-regulated (repressed) *Daphnia* genes were identified.

Methods

Male Production in Response to Methyl Farnesoate In order to demonstrate that methyl farnesoate induces male production in *D. magna* during their embryonic development stage, 20 7- to 8-day-old *D. magna* were exposed to 200 nM methyl farnesoate for 24 hours. The methyl farnesoate solution was made by mixing 200 µl methyl farnesoate stock solution into 800ml fresh Daphnia media. After each exposure, the sex of individual newborn *Daphnia* offspring were examined under a dissection microscope (10x magnification) according to their differences in mouth shapes (Olmstead and LeBlanc 2000).

cDNA Microarray comparing differentially expressed genes before and after methyl farnesoate exposure In a different group, 20 7- to 8-day-old *D. magna* were exposed to 200nM methyl farnesoate for 24 hours. Immediately after the exposure and before they started to produce babies, all live *D. magna* determined by mobility were collected for microarray study. RNAs were isolated from the same numbers of unexposed *D. magna* at the same time, in order to be used as control for the microarray experiments. For RNA isolation, *D. magna* were harvested by gently removing each *Daphnia* from the culture individually and immediately grinding them in liquid nitrogen using a pestle and mortar. RNA was isolated using the standard Trizol (Invitrogen, Carlsbad, CA, USA) method. Three replicate exposures were performed for each chemical treatment time point. cDNAs were synthesized using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) from isolated RNA. Fluorescence labeling was

proceeded by incubating the amino-allyl labeled cDNA with *Cy5* or *Cy3* fluorescent dyes (Amersham Biosciences, Piscataway, NJ, USA), to give each group of RNA a distinguished fluorescent signal intensity during dye-coupling. For each exposure experiment, the RNA from the exposed and unexposed *D. magna* were hybridized twice to two microarray glass slides, each containing the complete cDNA genomic library of *D.manga*. A total of six replicates were done for each methyl farnesoate exposure. The dyes were switched for each technical replicate so that the control cDNA once labeled with *Cy3* in one hybridization would be labeled with *Cy5* in the other. The microarray slides were scanned using ArrayWorx Biochip Reader (Axon Instruments, Morgan Hall Room 58), which is used to detect the surface-generated fluorescence by dyed cDNA samples. Single gene spots were identified using GenePix 6.0 (Axon Instruments, Morgan Hall Room 58), a software program developed for the usage of signal intensity quantifications in cDNA microarray image processing. Candidate differentially expressed cDNAs were identified using the α-outlier-generating model and the outlier regions approach based on methods developed by Loguinov *et al.* Differentially expressed genes were sequenced at the UC Berkeley Sequencing facility. Gene expression profiles were used to determine genes that were differentially expressed during the methyl farnesoate treatment based on a homology search.

Results

Male Production in Response to Methyl Farnesoate When 20 7- to 8-day-old *Daphnia* were exposed to effective concentration of methyl farnesoate added media for 24 hours, no male progeny were produced in the control group. While in the methyl farnesoate exposed group, male offsprings were produced two days after the exposure and this male production lasted for four days (Table 1). In the exposed group, the percentage of male offsprings in the new broods increased with the exposure time, until ≥120 hours (Table 1b, Fig. 1).

Table 1a. Stimulation of male production after 24-hour 0nM methyl farnesoate exposure, n=20. In the control group, no male progeny was found in the neonates.

		0-24	24-48	48-72	72-96	96-120	120-144	144-168
Male	Numbers	0	0	0	0	0	0	0
	%	0	0	0	0	0	0	0
Female	Numbers	5	32	28	28	36	13	4
	%	100	100	100	100	100	100	100

Table 1b. Stimulation of male production after 24-hour 200nM methyl farnesoate exposure, n=20. In the exposed group, male offspring was produced within 48-144 hours after the exposure.

		0-24	24-48	48-72	72-96	96-120	120-144	144-168
Male	Numbers	0	0	14	11	16	1	0
	%	0	0	52	58	80	33	0
Female	Numbers	23	4	13	8	4	2	7
	%	100	100	48	42	20	67	100

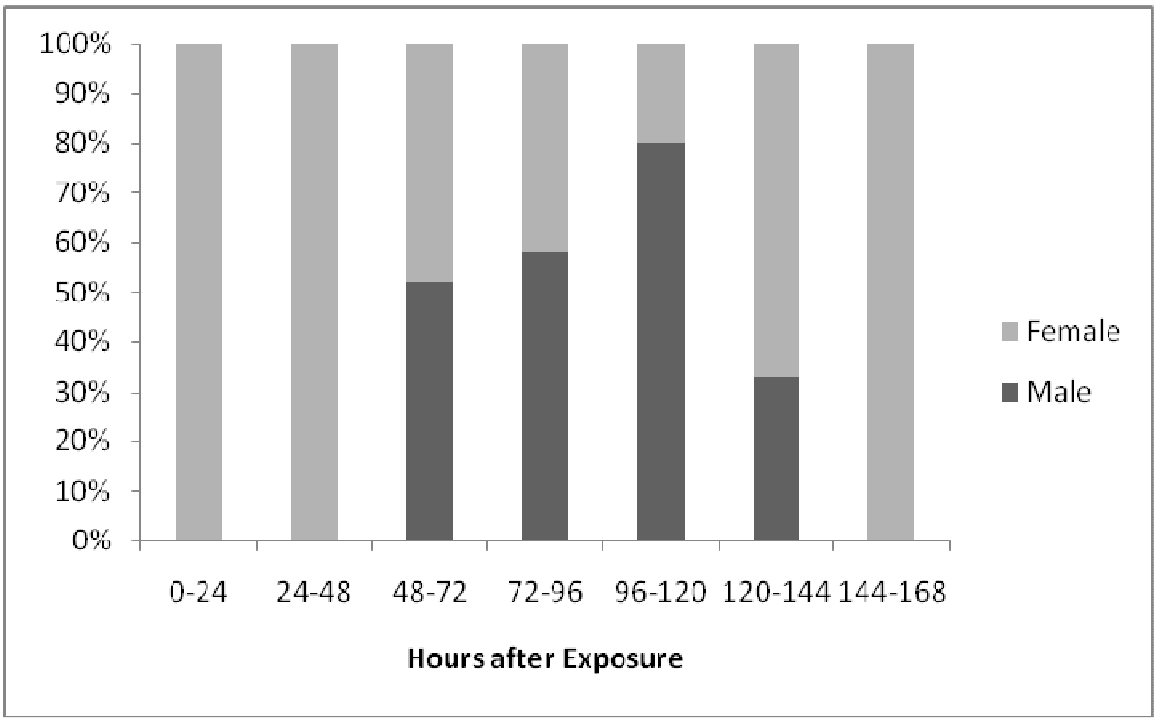

Figure1. Stimulation of male production after 24-hour 200nM methyl farnesoate exposure, n=20. Male offspring percentage in neonates population increased 48-120 hours after the exposure, decreased thereafter, till male production returned to zero 144 hours after the exposure.

Differentially expressed before and after methyl farnesoate exposure Overall, 129 of the 5000 gene fragments were differentially expressed after methyl farnesoate exposure. Homologies were found for 7 of them by referring to *Daphnia* gene lists prepared by Vulpe lab, UC Berkeley (Table 2). Considering selected gene information, 4 up-regulated and 3 down-regulated genes were found.

Table 2. Predicted differentially expressed genes after 24-hour 200nM methyl farnesoate exposure in *Daphnia magna*.

Gene Regulation	Accession Clone	Gene Most Similar To	Protein Function
Up-	DV 437806	Protein Phospohtase	Cell Signaling Pathway
Up-	DV 437853	Trypsin	Protease
Up-	DV 437800	Protein Phospohtase	Cell Signaling Pathway
Up-	DV 437809	GST	Oxidative Stress Pathway
Down-	DV 437828	Protein Kinase	Cell Signaling Pathway
Down-	DV 437832	Ras-related Protein	Cell Signaling Pathway
Down-	DV 437886	Cuticle Protein	Exoskeleton Related Protein

Discussion

Olmstead and LeBlanc (2000) successfully demonstrated the ability of methyl farnesoate as a sex differentiating agent in *D. magna,* by identifying several male secondary sex characteristics in *Daphnia* development after acute methyl farnesoate exposure. My results are consistent with their finding: methyl farnesoate could stimulate the production of male progeny in sexually matured *Daphnia* immediately after the 24-hour exposure (Fig. 1b). By incorporating toxicogenomic methods, especially cDNA microarray techniques that are now available after oligonucleotide-based DNA microarray has been developed for *D. magna* (Watanabe *et al.* 2007), I was able to identify several functionally known genes that are especially susceptible to methyl farnesoate exposures.

Effects of methyl farnesoate on sexual differentiation It has been demonstrated that the juvenile hormone analogue (JHA) methyl farnesoate could stimulate production of male progeny among neonates (Olmstead and LeBlanc 2000). The exposure of sexually matured maternal *Daphnia* to methyl farnesoate was reported to have altered sex ratios of offspring in favor of males (Olmstead and LeBlanc 2001). However, I previously found that methyl farnesoate could not stimulate male production outside a narrow developmental window (7- to 8-day-old), which overlaps with oocyte maturation period in *D.magna*. This indicates that methyl farnesoate might initiate the male production pathway by stimulating oocyte maturation, which is a common trait shared by different classes of Crustacean JHAs.

While the production of male progeny stopped 144 hours after the exposure in methyl farnesoate exposed population, no male progeny was found in the control population (Table 1a, Fig. 1) throughout the experiment. This observation demonstrated that the production of male progeny is not generated in response to other detectable environmental changes, such as reduced photoperiod or food recourses, since these variables were held same for both populations.

Furthermore, although it has been suggested that methyl farnesoate may affect sex differentiation indirectly by affecting the overall growth of *D. magna* (Olmstead and LeBlanc 2003), I did not observe significant abnormalities associated with other aspects of *Daphnia* reproduction, such as reduction in brood size, time of the first brood, length of mothers and length of babies etc. Thus, whether the stimulation of male production is one independent downstream event in the methyl farnesoate signaling pathway or not is still unknown. Further studies could emphasize more on other phenotype changes in maternal *Daphnia* after methyl farnesoate exposure, in the attempt to establish the relationship between the production of male offsprings and other methyl farnesoate mediated growth or reproductive changes in *D. magna*.

Methyl farnesoate induced gene profiles I hypothesized that differentially expressed genes in methyl farnesoate exposure were involved in sex determination mechanism in *D. magna*. This hypothesis was neither fully supported nor refuted by my results on gene expression changes.

Among all identified differentially expressed *Daphni*a genes in methyl farnesoate exposure, four played a role in *Daphnia* cell signaling pathway, including a down-regulated ras-related gene usually associated with cell proliferation (Table 2). In the crayfish *Cherax quadricarinatus*, it was found that methyl farnesoate stimulates ovarian protein kinase C (PKC) (Soroka *et al*. 2000). It was also previously found that thymidine incorporation by ovaries also increased in response to methyl farnesoate exposure, suggesting that DNA synthesis was stimulated in this duration (Soroka *et al* 1997). Since most functions methyl farnesoate play in Crustaceans reproduction have been linked to its stimulatory function in ovarian maturation (Olmstead and LeBlanc 2003), it is very likely that this methyl-farnesoate-mediated sex determination is integrated into other cell growth and proliferation pathways.

It was also noticed that one up-regulated gene played a role in the oxidative stress pathway (Table 2), which involves changes in energy usage before and after the exposure. This is

consistent with the finding that the amount of energy distributed towards growth and reproduction is reduced due to external chemical stresses (Soetaert et al., 2007). Thus, it is reasonable to assume that generally genes related to some anabolic pathways would be differentially expressed in response to EDC exposures.

Other identified genes included an up-regulated protease gene and a down-regulated cuticle gene, which respectively played a part in *Daphnia* digestive system and exoskeleton function (Table 2). How these genes could interfere with the *Daphnia* sex determination pathway is yet unknown.

Takac *et al.* (2003) suggested that methyl farnesoate acted directly on ovarian maturation as a result of a methyl farnesoate receptor, methyl farnesoate binding protein or MFBP, on the ovarian tissue. But no receptor that is responsible for methyl farnesoate signal transduction has been identified (Olmstead 2007). Since RXR (Retinoid X Receptor) has been implicated as a receptor for juvenile hormone in *Drosophila* (Jones and Sharp 1997), it is possible for Crustacean RXR to play a similar role in *D. magna* in response to methyl farnesoate. However, I was undetermined based on available information of *Daphnia* genome whether any gene identified is a downstream gene of RXR in *D. magna*. Thus, I was not able to predict the presence or the functions of RXR or MF binding protein in *D. magna.*

Olmstead and LeBlanc (2000) also suggested the regulation of male offspring production of methyl farnesoate implied that *Daphnia* possess sex determining genes, such as those similar to sex-1 genes in *C. elegans* or dsx genes in *Drosophila*. However, I was not able to identify these genes in my cDNA microarray analysis.

cDNA microarray as a powerful tool in studying endocrine disruption chemicals (EDCs) in *D.magna* Despite that toxicogenomic approach has been applied to the study of model mammals (Watanabe *et al*. 2007), its uses on aquatic Crustaceans have not been adequately studied.

Although acute and chronic toxicity tests on *Daphnia* have been used widely for aquatic toxicology (Iguchi *et al* 2006), conflicting results on the molting frequency, brood sizes, physical characters of *D. magna* adults and neonates following exposure to endocrine disruptive chemicals have been reported (Caspers 1998), making the specific detrimental effects of EDCs

hard to analyze. Thus, profiling of DNA transcripts, protein products, and metabolites which are helpful in generalizing the modes of action of EDCs should be effectively provided (Iguchi *et al*. 2006) for understanding the molecular basis of how they work in invertebrates. In this study, cDNA microarray techniques were selected as the study tool due to their ability to analyze the expression of a large number of genes simultaneously (Iguchi *et al*. 2006). It turns out that cDNA microarray is a very efficient technology in predicting mechanistic, diagnostic and predictive information by providing profiles of differentially expressed genes in *D.magna* after methyl farnesoate exposure (Table 2), which further substantiates the uses of cDNA microarray techniques in EDC toxicity studies.

It is also notable that cDNA microarray analysis took much less time compared to similar reproductive toxicological analysis. The general protocol for reproductive toxicity test takes 21 days for each concentration of any chemical, and provides no more insightful results than the effective concentration (EC_{50}) for the chemicals of interest in cDNA microarray analysis (Watanabe *et al*. 2007). The cDNA microarray takes less than a week, and characteristic profiles of EDCs at the genetic level can be obtained (Table 2).

However, since the gene sequence information for *D. magna* has not been completed (Watanabe *et al.* 2007), my results could only be based on current information on *Daphnia* gene segments, making it hard to connect all information together in a productive way. For instance, although Olmstead (2003) suggests that methyl farnesoate receptors may regulate the sex determination as well as hemoglobin level, since gene sequence information for hemoglobin genes are not yet available, my research was unable to link these two inter-related parts together. Future research should address the inadequacies in gene sequence information of *D.magna.* cDNA library construction for a larger variety of environmental organisms, including Crustaceans, will help future research.

To summarize, cDNA microarray techniques have a clear value in elucidating molecular mechanisms of environmental contaminants and for the development of specific biomarker genes in exposure assessments. My identified methyl farnesoate responsive genes suggested that this endogenous hormone played various roles in *D. magna* metabolism, besides its involvement *Daphnia* sex determination mechanism. In addition, incorporating my identified methyl farnesoate sensitive genes as potential biomarkers will enable scientists further understand how

methyl farnesoate signaling pathway can be altered by adverse effects of EDCs. Moreover, my results also substantiated the usage of cDNA microarray as a powerful analytical tool to study EDCs in aquatic Crustaceans, which could provide scientific researchers with a better evaluation of the toxic effects of certain specific chemicals on environmental organisms. Once the genomic library construction for *Daphnia* is completed, it could be used to examine the molecular effects of various toxicants and predict their modes of actions based on associated gene expression patterns.

Acknowledgement

I would like to take this chance to thank Professor Chris Vulpe, all members in Vulpe Lab but especially Dr Seonock Woo, Dr Helen Poynton, Rich Zuzow and William Jo, who have given me numerous supports at different stage of this project; I would like to thank instructors of ES 196, who supervised this project from the beginning till this point with their brilliant comments and suggestions; I would also like to thank URAP staff, my ES advisor Susan Kishi, my MCB faculty advisor Professor Robert Beatty, Professor Karsten Weis, Professor Leonard Bjeldanes and all other people who have generously helped me to work on this project.

References

Hebert, P.D. 1978. The population biology of Daphnia Crustacea Daphnidae. Biological Reviews of the Cambridge Philosophical Society. 53:3:387-426.

Iguchi, T., Watanabe, H., and Katsu, Y. 2006. Application of Ecotoxicogenomics for Studying Endocrine Disruption in Vetebrates and Invertebrates. Environmental Health Prospect. 114:101-105

Jones, G. and Sharp, P. 1997. Ultrapiracle: an invertebrate nuclear receptor for juvenile hormones. Proceedings of the National Academy of Sciences of the United States of America. 94:25:13499-13503.

Kashian, D. and Dodson, S. 2002. Effects of common-use pesticides on developmental and reproductive processes in Daphnia. Toxicology and industrial health. 18:225-235.

Kavlock, R.J. 1999. Overview of endocrine disruptor research activity in the United States. Chemosphere 39:8, 1227-1236

Kendall, R., Dickerson, R., Giesy, J., Suk, W.(Eds). 1998. Principles and processes for evaluating endocrine disruption in wildlife. SETAC Technical Publications. SETAC Press. 491.

Kilham, S., Kreeger, A., Lynn, S., Goulden, C. and Herrera, A. 1998. COMBO: a defined freshwater culture medium for algae and zooplankton. Hydrobiologia 377:147-159.

LeBlanc, G.A. 2007. Crustacean endocrine toxicology: a review. Ecotoxicology 16:61-81

Loguinov, A., Mian, I. and Vulpe, C. 2004. Exploratory differential gene expression analysis in microarray experiments with no or limited replication. GenomeBiology.com 5: 18-52.

Nuwaysir, E.F., Bittner, M., Trent, J. Barrett, J.C., and Afshari, C.A. 1999. Microarray and toxicology: the advent of toxicogenomics. Molecular Carcinogenesis. 24:3:153-159.

Olmstead, A. and LeBlanc, G. 2003. Insecticidal juvenile hormone analogs stimulate the production of male offspring in the crustacean Daphnia magna. Environmental health perspectives 111:919-924.

Olmstead, A. and LeBlanc, G. 2002. Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean Daphnia magna. Journal of experimental zoology 293:736-739.

Poynton, H, Varshavsky, J., Chang, B., Chan, S. and Vulpe, C. 2007. *Daphnia magna* ecotoxicogenomics provides mechanistic insights into metal toxicity. Environmental science technology 41:1044-1050.

Riger C. and LeBlanc, G. Stress Signaling: Coregulation of Hemoglobin and Male Sex Determination through a Terpenoid Sigaling Pathway in a Crustacean. 2004. The Journal of Experimental Biology 208:15-23

Rodriguez, E.M., Medeani, D.A., and Fingerman, M. 2007. Endocrine disruption in crustaceans due to pollutants: a review. Comparative Biochemistry and Physiology. 146:661-671.

Takac, M., Krylov, V., and Jonak, J. 2003. The c-src1 gene visualized by in situ hybridization on *Xenopus laevis* chromosomes. Cytogenetic and Genome Research. 103:1-2:169-172.

Trudeau, V. and Tyler C. 2007. Endocrine Disruption: Discussion. General and Comparative Endocrinology. 153: 13-14

Tyler, C., Jobling, S., and Sumpter, J. 1998. Endocrine Disruption in Wildlife: A Critical Review of the Evidence. Critical Reviews in Toxicology 28:4, 319-361

Soetaert, A., Ven, K., Moens, L., Vandenbrouck, T., Remortel, P., and De Coen, M. *Daphnia* Magna and Exotocicogenomics. 2007. Chemosphere 67:60-71

Watanabe, H., Takahashi, E., Nakamura, Y., Oda, S., Tatarazako, N., and Iguchi, T. Development of a *Daphnia Magna* DNA Microarray For Evaluating The Toxicity of Environmental Chemicals. 2007. Environmental Toxicology 26:4, 669-676