ABSTRACT

EYTCHESON, STEPHANIE ANN. The Role of the Nuclear Receptor E75 in the Molt Cycle of Daphnia magna and Consequences of its Disruption. (Under the direction of Dr. Gerald A. LeBlanc).

In this research, we investigated the role of E75 in regulating the timing of the molt cycle and impacts of its disruption by exogenous sources of nitric oxide. The hypothesis tested was that the nuclear receptor E75 is an early regulator of the molt cycle and its disruption can have adverse population-level impacts. This hypothesis was evaluated by testing the following sub-hypotheses: 1) proteins can be effectively targeted and suppressed in D. magna using a dsRNA feeding approach, 2) suppression of E75 results in disruption of the molt cycle and may adversely impact reproduction and development, and 3) exogenous sources of NO adversely affect daphnid population dynamics through interaction with E75. In order to determine the function of E75 in the molt cycle, a novel RNAi approach that involved feeding E. coli expressing E75 dsRNA was used to suppress E75 levels in D. magna. Hemoglobin was targeted to develop the procedure because changes in hemoglobin levels result in distinct color changes in the organism that could be monitored during feeding the dsRNA. Exposure of daphnids to pyriproxyfen increased hemoglobin protein levels and resulted in copper coloration of the animals. Feeding daphnids E.coli that expressed the dsRNA targeting hemoglobin suppressed hemoglobin mRNA levels and blocked this coloration. We also demonstrated that daphnids with suppressed hemoglobin levels were sensitized to the toxicity of nitrite. We next examined the sequence of mRNA accumulation of gene products hypothesized to be involved in regulating the molt rhythm, some of which may be regulated by E75. Consistent with our model, the sequence of maximum RNA accumulation occurred in the order of E75, HR3, then FTZ and CYP18a1. Suppression of E75 had no effect on mRNA levels of HR3, increased levels of FTZ, and
suppressed levels of CYP18a1. Further, E75 suppression increased the length of the molt cycle and reduced the number of offspring produced. Finally, we evaluated the effects of exogenous sources of nitric oxide on population dynamics of *D. magna*. Nitric oxide binds E75 and suppresses its activity. Thus, exposure to nitric oxide donors should mimic the effects of E75 suppression. Individual daphnids were first exposed to sodium nitroprusside or sodium nitrite to identify exposure concentrations that elicited subtle effects on the organisms. Results of these experiments were used to identify concentrations of these compounds that may not be recognized as toxic based upon effects on individuals, but combined effects would elicit significant adverse effects on populations. Exposed and control populations were periodically evaluated for numbers of individuals in sorted size classes using convolutional neural networks and machine learning. Contrary to our predictions, sodium nitrite elicited no significant adverse effect on population growth. The lack of effect was due, at least in part, to a positive effect of the chemical on expansion of the juvenile daphnid populations. Nitrite stimulated algae growth, providing increased nutrition to the organisms. Exposure to sodium nitroprusside resulted in a reduction in growth rate and carrying capacity of daphnid populations. Results of this research program support the hypothesis that E75 is an early regulator of the molt rhythm and that disruption of E75 can elicit deleterious effects on individual daphnids that may result in population-level consequences.
The Role of the Nuclear Receptor E75 in the Molt Cycle of *Daphnia magna* and Consequences of its Disruption

by

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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Toxicology

Raleigh, North Carolina 2018

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DEDICATION

I dedicate this work to my family.
BIOGRAPHY

Stephanie Ann Eytcheson was born December 31, 1989, in Peoria, Illinois, to Madea Everette and Tom Eytcheson. Eventually her family grew to include her brother Adam, her step-father Randy, her step-mother Dawn, and her step-sister Taylor. Stephanie spent the first years of her life in Illinois and Indiana until she moved to South Carolina in third grade. Throughout her schooling Stephanie was interested in all subjects, but in high school she discovered her real passion for science. It was during this time that her chemistry teacher, Mrs. Gilstrap, inspired her to pursue her interests in chemistry. Having taken every science class offered, Stephanie won an award at the end of her senior year for having the highest science GPA among the students. Following graduation, Stephanie moved to Waco, Texas, to attend Baylor University. While at Baylor, Stephanie was afforded the opportunity to pursue all of her interests. In her junior year she sought a laboratory to gain experience and met Dr. Bryan Brooks. It was in his lab where she learned about aquatic toxicology and decided it would be a good area to apply her background in chemistry. With the help of Dr. Brooks and her lab mates at Baylor, Stephanie was able to present a poster at the North American SETAC meeting in Boston in 2011. It was here that she met Dr. David Buchwalter and learned about the Toxicology Program at NCSU. After graduating from Baylor with a Bachelor of Science in Chemistry with a minor in Environmental Studies, Stephanie moved to Raleigh, NC, to pursue her Ph.D. in Environmental Toxicology. In January 2013, Stephanie officially joined Dr. LeBlanc’s lab. Stephanie hopes to use the experiences and knowledge gained during her time in the lab to advance our understanding of toxicology to improve the environment for future generations and provide clean water worldwide.
ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Gerald A. LeBlanc for his guidance and support over the last few years. I remember meeting him for the first time when he picked me up during my visit to NC State like it was yesterday. I had no idea what the next years would entail, but I am grateful for everything I have learned from him.

I would also like to thank my committee members: Dr. David Buchwalter, Dr. Michael Bereman, Dr. Mike Roe, and Dr. Kevin Flores for their input and guidance on this project.

I would like to extend a special thanks to Gwijun Kwon for her patience and kindness while offering me technical and moral support.

Thank you to all the past and present members and visitors to the LeBlanc lab for support and encouragement. Thanks go to Lihui An, Steph Street, Allison Camp, Jeonga Yun, Junmin Gao, Helen Yang, Maher Haeba, Huahong Shi, Charisse Holmes, Elizabeth Medlock Kakaley, and the undergraduate students Julia Roberson, Elizabeth Keel, Alexis Cacchione, and Zakkiyah Majeed.

Thank you to the teachers who have inspired me along the way, especially Mrs. Gilstrap and Dr. Bryan Brooks, for helping shape my interests in chemistry and aquatic toxicology.

Without the encouragement and support from my parents, my grandparents, my brother and stepsister, and the rest of my family I would not be who I am or where I am today. Thank
you all for your love over the years. Also, thank you to Percival for being a belly to cry on in times of stress and a comforting purr to cheer me up, and to Oketa for making me laugh with her sink antics.

Finally, I would like to thank TJ Kelly for his love and encouragement over the last three years. Thank you for listening to me talk science, tolerating my occasional grumpiness, and always cheering me up!
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INTRODUCTION

Endocrine signaling and disruption

The endocrine system is essential to control a variety of physiological functions such as metabolism, production of cells, stress response, reproduction, and development. The endocrine system controls these functions by producing hormones which serve as messengers that inform cells using receptor-mediated processes.

Because the endocrine system is responsible for regulating critical physiological processes, it is important to define endocrine signaling pathways as well as critical targets within these pathways which may be impacted by endocrine disrupting chemicals (EDCs). The World Health Organization defines an EDC as “an exogenous substance or mixture that alters functions of the endocrine system, and, consequently, causes adverse health effects in an intact organism, or its progeny, or (sub)populations”. EDCs can interfere with hormone signaling in a variety of ways, such as activating or inactivating hormone receptors or by altering synthesis or inactivation/elimination of hormones. The US EPA’s Endocrine Disruptor Screening Program was implemented in 1998 to screen drugs and chemicals for their ability to disrupt estrogenic, androgenic, and thyroid hormone-regulated processes; however, environmental contaminants may interact with other hormone signaling processes. Additional hormone/receptor pathways shown to be susceptible to endocrine disruption include the retinoid signaling pathway, vitamin D signaling pathway, peroxisome proliferator-activated receptor (PPAR) pathway, and the ecdysteroid signaling pathway. Additional axes that may be susceptible to endocrine disruptors include the hypothalamic:pituitary:adrenocortical axis, the hypothalamus:pituitary:gonad axis, hypothalamus:pituitary:thyroid axis, and the somatotropic axis.

Nitrogen oxide as a signaling molecule
Recent studies have shown that nitric oxide plays an important role in the endocrine and other systems. Nitric oxide is a potent signaling molecule that plays a role in almost every biological system and, as such, needs to be tightly regulated. In fact, the relationship between nitrate, nitrite, and nitric oxide has been described as its own endocrine system to maintain nitric oxide bioavailability with or without classical nitric oxide synthase activity. Nitric oxide acts like a hormone as it is produced in tissues then transported to target cells in the form of nitrite or S-nitrosothiol. Nitric oxide has been shown to be an endocrine disruptor in zebrafish by down regulating tyrosine hydroxylase through its interaction with estrogen receptors in dopaminergic neurons. Further, it has been shown that testicular and adrenal steroidogenesis are negatively regulated by nitric oxide. Nitric oxide also plays a role in vasodilation, neural transmission, reproduction, apoptosis, and lipolysis.

Some nuclear receptors (e.g. rev-erb, RXR) utilize heme as a ligand, and binding of nitric oxide to the heme regulates receptor activity. RXR is able to heterodimerize with nuclear receptor partners to activate enhancer regions containing direct repeat motif sequences; however, heme can bind to the ligand binding domain of RXR to suppress its ability to activate target gene expression. A role for nitric oxide in the regulation of RXR via its heme moiety has yet to be established. Nitric oxide may function as a negative regulator of gene expression by inhibiting the DNA binding activity of transcription factors containing zinc-fingers, cysteine residues near or in the DNA-binding domain, or redox-sensitive transcription factors. In general, disruption of zinc finger domains by nitric oxide results in loss of function. While nitric oxide may not meet the formal definition of a hormone, it certainly functions as an important regulator of endocrine function.
Environmental nitrate and nitrite

Nitrogen comprises seventy-eight percent of the Earth’s atmosphere. Most organisms lack the ability to break the bonds of $N_2$, thus most atmospheric nitrogen is biologically unavailable\textsuperscript{12}. Nitrifying bacteria are able to process atmospheric nitrogen into biologically active forms by “fixing” $N_2$ to $NH_4^+$ which can subsequently be oxidized to nitrite ($NO_2^-$) and then nitrate ($NO_3^-$)\textsuperscript{13}. These compounds are assimilated by plants or converted back to atmospheric nitrogen by denitrifying bacteria; however, the increase in nitrogenous compounds in the environment through anthropogenic activities has altered the nitrogen cycle resulting in increased amounts of biologically available nitrogen\textsuperscript{14–16}.

Levels of nitrogenous compounds are on the rise due to anthropogenic sources such as the use of nitrogen-based fertilizer, the mining and burning of fossil fuels, and runoff from concentrated animal feeding operations (CAFOs)\textsuperscript{17–19}. As of 2001, 83 million tons of nitrogen were fixed by anthropogenic means for use as fertilizer\textsuperscript{20}, and it was predicted that 135 million metric tons would be released by 2030 in response to the demand for food production\textsuperscript{17}. In 2007, it was reported that 133 million tons of manure were released from CAFOs annually\textsuperscript{21}; however, this estimate has increased to hundreds of millions of tons as there are more than 60,000 CAFOs in the United States\textsuperscript{22}.

Limits have been set by the World Health Organization as well as by individual countries to protect sensitive subpopulations from acute exposure to nitrates and nitrites. The WHO drinking water limits for nitrate and nitrite are 50 mg/L and 3.0 mg/L, respectively\textsuperscript{23}. In the United States, the EPA has set similar limits with the maximum allowable limit of nitrate being 10 mg N/L (about 45 mg nitrate/L) and nitrite being 1 mg N/L (about 3 mg nitrite/L)\textsuperscript{24}. These limits have been set to protect human health, but excess nitrate and nitrite have deleterious
effects on the environment as well such as the stimulation of eutrophication and the production of dead zones\textsuperscript{12,25,26}.

\textit{Mechanisms of Nitrite Toxicity}

Humans are exposed to nitrates and nitrites via ingestion of water, food, and pharmaceuticals. Once ingested, nitrate can be converted to nitrite by microorganisms along the gastrointestinal tract\textsuperscript{27}. The primary mechanism of toxicity of nitrite is methemoglobinemia. Nitrite is able to bind to hemoglobin resulting in reduction of the iron in the heme moiety from the ferrous form of the ion to the ferric ion thus becoming methemoglobin. Oxygen is unable to bind to the ferric form of iron resulting in reduced oxygen transport to cells, asphyxiation, and death. Children are susceptible to methemoglobinemia due to their low levels of NADH-cytochrome \textit{b5} reductase which converts methemoglobin back to hemoglobin\textsuperscript{28}. In addition to methemoglobinemia, nitrates and nitrites are implicated in other human health ailments such as cancers (oral, gastrointestinal, etc.), Alzheimer’s disease, dementia, multiple sclerosis, and thyroid issues\textsuperscript{19}.

These health impacts are significant, and exposure limits have been set, based primarily upon methemoglobinemia, to protect humans against nitrate and nitrite toxicity; however, these limits are often exceeded in water and do not necessarily protect organisms residing in aquatic environments. As such, the EPA has established ecoregional nutrient criteria to protect against loss of diversity among biological communities, loss of productivity and stability, and prevention of eutrophication\textsuperscript{29}. The United States is divided into fourteen ecoregions with the recommended EPA criteria for total nitrogen (mg/L) ranging from 0.10 to 1.27 in lakes and reservoirs and 0.12 to 2.18 in rivers and streams\textsuperscript{30}. 
Impacts of nitrogen oxides on heme-containing nuclear receptors have been largely unexplored. Several studies indicate that exposure to nitrogen oxides results in disruption to reproductive endocrinology. Guillette and associates showed that alligators residing in lakes containing excess levels of nitrate had reduced plasma testosterone levels. Environmentally relevant levels of nitrate have also been linked to smaller ovarian follicles and suppressed ovarian steroid synthesis in *Xenopus laevis*. Further, reproductive toxicity was observed in *Ceriodaphnia dubia* at concentrations less than the allowable limits set by the WHO and US EPA with EC50 values of 21 mg N/L and 0.22 mg N/L of nitrate and nitrite respectively. A correlation between reproductive impairments in mosquitofish *Gambusia holbrooki* and exposure to nitrate has also been reported.

Toxicity from nitrate and nitrite in the environment can occur either indirectly or directly. Indirect toxicity to aquatic organisms occurs when nitrate and nitrite alter the environment in which these organisms live. Excess nitrogen resulting from high levels of nitrate and nitrite can result in massive algae blooms (eutrophication). Death of the algae stimulates the growth of microorganisms increasing the biological oxygen demand of the ecosystem and causing a decline in dissolved oxygen levels. These reduced dissolved oxygen levels can result in dead zones where resident organisms either evacuate or die. Today there are over 400 dead zones worldwide. Another consequence of environmental nitrates and nitrites is acidification of aquatic ecosystems which impacts organisms living in these environments by altering the solubility of important minerals. Specifically, acidification of aquatic ecosystems impacts the concentration of calcium carbonate and can subsequently reduce calcium deposition in the shells of some mollusk species. Further, it has been shown that reduced availability of carbonate results in disorientation of calcite crystals in the shells of mussels which reduces the structural
integrity of the shell and limits its ability to protect against predators and environmental changes. Additionally, the reduction of calcium levels during periods of acidification is linked to the decline of daphnid populations due to reduced survival and fecundity.

Direct toxicity of nitrate and nitrite might present as mortality in response to exposure to these chemicals or as a reduction in general health, fecundity, or developmental abnormalities in offspring. These consequences may occur as a result of nitrogen oxides acting as endocrine disrupting chemicals. An important mechanism by which nitrate and nitrite may act as EDCs is the conversion of these nitrogen oxides to nitric oxide, an important signaling molecule.

Endogenous nitric oxide is produced by nitric oxide synthase from L-arginine and oxygen. Nitric oxide plays a role in many biological processes including immunity, vasodilation, and circadian rhythm. Among these processes there are numerous targets to which nitric oxide can bind, including heme moieties, iron and zinc clusters, or cysteine residues; thus, regulation of nitric oxide levels is vital for proper biological function. Exposure to sufficiently high levels of nitrates or nitrites and their conversion to nitric oxide may disrupt these processes.

In addition to in vivo conversion to nitric oxide, nitrite can be converted to nitric oxide in anoxic or low pH conditions. As previously mentioned, nitrogen oxides contribute to eutrophication which can result in hypoxia, thus facilitating the conversion of nitrate/nitrite to nitric oxide. Further, environmental nitrogen oxides aid in acidification of aquatic ecosystems which results in increased nitric oxide levels.

*The Ecdysteroid Signaling Cascade*

Ecdysteroids have been shown to induce gene expression of the nuclear receptors HR3 and E75. The ecdysteroid signaling cascade begins with a pulse of the ecdysteroid 20-
hydroxyecdysone (20E). The decrease in 20E is responsible for initiating exuviation and brood release in daphnids. This ecdysteroid pulse also induces gene expression of the nuclear receptors E75 and HR3 in daphnids and *Drosophila*. In *Daphnia magna* HR3 and E75 dimerize, and E75 serves as a negative regulator for HR3. In *Drosophila* it has been shown that nitric oxide can free HR3 from its dimer with E75 by binding the heme moiety of E75. We propose that nitric oxide functions similarly in *D. magna* to release HR3 from its dimer partner E75. We further propose that the pulse of nitric oxide responsible for the release of HR3 occurs in response to environmental cues to adjust the duration of the intermolt period as necessary based upon environmental conditions to ensure that embryos have the appropriate time to develop. Upon liberation from the dimer with E75, HR3 is able to serve as a transcription factor responsible for the progression of the molt cycle (Figure 1). It has been shown that daphnids are able to convert exogenous sources of nitrogen into nitric oxide which is important given the potential for nitric oxide to interact with the nuclear receptor E75.

**E75/rev-erb**

The nuclear receptor E75 is an important component of the ecdysteroid signaling cascade. We hypothesize that E75 dictates the duration of the ecdysteroid signaling cascade in response to nitric oxide cues. *Drosophila* E75 is a thiolate hemoprotein which is responsive to nitric oxide and carbon monoxide. E75 also contributes to regulating circadian rhythms in *Drosophila*, with both knockdown or overexpression of E75 disrupting these rhythms. In *Drosophila*, E75 regulates genes that encode the enzymes required for biosynthesis of ecdysteroid which may be linked to alterations in circadian rhythms. It has been proposed that heme binds to and activates E75’s ability to regulate transcription. According to this model,
nitric oxide binds to heme causing its release from the E75 ligand-binding domain. An alternative model posits that heme normally resides in the E75 ligand-binding pocket and binding of nitric oxide to the heme moiety results in a conformational change that prevents dimerization with HR3 \(^{48,50}\).

E75 is a homolog of the receptor rev-erb which has two forms—rev-erb\(\alpha\) and rev-erb\(\beta\). Rev-erb\(\alpha\) and rev-erb\(\beta\) are heme-responsive nuclear receptors which play a role in circadian rhythms including behavior and metabolism \(^{53,54}\). The circadian cycle starts with dimerization of two proteins Bmal1 and Clock which activate the downstream clock genes Period (per) and Cryptochrome (cry) \(^{55}\). The circadian cycle functions as a negative feedback loop in which per and cry bind to bmal-clock to inhibit their own transcription. Rev-erb\(\alpha\), a negative regulatory component of the circadian cycle, acts as an additional negative feedback loop to repress Bmal1 during the “night” portion of the circadian cycle \(^{55}\). While both rev-erb\(\alpha\) and rev-erb\(\beta\) are negative regulators of the circadian rhythm, it has been shown that dysregulation of rev-erb\(\beta\) expression effects biological processes linked to cancer and metabolic disorders \(^{53}\).

Due to the homologous nature of rev-erb and E75, we hypothesize exposure of daphnids to environmental nitrites and nitrates and subsequent conversion to nitric oxide may disrupt the ecdysteroid signaling cascade via binding to the heme moiety of E75 resulting in alteration of the expression of genes downstream in the cascade similar to dysregulation of the circadian rhythm by disruption of rev-erb.

\textit{Daphnia magna}

The role of E75 as a regulator of an infradian rhythm was investigated using the model organism \textit{D. magna}. This is an ecologically important organism due to its role in controlling
algae levels and serving as a food source for macroinvertebrates and fish\textsuperscript{56,57}. Further, daphnids serve as a sentinel for freshwater toxicity, and as such, it is important to understand how this species may be targeted for toxicity by environmental contaminants.

Daphnids have been extensively used as a model species in various applications\textsuperscript{58}. \textit{D. magna} was selected for use in this research program due to its amenability to laboratory culture, the periodicity of its molt cycle (~every three days in adults), and its rapid time to reproductive maturity and high fecundity. Further, under suitable environmental conditions, daphnids reproduce clonally via parthenogenesis producing genetically identical female offspring\textsuperscript{59,60}, thus reducing variability in responses among individuals. Finally, the genome of \textit{D. magna} has been sequenced and annotated, greatly facilitating the identification of genes and their corresponding mRNAs (http://daphnia.cgb.indiana.edu/).

\textit{Research Outline}

The goal of this research program was to test the hypothesis that expression and activity of E75 is a critical early event that regulates the timing of the molt cycle in \textit{D. magna}. Further, we hypothesized that the disruption of E75 by nitrate and nitrite, as sources of nitric oxide, could adversely impact daphnid population dynamics.

Our first objective was to establish a method to suppress E75 levels in daphnids. Chapter One describes the development and use of a novel methodology to knock-down targeted gene expression by feeding bacteria containing dsRNA to the daphnids. We target hemoglobin gene expression for this methods-development stage as the production of hemoglobin can be induced in daphnids, and hemoglobin induction or suppression is visually evident by the change in
coloration of the daphnids. We were successful in knocking-down hemoglobin levels and demonstrated that tolerance of daphnids to the toxicity of nitrite correlated to hemoglobin levels.

We then applied the above siRNA procedure to suppress E75 and evaluate the concomitant impacts on the timing of the molt cycle and on offspring production (Chapter 2). We were successful in specifically inhibiting the expression of E75, and found that E75 suppression significantly increased the duration of the molt cycle, while having no effects on neonatal development. However, we speculated that increasing the duration of the molt cycle would reduce the number of broods produced in the lifetime of a daphnid, thus negatively impacting population growth.

Next, we tested this speculation by evaluating the population-level impacts of exposure to the nitric oxide donors sodium nitrite and sodium nitroprusside (Chapter 3). According to our model for the role of E75 in regulating the duration of the molt cycle, nitric oxide donors would delay the molt cycle as did the suppression of E75. We utilized microcosms in conjunction with convolutional neural networks and machine learning to evaluate the impacts of these nitric oxide donors on daphnid population dynamics.

Conclusion

This goal of this research project was to investigate the role of E75 in the ecdysteroid signaling cascade and the potential for nitric oxide to disrupt two processes linked to this cascade: reproduction and molting. By suppressing E75 we showed that the intermolt duration is dictated by this nuclear receptor; however, no effects were observed on neonatal development. With the microcosm experiments, we were able to rapidly determine the effects of the nitric
oxide donors sodium nitroprusside (SNP) and sodium nitrite (NaNO₂) on the population dynamics of *Daphnia magna*.

Overall, results from this study show that targeted gene knockdown is an effective tool to investigate gene function in *Daphnia magna*. Further, allowable limits of environmental nitrogen should be reevaluated as exogenous sources of NO may interact with the nuclear receptor E75, which is vital for regulating the ecdysteroid signaling cascade. Disruption of this cascade results in altered duration of the molt cycle and may have consequences on population growth. These results advance our understanding of the ecdysteroid signaling cascade in *Daphnia magna* as well as the potential for environmental nitrate and nitrite to act as endocrine disruptors.
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doi:10.1016/j.envint.2006.05.002


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Figure 1. Proposed model for the molecular events that dictate the duration of the molt cycle in daphnids. A pulse of 20-hydroxyecdysone initiates the cycle with induction of the nuclear receptors E75 and HR3. These proteins spontaneously dimerize. In response to environmental cues, a pulse of nitric oxide is produced that binds to the heme moiety associated with E75 causing the release of HR3. Free HR3 stimulates the expression of the transcription factor FTZ which induces several CYPs responsible for synthesizing and inactivating the next pulse of 20E. This 20E pulse initiates molting which denotes the end of the molt cycle.
CHAPTER ONE: HEMOGLOBIN LEVELS MODULATE NITRITE TOXICITY IN DAPHNIA MAGNA

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This manuscript has been published by the journal Scientific Reports and is in the format required of that journal.
Abstract

Nitrogenous compounds enter the environment through various anthropogenic sources. Among these are nitrate (NO$_3^-$) and nitrite (NO$_2^-$) which can oxidize the heme moiety of hemoglobin and reduce the oxygen-carrying capacity of the molecule resulting in toxicity. Of the two anions, nitrite is more toxic. Hemoglobin levels are influenced by environmental conditions; thus, we hypothesized that hemoglobin levels would influence the toxicity of nitrite with low hemoglobin levels resulting in enhanced toxicity and high hemoglobin levels resulting in reduced toxicity. We tested this hypothesis by elevating hemoglobin levels with pyriproxyfen treatment and lowering hemoglobin levels using siRNA in Daphnia magna. Exposure to pyriproxyfen significantly elevated hemoglobin mRNA levels and induced copper coloration of the organisms, indicative of increased hemoglobin protein accumulation. siRNA treatment significantly reduced hemoglobin mRNA levels in both untreated and pyriproxyfen-treated organisms and attenuated copper coloration. Pyriproxyfen treatment increased the tolerance of daphnids to the acute toxicity of nitrite approximately 2-fold while siRNA treatment significantly decreased the tolerance of daphnids to nitrite toxicity. Results indicate that increased hemoglobin levels increase the tolerance of daphnids to nitrite toxicity which may serve to protect daphnids in environments subject to hemoglobin-elevating hypoxia or elevated temperatures.
Introduction

Seventy-eight percent of the earth’s atmosphere is comprised of nitrogen, most of which is biologically unavailable due to the inability of most organisms to break the bonds of N\textsubscript{2}. Atmospheric nitrogen is processed into biologically available forms by nitrifying bacteria. These bacteria “fix” atmospheric nitrogen to NH\textsubscript{4}\textsuperscript{+} which can be oxidized to NO\textsubscript{2}\textsuperscript{−} and then NO\textsubscript{3}\textsuperscript{−}. Levels of nitrite and nitrate in the environment are typically regulated through assimilation by plants or through conversion back to atmospheric nitrogen by denitrifying bacteria\textsuperscript{2}. The US Environmental Protection Agency has set the drinking water standard for nitrate at 10 mg N/L (about 45 mg nitrate/L) and nitrite at 1 mg N/L (about 3 mg nitrite/L)\textsuperscript{3} while the World Health Organization has set limits at 50 mg/L for nitrate and 3 mg/L nitrite; these limits are set for short-term exposures\textsuperscript{4}. Though limits have been set to protect sensitive subpopulations from the acute toxic effects of nitrate and nitrite, contamination with these nitrogen oxides remains problematic with levels in freshwaters often exceeding allowable limits\textsuperscript{5}. Nitrogenous compounds are introduced into the environment by various anthropogenic activities such as the use of concentrated animal feeding operations, nitrogen-based fertilizers, and the burning of fossil fuels. These activities have altered the nitrogen cycle resulting in increased levels of biologically available nitrate and nitrite\textsuperscript{6,7}. Increasing levels of these biologically available nitrogen oxides have consequences both environmentally (harmful algal blooms, hypoxic waters, ocean acidification) and on human health (methemoglobinemia, birth defects, cancer)\textsuperscript{5,6}.

One potential mechanism of nitrite toxicity is oxidation from the ferrous to the ferric state of the heme moiety of hemoglobin\textsuperscript{8,9}. Hemoglobin or hemoglobin-like molecules are found in all kingdoms of organisms, from Archaea and Bacteria to Plantae and Animalia\textsuperscript{10–13}. 
Hemoglobin levels are often regulated by environmental factors. For example, hemoglobin synthesis can be induced by hypoxia or elevated temperature\textsuperscript{14,15}. Hemoglobin functions primarily in two important ways: 1) to sequester and deliver oxygen to the organism and 2) to transport other gaseous signaling molecules\textsuperscript{16}. Hemoglobin is able to transport such molecules via reversible binding to iron which is coordinately bound to a protoporphyrin IX (heme group)\textsuperscript{17}. Oxygen is able to bind to the ferrous form of iron (Fe\textsuperscript{2+}) in the heme group\textsuperscript{18}. Exposure to nitrite results in oxidation of the ferrous ion into the ferric ion (Fe\textsuperscript{3+}) creating methemoglobin to which oxygen is unable to bind\textsuperscript{19,20}.

We tested the hypothesis that hemoglobin levels modulate the tolerance of \textit{D. magna} to nitrite toxicity. We proposed that elevated hemoglobin levels would offset the oxidation of heme moieties by nitrite thus leaving sufficient unaltered hemoglobin to support normal function. Conversely, we proposed that reduced hemoglobin levels would reduce the tolerance of daphnids to the toxicity of nitrite by providing insufficient hemoglobin reserves for the transport of oxygen. Here, we increased hemoglobin levels in daphnids by treatment with the insecticide pyriproxyfen, which induces hemoglobin production via activation of the methyl farnesoate signaling pathway\textsuperscript{21}, and decreased hemoglobin levels using siRNA.

\textbf{Results}

\textit{siRNA Optimization}

Exposure of daphnids to 3.0 nM pyriproxyfen significantly increased the level of hemoglobin Dhb2 mRNA (Fig. 1). Feeding organisms \textit{E. coli} that expressed dsRNA to Dhb2 at $7.2 \times 10^7$ cells/100 mL medium significantly (~50\%, $p < 0.05$) reduced Dhb2 mRNA levels in pyriproxyfen-exposed daphnids. We next investigated the minimum amount of time of
feeding dsRNA required to reduce Dhb2 mRNA levels in daphnids. Dhb2 mRNA levels were significantly reduced after fourteen days of feeding (p < 0.05, Fig. 2).

*Modulation of Hemoglobin*

We evaluated the specificity of hemoglobin induction by pyriproxyfen and suppression by siRNA by evaluating levels of Dhb1, Dhb2, and EcR-A (ecdysteroid receptor) mRNA following treatments. Daphnids were provided the vector-containing bacteria for 14 days then exposed to pyriproxyfen for 4 days (with continued provision of the bacteria). Pyriproxyfen (6.0 nM) significantly elevated Dhb1 and Dhb2 mRNA levels in daphnids fed bacterial cells containing empty vector (Fig. 3). This elevation was significantly (p < 0.05) attenuated among daphnids provided bacteria expressing dsRNA (Fig. 3). EcR-A mRNA levels were not modulated by pyriproxyfen or siRNA. Dhb1 and Dhb2 nucleotide sequences are highly similar and distinct from that of EcR-A. Results indicate that the Dhb2 dsRNA also annealed to Dhb1. Modulation of hemoglobin levels resulted in a phenotype wherein daphnids exposed to pyriproxyfen developed increased copper-coloration which was attenuated by feeding of dsRNA targeting hemoglobin.

*Modulation of Nitrite Toxicity by Pyriproxyfen and siRNA*

The ability of pyriproxyfen to modulate the toxicity of nitrite was evaluated. We hypothesized that increased hemoglobin from pyriproxyfen treatment would reduce the ability of nitrite to elicit toxicity. Daphnids (5 days old) were exposed, or not (control), to 3.0 nM pyriproxyfen and then exposed to a concentration series of sodium nitrite. Mobility of individual daphnids was assessed after 48 hours of exposure to sodium nitrite. The 48-hour
EC50 of nitrite with daphnids that were not exposed to pyriproxyfen was 23 mg N/L (95% confidence interval (CI): 21–25 mg N/L). The 48-hour EC50 for nitrite nearly doubled among daphnids pre-exposed to pyriproxyfen (55 mg N/L, 95% CI: 50–59 mg N/L). The no observed effect concentration (NOEC) for nitrite increased from 6.5 mg N/L to 18 mg N/L with pyriproxyfen exposure (Fig. 4). Results were consistent with the hypothesis that increased hemoglobin levels reduced the toxicity of nitrite. We next sought to further test the hypothesis by evaluating the toxicity of nitrite following the induction (pyriproxyfen treatment) and suppression (siRNA treatment) of hemoglobin.

We chose a concentration of sodium nitrite that would immobilize ~50% of control animals (i.e. ethanol control and fed bacteria containing empty vector) so as to detect either increased or decreased tolerance to nitrite. No toxicity occurred among control organisms or other groups not exposed to nitrite (Fig. 5). Nitrite exposure elicited ~50% immobility among control daphnids. dsRNA feeding alone resulted in a significant reduction in tolerance to nitrite toxicity ($p = 0.0083$). Pyriproxyfen treatment alone significantly increased tolerance to nitrite toxicity when compared to organisms not exposed to pyriproxyfen ($p = 0.0001$). Nitrite toxicity was significantly reduced among daphnids fed dsRNA and exposed to pyriproxyfen as well as nitrite when compared to organisms fed dsRNA and exposed to nitrite without pyriproxyfen exposure (from 70% to 30%, $p < 0.0001$). Overall, tolerance to the toxicity of nitrite was directly related to hemoglobin levels.

**Discussion**

In the present study we hypothesized that hemoglobin levels modulate tolerance to nitrite toxicity in the model organism *D. magna*. Under all scenarios evaluated, tolerance of
daphnids to nitrite toxicity tracked with hemoglobin levels. We propose that high hemoglobin levels allow for the oxidation of the heme moiety by nitrite to occur with sufficient unaltered hemoglobin remaining for normal oxygen transport. Conversely, we propose that low hemoglobin levels result in oxidation of heme groups to an extent which depresses oxygen provision to cells.

Hemoglobin levels were suppressed in this study using siRNA. The protocol used to knock down hemoglobin gene expression via feeding dsRNA was based upon that described by Schumpert, et al.\textsuperscript{22} for the suppression of phenoloxidase. In their study, smaller daphnid species were used (\textit{D. pulex}, ≤ 3.0 mm, \textit{D. pulicaria}, ≤ 3.0 mm, and \textit{D. melanica}, ≤ 2.0 mm); thus, we optimized feeding for \textit{D. magna} (≤ 5.0 mm)\textsuperscript{23–25}. The lowest concentration of bacterial cells (2.4 × 10\textsuperscript{7} cells/mL) used in this study was the optimal concentration used by Schumpert \textit{et al.}\textsuperscript{22}. With \textit{D. magna}, maximum suppression of Dhb2 mRNA levels was observed at a concentration of 7.2 × 10\textsuperscript{7} cells/100 mL medium. At this concentration, we observed a 50% reduction in mRNA abundance. The reason for our inability to knock down more than 50% of the Dhb1 and Dhb2 mRNA is unknown but may be due to toxicity associated with feeding higher concentrations of \textit{E. coli}. Overall performance of daphnids has been shown to decrease with increasing ratio of \textit{E. coli}/algal cells in the medium\textsuperscript{26}. Further, 12 × 10\textsuperscript{7} cells \textit{E. coli}/100 mL medium was previously shown to be toxic to daphnids\textsuperscript{22}. We suspect that food consumption by \textit{D. magna} may have decreased with increasing \textit{E. coli} concentration resulting in reduced dsRNA delivery and commensurate loss of the suppressive action of the siRNA.

In mammals, nitrite toxicity presents primarily as methemoglobinemia. Typically, mammals are exposed to nitrate-contaminated food or water. The nitrate is converted to nitrite
which oxidizes ferrous ion in hemoglobin to the ferric state which is incapable of transporting oxygen. Toxicity results from oxygen deprivation of cells. We propose a similar mechanism of acute toxicity of nitrite to daphnids. However, *D. magna* possess at least 7 hemoglobin-producing genes\(^{27}\) and are capable of rapid and significant modulation of hemoglobin levels\(^{15}\) in order to meet the oxygen requirements of the organism\(^{28}\). Here, we demonstrate that these variations in hemoglobin levels can affect tolerance of the organisms to the toxicity of nitrite.

Hemoglobin levels in daphnids are induced by at least two different pathways. The hypoxia signaling pathway results in increased hemoglobin production in response to low oxygen concentrations\(^{29}\). The methyl farnesoate signaling pathway increases hemoglobin production in response to other environmental cues such as photoperiod and temperature\(^{30}\). We have observed that some insect growth-regulating insecticides stimulate hemoglobin induction via the methyl farnesoate signaling pathway\(^{21,31,32}\). Pyriproxyfen, used in this study to elevate hemoglobin, is one such insecticide.

Human-generated hypoxia in aquatic habitats has become a major threat to the environment. Overuse of nitrogen-based fertilizers has resulted in the global occurrence of eutrophication and a growing number of hypoxic “dead” zones, now numbering in excess of 400\(^{33}\). Biota within these zones typically relocate or die\(^{34}\). Hypoxia is primarily responsible for the demise of inhabitants of these regions. In contrast, eutrophication at a level that does not induce lethally hypoxic condition tends to increase productivity at higher trophic levels\(^{35,36}\). Conceivably, the induction of hemoglobin at these margins of hypoxia may confer tolerance of some species to the toxic effects of nitrate and nitrite in these regions. Blue crab (*Callinectes sapidus*) and various other crustacean species have been shown to be tolerant of
low oxygen conditions as they are capable of regulating oxygen transport presumably due to induction of oxygen-transporting proteins. Fish have been shown to increase hemoglobin levels in response to low oxygen conditions.

Elevated temperatures can increase hemoglobin in aquatic species presumably due to the increasing oxygen demand of the organism coupled with the reduced oxygen solubility in water with increasing temperature. Further, increasing temperatures associated with climate change has been linked to an increase in the flux of nitrogen oxides into the environment. Increased hemoglobin levels associated with climate change may prove to be protective against the commensurate accumulation of nitrate and nitrite in the environment.

Materials and Methods

*Daphnia magna*

*D. magna* used in these experiments have been cultured in our lab at North Carolina State University for over 20 years under conditions suitable for parthenogenetic reproduction as described previously. In culture, daphnids were fed 2 mL (1.4 × 10^7 cells) of green algae (*Pseudokirchneriella subcapitata*) and 1 mL (~4 mg dry weight) of Tetrafin® (Tetra, Spectrum Brands, Blacksburg, VA) fish food suspension twice daily. Preparation of the fish food suspension is described elsewhere. During experiments, daphnids were fed 3.5 × 10^5 cells of green algae once daily without fish food suspension.

*dsRNA*

The L4440 vector, a gift from Dr. Andrew Fire (Addgene plasmid #1654), contains two T7 promoters which can be induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) to produce dsRNA of the sequence ligated between these promoters (Fig. 6a). Primers were
designed to clone a target sequence of 315 base pairs chosen from the nucleotide sequence of *D. magna* hemoglobin Dhb2 (Fig. 6b) and were prepared with the XbaI and KpnI restriction enzyme sites. The primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The desired sequence with the restriction enzyme sites was amplified according to the Phusion Hot Start II High-Fidelity PCR Master Mix protocol (ThermoFisher Scientific, Waltham, MA). The amplified target and L4440 plasmid vector were then double digested and ligated. The L4440 constructs were transformed into GC5 cells and plated on LB plates containing ampicillin (100 μg/mL, Sigma-Aldrich Corp., St. Louis, MO) and tetracycline (12.5 μg/mL, Sigma-Aldrich). Individual colonies were selected from LB plates and PCR was performed to assess whether ligation of the insert into the L4440 vector was successful. Successfully ligated vectors were sequenced (Eton Bioscience, San Diego, CA). After sequencing, the L4440 constructs were transformed into competent HT115 (DE3) cells obtained from the *Caenorhabditis* Genetics Center (funded by NIH Office of Research Infrastructure Programs (P40 OD010440)). Following transformation of HT115 cells containing the constructs, the bacterial cells were grown to OD$_{595} = 0.04$ ($2.8 \times 10^8$ cells/mL), and frozen at −80 °C. A dilution series of cells was prepared, and the optical density of each dilution at 595 nm was measured and used to generate a standard curve against cell count.

For experiments, transformed cells were grown overnight in LB medium containing ampicillin and tetracycline. IPTG (Sigma-Aldrich) was added at a concentration of 2 mM to induce production of the T7 RNA polymerase and subsequent production of dsRNA of the target sequence. At the time of feeding, optical density of the culture at 595 nm was measured and used to determine the number of cells per milliliter. Cells were centrifuged for 10 minutes
at 3000 rpm and room temperature. LB medium was decanted and cells were resuspended in daphnid medium for feeding.

*mRNA Analyses*

At the end of experiments, 3–4 daphnids per treatment were stored in 100 μL RNAlater (Invitrogen, Carlsbad, CA) in 1.7 mL tubes. Samples were stored at 4 °C for 24 hours then transferred to −80 °C until RNA extraction. Daphnids were homogenized using a Bullet Blender (Next Advance, Inc., Troy, NY), and RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI). RNA concentration was determined by measuring absorbance at 260 nm using Nanodrop-1000 spectrophotometer (ThermoFisher). cDNA was prepared from extracted RNA using ImProm-II™ Reverse Transcription System (Promega) with oligo (dT) primers.

mRNA levels were measured by quantitative RT-PCR. Preparation of primers for *D. magna* actin, gapdh, Dhb1, Dhb2, and EcR-A (Fig. 6c) have been described previously. Actin and gapdh were used to normalize Dhb1, Dhb2, and EcR-A mRNA levels. PCR was performed using the ABI PRISM ® 7000 Sequence Detection System with SYBR®Green PCR Mastermix (ThermoFisher) or iTaq Universal Sybr Green Supermix (BioRad, Hercules, CA) in 96-well plates (Olympus Plastics, Genesee Scientific, San Diego, CA) sealed with ThermalSeal (Excel Scientific, Inc., Victorville, CA). mRNA levels were calculated from raw data using Genex software from BioRad which utilizes algorithms to normalize mRNA levels to multiple housekeeping genes.
Optimization of hemoglobin suppression using siRNA

The siRNA methodology used in the study was based upon that described by Schumpert, et al.\textsuperscript{22} The optimal concentration of dsRNA-expressing bacterial cells required to effectively knock down expression of hemoglobin in daphnids consuming the cells was determined. Daphnids were provided $2.4 \times 10^7$, $7.2 \times 10^7$, or $12 \times 10^7$ bacterial cells (per 100 mL medium) containing either empty vector or vector expressing Dhb2 dsRNA. At day 10, organisms were exposed to 3.0 nM pyriproxyfen (Sigma-Aldrich). Feeding of bacteria and exposure to pyriproxyfen continued until copper-coloration was observed among daphnids exposed to pyriproxyfen and provided empty vector (Fig. 6d). Daphnids were then collected and stored in 100 μL RNAlater at 4 °C for 24 hours and then moved to −80 °C until RNA was extracted.

Hemoglobin modulation

Hemoglobin levels were elevated by exposing daphnids to 6.0 nM of the insecticide pyriproxyfen\textsuperscript{31}. Pyriproxyfen was dissolved in 100% ethanol for delivery into the exposure solutions. The concentration of ethanol in all exposure solutions, including control was 0.05%. Hemoglobin levels were suppressed by exposing daphnids to bacteria containing Dhb2 dsRNA. Daphnids consume the bacteria resulting in the distribution of the siRNA throughout the organisms\textsuperscript{22}. Exposure regimens for the various experiments are depicted in Fig.6e. All exposures were conducted with animals <24 hours old, unless stated otherwise, in 40 mL medium in 50 mL beakers. Experiments were conducted in 16:8 hour light: dark cycle at 20 °C. Medium was changed every other day. Animals were fed 50 μL ($3.5 \times 10^5$ cells) green algae and $7.2 \times 10^5$ cells/mL bacteria daily.
Nitrite Toxicity

The impact of hemoglobin levels on the toxicity of nitrite was evaluated. First, the toxicity of nitrite to daphnids in which hemoglobin levels were not modulated was evaluated. Daphnids (7 days old) were exposed to concentrations of sodium nitrite ranging from 0–83 mg N/L for 48 hours after which immobility was determined. A daphnid was deemed immobile if it was on the bottom of the vessel and did not move for five seconds upon placing a pipet tip adjacent to it. Each treatment consisted of 10 daphnids isolated in 50 mL beakers containing 40 mL daphnid media. The experiment was conducted in 16:8 hour light: dark cycle at 20 °C. Similar experiments were performed concurrently where daphnids were exposed to 3.0 nM pyriproxyfen for 48 hours prior to initiating the exposure to sodium nitrite (Fig. 6e-3). EC50 values were calculated by preparing a non-linear fit of log-dose vs response in GraphPad Prism.

Subsequent experiments evaluating the toxicity of nitrite to daphnids pre-exposed to siRNA and/or pyriproxyfen were performed at a nitrite concentration of 25 mg N/L using methods as described above and the exposure regimen depicted in Fig. 6e-4. Each treatment consisted of nine organisms with two replicates. Results were analyzed by One-way Analysis of Variance with Tukey’s Multiple Comparisons Test using GraphPad Prism. Homogeneity of variances was confirmed with the Brown-Forsythe test.
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   doi:10.1016/j.envint.2006.05.002


Acknowledgments

This work was supported by the National Science Foundation grant IOS-1350998 to GAL.

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Contributions

GAL and SAE conceived and designed the experiments. SAE conducted the experiments. SAE and GAL performed data analyses and manuscript preparation.

Competing interests

The authors declare no competing financial interests.

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Figure 1. Relative abundance of Dhb2 mRNA in *D. magna* provided low (2.4x10^7 cells/100 mL), medium (7.2x10^7 cells/100mL), and high (12x10^7 cells/mL) concentrations of bacterial cells containing the L4440 vector or L4440 vector expressing Dhb2 siRNA with or without pyriproxyfen treatment. Data are presented as the mean and standard deviation (n=3). Results are normalized to the control group (no pyriproxyfen and siRNA) fed the low concentration of bacterial cells. An asterisk denotes a significant (p<0.05) difference between the connected data (One-way Analysis of Variance, Tukey’s Multiple Comparisons test).
Figure 2. Time course for the reduction of Dhb2 mRNA levels in *D. magna* fed bacteria producing the dsRNA targeting Dhb2. Daphnids were fed bacteria containing either L4440 vector (light gray) or L4440 vector containing the Dhb2 siRNA (dark gray) for the indicated number of days then evaluated for Dhb2 mRNA abundance. Abundance of mRNA in organisms fed Dhb2 siRNA was normalized to mRNA levels in organisms fed empty vector for each day. An asterisk denotes a significant (p<0.05) difference between the connected data (One-way Analysis of Variance, Bonferroni’s Multiple Comparisons test).
Figure 3. Relative abundance of Dhb1 (A), Dhb2 (B), and EcR-A (C) mRNA in *D. magna* provided L4440 vector or L4440 vector expressing Dhb2 siRNA with or without pyriproxyfen treatment. Data are presented as the mean and standard deviation (n=3). Results for each gene are normalized to the control (no siRNA and pyriproxyfen). An asterisk denotes a significant (p<0.05) difference between the connected data (One-way Analysis of Variance, Tukey’s Multiple Comparisons test).
Figure 4. Concentration-response of *D. magna* reared in the absence (circles) and presence (squares) of 3.0 nM pyriproxyfen for 48 hours prior to exposure to various concentrations of sodium nitrite. Data points are presented as the percentage immobilized daphnids (n=10) at each concentration of NaNO$_2$. Confidence intervals (95%) are depicted by dotted lines.
Figure 5. Immobilization of *D. magna* exposed to sodium nitrite following treatment with 

**Dhb2 siRNA and/or pyriproxifen.** Data are presented as the percentage immobilized daphnids (9 daphnids per replicate, 2 replicates per treatment). Treatments denoted with different letters were significantly (p<0.05) different (One-way Analysis of Variance, Tukey’s Multiple Comparisons Test).
Figure 6. Methodological details. (a) Bacteria were transformed with the L4440 vector with or without the dsRNA target sequence. Location of the target sequence is indicated in red between two T7 promoters. The black region designates the ampicillin resistance gene. (b) The nucleotide sequence cloned into the L4440 vector for the production of dsRNA. Primers designed to clone the target sequence of Dhb2 are shaded in gray. These primers also amplified the paralogous sequence for Dhb1. (c) Primer sequences used for quantitative RT-PCR. (d) Coloration of daphnids reared in: 1) control media and fed bacteria containing empty L4440 vector, 2) control media and fed bacteria containing siRNA-containing L4440 vector, 3) media containing 6.0 nM pyriproxyfen and fed bacteria with empty L4440 vector, 4) media containing 6.0 nM pyriproxyfen and fed bacteria with siRNA-containing L4440 vector. (e) Treatment regimens used: 1) days of feeding siRNA required to knockdown targeted gene, 2) modulation of hemoglobin levels by exposure to pyriproxyfen and feeding siRNA, 3) sodium nitrite concentration-response assessment, and 4) toxicity of nitrite with modulated hemoglobin levels.
A) L4440 Vector

B)  

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C)  

D) 1) 2) 3) 4)

E) 1) Clean media + siRNA  Collect day 10/12/14  
2) Day 0-14 Clean media + siRNA  Day 14-20 Pyniproxyfen +/or siRNA  Collect Day 20  
3) Day 0-5 Clean media  Day 5-7 Pyniproxyfen  Day 7-10 NaNO₂  Assess immobility  
4) Day 0-14 Clean media +/or siRNA  Day 14-16 Pyniproxyfen +/or siRNA  Day 16-18 NaNO₂  Assess immobility
CHAPTER TWO: SUPPRESSION OF NUCLEAR RECEPTOR E75
PROLONGS THE TIMING OF THE MOLT CYCLE IN THE
CRUSTACEAN DAPHNIA MAGNA

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Abstract

Biological rhythms regulate innumerable physiological processes, yet little is known of factors that regulate many of these rhythms. In particular, the regulation of most infradian rhythms is poorly understood. We hypothesized that the nuclear receptor E75 is a primary regulator of the molt/reproduction infradian rhythm in the crustacean *Daphnia magna*. We addressed this hypothesis by knocking down the expression of E75 *in vivo* using siRNA and evaluating consequences on mRNA levels along the putative regulatory cascade of this rhythm along with physiological endpoints that are controlled by the rhythm. E75 was effectively suppressed by feeding daphnids *E. coli* that expressed dsRNA targeting E75. Suppression of E75 had no significant effect on mRNA levels of the nuclear receptor HR3 which is co-regulated with E75. However, mRNA levels of FTZ, a nuclear receptor expressed subsequent to the expression of E75 and HR3 were elevated. mRNA levels of several cytochrome P450 enzymes that contribute to the synthesis of 20-hydroxyecdysone, the penultimate event in the molt/reproduction cycle, were not altered in response to E75 suppression. However, CYP18a1 mRNA levels were suppressed by E75 suppression. CYP18a1 is responsible for inactivation of 20-hydroxyecdysone. Results support our hypothesis that E75 binds and suppresses the activity of HR3 and that free HR3 regulates FTZ expression. The suppression of E75 caused the accumulation of free HR3 resulting in the overexpression of FTZ. Results also suggest that this E75-initiated cascade of events regulates the expression and pulsatile accumulation of 20-hydroxyecdysone via modulation of CYP18a1. This pulse is responsible for initiating the ultimate events of molting and brood release. Consistent with these molecular responses, the suppression of E75 increased the duration of the molt/reproduction cycle and reduced the number of offspring produced by maternal organisms.
Introduction

Biological rhythms regulate the timing of many physiological processes, and often reflect organismal adaptation to rhythmic changes in the environment \(^1\). Many biological processes are regulated by an internal circadian clock \(^2\). The core loop of this clock consists of the proteins Clock (Clk) and Bmal1 which dimerize and stimulate transcription of three Period (Per) and two Cryptochrome (Cry) genes \(^3,4\). Subsequently, a Per-Cry complex translocates to the nucleus and inhibits their own transcription by binding to the Clk and Bmal1 dimer \(^5\). There exists a secondary loop involving the nuclear receptors rev-erba (NR1D1) and retinoid-related orphan receptor (ROR) which link the circadian clock to many biological processes \(^3,6\). Transcription of Rev-erba and ROR are activated by Clk-Bmal1; however, Rev-erba acts as a transcriptional repressor of Bmal1 while ROR serves to activate transcription of Bmal1 \(^5,7\). Rev-erb and ROR compete for the ROR response element (RORE) binding sites to regulate timing of the circadian rhythm \(^8\). Interference of the timing of transcription of rev-erb results in circadian disruption and metabolic disorders \(^2,9\). Some genes involved in regulating the mammalian circadian rhythm are also involved in some ultradian (shorter than 24 hours) and infradian (longer than 24 hours) rhythms, although their roles in maintaining these rhythms are poorly understood \(^10\)–\(^12\).

The rev-erb ortholog E75 has been identified in a number of arthropod species \(^13\)–\(^17\). This nuclear receptor is a thiolate hemoprotein and has been shown to be responsive to nitric oxide \(^18,19\). Nitric oxide regulates the activity of E75 by binding to its heme component causing the loss of the heme or other conformational change in the protein causing dissociation from its heterodimeric partner, the ROR ortholog HR3 \(^18\)–\(^20\). Suppression or overexpression of E75 in Drosophila disrupts rest: activity rhythms \(^7\).
The molt/reproduction cycle in crustaceans consists of an environmentally entrained infradian rhythm\textsuperscript{21}. This rhythm is closely aligned with physiological processes critical to population sustainability such as growth, maturation, and reproduction\textsuperscript{22–24}. Accordingly, elucidating the molecular events that dictate this rhythm will enhance our understanding of the susceptibility of the rhythm to environmental changes.

We hypothesized that expression of E75 in the molt/reproduction cycle serves to bind and sequester HR3. Nitric oxide, we propose, is produced in response to environmental cues, and binds to E75 causing the release of HR3. HR3 then induces FTZ which induces cytochrome P450 enzymes responsible for the next pulse of 20-hydroxyecdysone. This pulse of 20-hydroxyecdysone stimulates the maternal organism to release offspring from the brood chamber and molt. Presently, we hypothesized that suppression of E75 would have no effect on HR3 mRNA levels (though free protein levels would increase), FTZ expression would be elevated and prolonged due to elevated free HR3 levels, and cytochrome P450 enzymes would be expressed in an altered manner similar to FTZ. We also hypothesized that these molecular alterations in the timing of the molt/reproductive cycle would have discernible consequences on the timing of exuviation and reproduction.

**Materials and Methods**

*Daphnia magna*

*D. magna* used in these experiments have been cultured for over 20 years in our lab under conditions suitable for parthenogenetic reproduction as described previously\textsuperscript{25}. Daphnid cultures were maintained in 1 L beakers and were provided $1.4 \times 10^7$ cells of green algae (*Raphidocelis subcapitata*) and ~4 mg dry weight of Tetrafin ® (Tetra, Spectrum Brands,
Blacksburg, VA) fish food suspension twice each day. Algae was cultured and fish food suspension was prepared as described previously \(^{26,27}\).

**Gene Expression during the Infradian Molt/Reproduction Cycle**

Daphnids (<24 hours old) were reared in 1L beakers for 10 days under the same feeding regimen as described for cultures. Individual daphnids were then each placed into a 50 mL beaker containing 40 mL media and fed \(1.4 \times 10^6\) cells *Raphidocelis subcapitata* and ~0.4 mg dry weight fish food suspension daily. Individual daphnids were observed every two hours for molting. Upon molting, designated time zero, daphnids were assigned to a group (15 daphnids/group) designated to be sampled at a specific time during the infradian rhythm. Sampling times were 0, 36, 48, 60, 72, 78, 84, and 90 hours post-molt. Upon sampling, daphnids were placed, in groups of five, in RINAlater for 24 hours at 4°C and then stored at -80°C until mRNA extraction as described below.

dsRNA Preparation

Oligonucleotide primers were designed to produce dsRNA targeting *D. magna* E75. Primers were synthesized (Integrated DNA Technologies, Inc. (Coralville, IA)) flanked by the XbaI and KpnI restriction enzyme sites. Each primer was amplified with the restriction enzyme sites according to the Phusion Hot Start II High-Fidelity PCR Master Mix protocol (ThermoFisher Scientific, Waltham, MA). The amplified target sequence was double digested and ligated with the L4440 plasmid vector, a gift from Dr. Andrew Fire (Addgene plasmid #1654). The L4440 construct was then transformed into GC5 cells and plated on LB plates containing ampicillin (100 µg/mL, Sigma-Aldrich Corp., St. Louis, MO) and tetracycline (12.5
μg/mL, Sigma-Aldrich). Individual colonies were selected and PCR was performed to identify inserts where ligation into the vector was successful. Successfully ligated inserts were sequenced (Eton Bioscience, San Diego, CA), and constructs were transformed into competent HT115 (DE3) cells obtained from the *Caenorhabditis* Genetics Center (funded by NIH Office of Research Infrastructure Programs (P40 Od010440)). Following transformation of HT115 cells containing the construct, stocks were prepared by growing the bacterial cells to OD<sub>595</sub>=0.040 (2.84x10<sup>8</sup> cells/mL) and freezing with 25% glycerol at -80°C.

The transformed cells were grown overnight in LB medium containing ampicillin and tetracycline for use in E75 knock-down experiments. Isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich) was added at a concentration of 2.0 mM to induce production of the T7 RNA polymerase and subsequent production of dsRNA of the target sequence. The concentration of transformed bacterial cells was determined by measuring the culture’s optical density at 595 nm<sup>28</sup>. Cells were concentrated by centrifuge at room temperature for 10 minutes at 3000 rpm, LB medium was decanted, and cells were resuspended in daphnid medium. The concentration of cells was determined according to a standard curve, and the volume required to feed 7.2x10<sup>7</sup> cells/100mL of medium was calculated.

*Suppression of E75 via RNAi*

Daphnids (< 24 hours old) were used to initiate experiments aimed at the suppression of E75. Individual daphnids were isolated in 40 mL of media and fed 2.88x10<sup>7</sup> cells *E. coli* bacteria containing either the empty L4440 vector (no dsRNA insert) or containing the L4440 vector with the insert targeting E75. Daphnids were also provided 3.5x10<sup>5</sup> cells of green algae (*R. subcapitata*) as a nutrition source. Daphnids were evaluated every two hours beginning fourteen
days after initiation of feeding the daphnids *E. coli* for the presence of a molted exoskeleton. The first observed molt was designated time zero. Daphnids were then continually evaluated until their second molt in order to determine the length of the molt cycle and the number of offspring released with the associated brood.

Maternal daphnids were stored in RINAlater in groups of three for mRNA analyses. Previous use of this method of mRNA suppression revealed that a minimum of 14 days of feeding *E. coli* containing dsRNA provided maximum suppression of targeted mRNA\textsuperscript{28}.

**mRNA Analyses**

Sampled daphnids were homogenized using a Bullet Blender (Next Advance, Inc., Troy, NY) and RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI). RNA concentrations were measured by their absorbance at 260 nm using a Nanodrop-1000 spectrophotometer (ThermoFisher). cDNA was prepared from the RNA using the ImProm-II\textsuperscript{TM} Reverse Transcription System (Promega) with oligo (dT) primers.

Quantitative RT-PCR was performed to measure targeted mRNA levels. Preparation of primers for *D. magna* actin, gapdh, E75, HR3, and FTZ have been described previously\textsuperscript{29,31}. Primers for *D. magna* CYP18a1 and CYP314a1 were prepared from sequences published by Sumiya, et al\textsuperscript{32}. Primers for CYP315, CYP306, CYP307, and CYP302 were identified from sequences available in wFleaBase (http://wfleabase.org/). These primer sequences are listed in Table 1. Actin and gapdh were used to normalize targeted mRNA levels. PCR was performed using the ABI PRISM® 7000 Sequence Detection System with iTaq Universal Sybr Green Supermix (BioRad, Hercules, CA) in 96-well plates (Olympus Plastics, Genesee Scientific, San Diego, CA) sealed with ThermalSeal (Excel Scientific, Inc., Victorville, CA). BioRad Genex
software, which utilizes algorithms to normalize mRNA levels to multiple housekeeping genes \(^{33}\), was used to calculate mRNA levels from raw data.

**Statistics**

All statistical comparisons were made using Student’s t-test (p<0.05). Normality of the data was confirmed using the Shapiro-Wilk test.

**Results**

*Gene Expression during the Infradian Molt/Reproduction Cycle*

We hypothesized that suppression of E75 levels would result in disruption of other downstream genes that may control the molt/reproduction cycle. We first determined timing of mRNA accumulation for E75 and associated genes in untreated organisms. E75 mRNA levels were elevated 36 to 60 hours post-molt (Fig. 1A). These levels were suppressed ~50% after ~14 days of feeding bacteria containing dsRNA (Fig. 1B). Fourteen days of feeding falls in the range of 36-60 hours post molt, when maximum E75 mRNA accumulation occurred.

HR3 mRNA levels exhibited a distinct peak in accumulation at 60 hours post-molt (Fig. 2A). HR3 mRNA levels were not suppressed by treatment with E75 dsRNA (Fig. 2B). The co-accumulation of E75 and HR3 mRNA is consistent with our model that these proteins are co-expressed and dimerize.

FTZ mRNA levels attained peak accumulation at 72 hours post-molt (Fig. 3A). FTZ mRNA levels increased after treatment with the E75 dsRNA (Fig. 3B). This increase was consistent with the suppression of E75 causing an increase in free HR3 which elevated expression of FTZ.
CYP18a1 mRNA also attained maximum mRNA accumulation at 72 hours post-molt (Fig. 4A). CYP18a1 mRNA levels were significantly reduced following treatment with E75 dsRNA (Fig. 4B). Cytochrome P450 gene products that contribute to 20-hydroxyecdysone synthesis were expressed at various times during the molt/reproduction cycle and, with the exception of CYP314a1, were not significantly affected by treatment with the E75 dsRNA (Fig. 5). In summary, suppression of E75 resulted in overall changes in the putative molt/reproduction cycle consistent with a lengthening of the rhythm.

Effects of E75 Suppression on Molting and Fecundity

We hypothesized that suppressing E75 would disrupt the timing of the infradian molt/reproduction rhythm. Suppression of E75 increased the duration of the molt cycle (Fig. 6A). In the control group fed empty vector, the average length of the molt cycle was approximately 72 hours and lengthened to approximately 75 hours in the group fed E75 siRNA. E75 suppression also reduced the mean number of offspring released by each maternal daphnid (Fig. 6B). The suppression of E75 had no discernible effects on offspring development (Fig. 6C).

Discussion

We hypothesized that the timing of the molt/reproduction infradian cycle is regulated by the nuclear receptor E75 and that suppressing this nuclear receptor would disrupt the timing of this rhythm. E75 and HR3 proteins are induced by 20-hydroxyecdysone (20E) in arthropods\textsuperscript{29,34}. The pulsatile secretion of 20E delineates the boundaries of the molt/reproduction cycle within the infradian rhythm. In species examined, E75 and HR3 dimerize which prevents HR3 from serving as a transcription factor\textsuperscript{19,35}. Nitric oxide can bind to the heme moiety of E75 and release
HR3 allowing it to function as an active transcription factor that ultimately leads to the synthesis of the next 20E pulse and initiating the next cycle within the rhythm\textsuperscript{19}. The timing of this cycle is critical for normal molting, growth, development, and reproduction\textsuperscript{36,37}.

Levels of E75 mRNA were suppressed by approximately 50\% via feeding siRNA which aligns with previous work from our lab\textsuperscript{28}. Concentrations of \textit{E. coli} greater than that used to attain 50\% suppression are harmful to the daphnids. In addition to mortality, surviving daphnids have reduced fitness and likely filter less bacteria, thus limiting the amount of siRNA taken up by the organisms\textsuperscript{28}.

Suppressing E75 using dsRNA had no effect on HR3 mRNA levels. This lack of effect was expected as this nuclear receptor is not known to be regulated by E75. We would expect HR3 free protein levels to increase with the suppression of its dimer partner E75. Protein levels were not evaluated in the present study.

Evidence with \textit{Drosophila} suggests that FTZ is regulated by HR3\textsuperscript{38}. The present study supports this relationship in daphnids as presumed elevated levels of free HR3 protein resulting from suppression of E75, increased FTZ mRNA levels. We propose that under normal conditions, nitric oxide synthesis is linked to environmental cues (e.g. temperature, food availability) that informs on the duration of the intermolt period required for proper development of offspring. The nitric oxide pulse causes the release of HR3 which stimulates the cascade of events required for the next 20-hydroxyecdysone pulse.

We found no evidence that this signaling cascade regulates the expression of cytochrome P450 enzymes involved in the synthesis of the terminal 20E pulse. 20E biosynthetic cytochrome P450s are induced by FTZ in some insects\textsuperscript{39–41}. Further, E75 has been shown to induce expression of some of these cytochrome P450s in \textit{Bombyx mori}\textsuperscript{16}. We did, however, provide
evidence that the 20E-inactivating cytochrome P450 18a1 is regulated by this signaling cascade in daphnids. Previous studies have shown that CYP18a1 is positively regulated by 20-hydroxyecdysone in Drosophila, and that its expression is positively correlated with that of FTZ in daphnids. It is noteworthy that CYP18a1 mRNA levels were measured at a single time point in the present study and the induction of CYP18a1 may have been missed. Nonetheless, results demonstrate the lability of CYP18a1 and accordingly, its importance in establishing the molt/reproduction infradian rhythm in daphnids.

Previous studies have shown that the disruption of E75 function (e.g. dimerization with HR3) by exposure of daphnids to a nitric oxide-generating compound or a pharmaceutical that targets E75 increased the duration of the molt cycle. Here, the suppression of E75 levels increased the duration of the molt cycle which provides further evidence for the role of E75 in regulating the molt cycle in daphnids. Further, we demonstrated that suppression of E75 reduced the number of offspring produced by female daphnids. E75 regulates aspects of oogenesis and vitellogenesis in some insects and the suppression of E75 reduces fecundity in insects. It is plausible that some component of the signaling pathway in which E75 is an early gene product also regulates these reproductive processes in crustaceans with the suppression of E75 having the observed negative outcome on reproductive output.

In conclusion, results of this study support the hypothesis that the timing of the molt/reproduction infradian rhythm in daphnids is regulated by the nuclear receptor E75 and that suppressing this nuclear receptor would disrupt the timing of this rhythm. Specifically, the suppression of E75 likely elevated and prolonged levels of free HR3 protein, which elevated and prolonged the expression of FTZ. This caused a delay in the terminal ecdysteroid pulse resulting in an increase in the duration of the inter-molt period and a reduction in fecundity of the
organisms. The existence of a similar pathway in economically important crustaceans would provide molecular targets that could increase growth rates and fecundity within aquacultural operations or may identify molecular targets by which environmental chemicals or other environmental factors (e.g. global climate change) could negatively impact these population-relevant parameters.

Acknowledgments
This work was supported by the National Science Foundation grant IOS-1350998 to GAL.

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Contributions
GAL, SAE, and SMS conceived and designed the experiments. SAE and SMS conducted the experiments. SAE and GAL performed data analyses and manuscript preparation.

Competing interests
The authors declare no competing financial interests.

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**Figure 1. E75 mRNA levels.** (A) Relative E75 mRNA levels among control daphnids during the molt infradian rhythm. Each data point represents a mean (n=3 samples, 5 daphnids per sample). Error bars represent the standard errors. (B) E75 mRNA levels after feeding bacteria containing siRNA targeting E75 for 14 days. Data are presented as mean relative mRNA levels (n=16). Error bars represent the standard errors. Significant difference (p=0.0007) is indicated with an asterisk.
Figure 2. HR3 mRNA levels. (A) Relative HR3 mRNA levels among control daphnids during the molt infradian rhythm. Each data point represents a mean (n=3 samples, 5 daphnids per sample). Error bars represent the standard errors. (B) HR3 mRNA levels after feeding bacteria containing siRNA targeting E75 for 14 days. Data are presented as mean relative mRNA levels (n=16). Error bars represent the standard errors.
Figure 3. FTZ mRNA levels. (A) Relative FTZ mRNA levels among control daphnids during the molt infradian rhythm. Each data point represents a mean (n=3 samples, 5 daphnids per sample). Error bars represent the standard errors. (B) E75 mRNA levels after feeding bacteria containing siRNA targeting E75 for 14 days. Data are presented as mean relative mRNA levels (n=16). Error bars represent the standard errors. Significant difference (p=0.0083) is indicated with an asterisk.
**Figure 4. CYP18a1 mRNA levels.** (A) Relative FTZ mRNA levels among control daphnids during the molt infradian rhythm. Each data point represents a mean (n=3 samples, 5 daphnids per sample). Error bars represent the standard errors. (B) E75 mRNA levels after feeding bacteria containing siRNA targeting E75 for 14 days. Data are presented as mean relative mRNA levels (n=16). Error bars represent the standard errors. Significant difference (p<0.0001) is indicated with an asterisk.
Figure 5. mRNA levels of cytochrome P450s involved in 20-hydroxyecdysone biosynthesis following treatment with siRNA targeting E75. Cytochrome P450 mRNA levels were measured after feeding bacteria containing siRNA targeting E75 for 14 days. Data are presented as mean relative mRNA levels derived from four separate experiments (n=16). Error bars represent the standard errors. Significant difference (p=0.0047) is indicated with an asterisk.
Figure 6. Impact of E75 suppression on (A) the duration of the molt cycle, (B) fecundity of maternal daphnids, and (C) incidence of developmental abnormalities among offspring. (A) Data are presented as the mean duration of the molt cycle and standard error (n=12 individual daphnids). (B) Data are presented as the mean number of neonates born per female and standard error (n=19-20 individual daphnids). (C) Data are presented as the mean percentage of abnormal neonates per maternal daphnid and standard error (n=19-20 individual daphnids).
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CHAPTER THREE: THE IMPACT OF NITRIC OXIDE DONORS
SODIUM NITROPRUSSIDE AND SODIUM NITRITE ON POPULATION
DYNAMICS OF DAPHNIA MAGNA

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Abstract

Levels of biologically available nitrogen in the environment are increasing despite limits set by the US Environmental Protection Agency and the World Health Organization. These limits are set to protect human health and wildlife populations in the environment; however, the effects on populations are not well studied. Environmental nitrogen oxide species (NO\textsubscript{x}) can serve as a source of nitric oxide, a potent signaling molecule. We have shown that exogenous sources of nitric oxide can disrupt the molt infradian rhythm of daphnids (Daphnia magna) with subtle adverse consequences on processes such as embryo development and fecundity. We hypothesize that low levels of nitric oxide donors will significantly alter the population dynamics of daphnids by targeting and disrupting these processes that together are critical to population growth and sustainability. We tested this hypothesis by exposing daphnid populations to non-lethal levels of sodium nitroprusside or sodium nitrite. Convolutional neural networks and machine learning were used to quantify population growth rates and size class distributions. Results revealed that concentrations below the recommended limits for environmental nitrogen impacted population dynamics in adult daphnids exposed to sodium nitroprusside but not sodium nitrite. Results suggest that recommended limits for NO\textsubscript{x} species in the environment are adequate for the protection of this model species; however, these limits may not suffice for contaminants which serve as more potent NO donors.
Introduction

Seventy-eight percent of the Earth’s atmosphere is comprised of nitrogen; however, most of this nitrogen is biologically unavailable due to most organisms inability to break the bonds of N$_2$\(^1\). Atmospheric nitrogen is processed into biologically active forms by nitrifying bacteria which “fix” N$_2$ to NH$_4^+$ which can be subsequently oxidized to nitrite (NO$_2^-$) and nitrate (NO$_3^-$)\(^2\). This nitrogen cycle is typically balanced by assimilation of these compounds by plants and conversion back to atmospheric nitrogen by denitrifying bacteria\(^3,4\). The large-scale introduction of nitrogenous compounds into the environment from anthropogenic activities has disrupted the nitrogen cycle with excess biologically available nitrogen accumulating in the environment\(^5\).

Nitrogenous compounds are introduced into the environment through a variety of anthropogenic sources, including the use of nitrogen-based fertilizers, runoff from concentrated animal feeding operations (CAFOs), and the mining and burning of fossil fuels\(^6-8\). As of 2001, 83 million tons of nitrogen were fixed annually to be used as fertilizer\(^9\), and it was predicted that this level would increase to 135 million tons released by 2030 in response to population growth and the demand for food production\(^6\). It was reported in 2007 that 133 million tons of manure are released from CAFOs annually in the United States\(^10\). Presently hundreds of millions of tons of manure released annually as there are more than 60,000 CAFOs in the United States today\(^11\).

Limits have been set to protect sensitive human subpopulations, such as infants, from acute exposure to nitrates and nitrites. Limits set by the World Health Organization are approximately 11 mg N/L (50 mg/L nitrate) and approximately 1 mg N/L (3.0 mg/L nitrite)\(^12\). Similarly, the United States EPA has set the maximum allowable limit of nitrate at 10 mg N/L (approximately 45 mg nitrate/L) and nitrite at 1 mg N/L (approximately 3 mg nitrite/L)\(^13\). These limits are set to protect human health; however, excess nitrate and nitrite also can have negative
consequences on the environment including stimulation of eutrophication and the subsequent production of dead zones \(^1,^{14,15}\). The US EPA also has established criteria to protect organisms residing in aquatic environments from nitrate and nitrite toxicity. These ecoregional nutrient criteria are set to protect against loss of diversity, loss of productivity and stability, and to prevent eutrophication \(^16\). The criteria for total nitrogen allowed ranges from 0.10 to 1.27 mg N/L in lakes and reservoirs and 0.12 to 2.18 mg N/L in rivers and streams \(^17\). Reported levels of nitrite in the environment vary. In Lahn River, Germany, levels of environmental nitrite range from 0.25 to 0.25 mg N/L, and concentrations ranging from 0.004 to 0.172 mg N/L were reported from six rivers in North Ireland \(^18,^{19}\). In Quebec, reported levels of nitrite in surface water located in an agricultural watershed range from 0.022 to 0.050 mg N/L, and nitrite in ground water in the Nile Delta in Egypt has been reported to fall in the range of 0.03 to 0.113 mg/L \(^20,^{21}\).

Excess environmental nitrogen can elicit toxicity indirectly or directly. Indirect toxicity occurs when the environment in which organisms live is altered. Excess nitrogen may cause eutrophication resulting in increased biological oxygen demand leading to reduced dissolved oxygen levels and formation of dead zones where resident organisms are forced to evacuate or face death. There are currently more than 400 dead zones worldwide \(^15\). Excess environmental nitrate and nitrite may also contribute to acidification of aquatic ecosystems and alteration of the solubility of important minerals \(^4,^{22}\). Direct toxicity of environmental nitrogen may present as a reduction of general health, fecundity, developmental abnormalities in offspring, or as mortality \(^5,^{23}\). These consequences may occur as a result of environmental nitrogen being converted to nitric oxide which then acts as an endocrine disruptor \(^24,^{25}\). Nitric oxide is an
important signaling molecule which plays a role in many biological processes including reproduction, development, and circadian rhythm\textsuperscript{26-28}.

Nitric oxide interacts with the nuclear receptor rev-erb (called E75 in arthropods) which is a thiolate hemoprotein that contributes to the regulation of circadian rhythm and metabolism\textsuperscript{29-32}. Disruption of rev-erb/E75, either by overexpression or suppression, results in disruptions in circadian rhythms and associated processes\textsuperscript{33}.

E75 is also responsible for regulating the genes that encode the enzymes required for ecdysteroid biosynthesis\textsuperscript{34}. The ecdysteroid signaling cascade is initiated by a pulse of the ecdysteroid 20-hydroxyecdysone (20E)\textsuperscript{35}. This pulse of 20E induces expression of E75 and HR3\textsuperscript{36,37} HR3 is the arthropod ortholog of mammalian ROR. E75 and HR3 can spontaneously dimerize where E75 negatively regulates the transcriptional activity of HR3\textsuperscript{38}. Nitric oxide can bind to the heme moiety of E75 and free HR3 from the dimer\textsuperscript{30}. We have hypothesized that the pulse of nitric oxide responsible for the dissociation of the E75-HR3 dimer in daphnids is linked to environmental cues that ensure a sufficient duration of the intermolt period for embryos to fully develop. Disruption of E75 expression or activity, we posit, increases the length of the molt cycle with adverse consequences on physiological processes that depend upon this cycle (Chapter 2)\textsuperscript{39,40}

In the present study, we hypothesized that low exposure levels of nitric oxide donors would elicit subtle effects on physiological processes which, in combination, would alter the population dynamics of \textit{Daphnia magna}. 
Materials and Methods

*Daphnia magna*

*D. magna* used in these experiments have been cultured in our lab for over 20 years. Daphnids were raised in 1L beakers under conditions suitable for parthenogenetic reproduction as described previously \(^{41}\). Cultures were fed \(1.4 \times 10^7\) cells of green algae (*Raphidocelis subcapitata*) and \(~4\) mg dry weight of Tetrafin® (Tetra, Spectrum Brands, Blacksburg, VA) fish food suspension twice daily. Fish food was prepared as described previously \(^{42}\).

Assessment of Responses of Individual Daphnids

Nitric oxide donors used in this study were sodium nitroprusside (purity ≥99%, Sigma Aldrich, St. Louis, MO) and sodium nitrite (purity ≥99.5%, Sigma Aldrich). Sodium nitroprusside spontaneously decomposes within organisms and liberates nitric oxide \(^{43}\). Sodium nitrite undergoes reductive metabolism to liberate nitric oxide \(^{44}\). Concentration-response experiments were performed to determine the appropriate exposure concentrations of sodium nitroprusside (SNP) and sodium nitrite (NaNO\(_2\)) in the population experiments. Individual daphnids were isolated in 50 mL beakers containing 40 mL of test media. Daphnids were fed \(7 \times 10^5\) cells algae and \(~0.2\) mg fish food suspension daily. Media was changed every other day during these experiments. Daphnids were observed daily for mortality and the number of offspring produced. Neonates were examined microscopically for the presence of developmental abnormalities. Each organism’s exposure was terminated after the release of its fourth brood, approximately 20 days.
Population Experiments

Experiments were performed to evaluate the subtle effects of nitric oxide donors on daphnid population structure and growth rate. Experiments were performed in 1L beakers (microcosms) containing 1000 mL media. Each beaker was seeded with twenty daphnids: five 8-day old, five 6-day old, five 4-day old, and five 2-day old organisms. Each treatment was evaluated using three replicate beakers. Media was changed Mondays, Wednesdays, and Fridays. Fifty neonates were collected from each beaker on Mondays and Fridays and evaluated microscopically for the presence of developmental abnormalities. Neonates were returned to the beaker from which they were derived. The total number of daphnids in the population and their distribution among size classes was determined, as described below, twice per week. Each beaker was initially provided $2.8 \times 10^7$ cells algae and ~8 mg dry weight fish food suspension daily. The amounts of algae and fish food provided to the beakers were increased as daphnid biomass increased (Table 1).

Population Structure and Growth Assessments

Daphnid population size and class structure was evaluated twice weekly using an artificial intelligence/machine learning approach. These methods were modified to be suitable identifying and classifying daphnids based on previously described image segmentation\textsuperscript{45,46}. Briefly, contents of a test beaker were collected by passing the media through a fine-mesh net and contents were transferred to a plexiglass tray (11.13 cm × 22.28 cm) containing 20 mL of the media. The contents of the tray were scanned an Epson Perfection V800/V850 desk-top scanner set to 48-bit color and 600 dpi. Four scans were collected per sample and the mean counts were reported as population sizes. Digitized scans were subjected to analysis using machine learning
software where daphnids were identified, counted, the numbers of individuals in each size class determined. Size classes, estimated from the major axis length, were: Class 1 immature daphnids, <2.2 mm long; Class 2 juvenile daphnids, 2.2 to 2.8 mm long; Class 3 adult daphnids, >2.8 mm long. Lengths reported were the major axis length, measured from the base of the tail spine to the top of the head capsule, above the eye.

Statistics

Statistical comparisons were made using One-way Analysis of Variance followed by Dunnet’s (Concentration response experiments) or Sidak’s multiple comparisons test (population growth assessments) using GraphPad Prism. Homogeneity of variances was confirmed with the Brown-Forsythe test.

Results

Assessment of Responses of Individual Daphnids

We hypothesized that low concentrations of the nitric oxide donors would elicit subtle effects on individual daphnids, which, in combination, would result in significant impacts on population structure and growth. Thus, we first performed concentration-response experiments to determine the effects of sodium nitrite and sodium nitroprusside on survival, development, and reproduction among individual daphnids to aid in choosing the concentration to use for population-level experiments.

Exposure of daphnids to 1.0 and 1.6 mg N/L was lethal to 100% of the exposed daphnids (Fig. 1) whereas exposure concentrations ≤0.60 mg N/L sodium nitrite had no significant effect on survival of the parental organisms. The number of offspring produced by the maternal
organisms exposed to 0.36 and 0.60 mg N/L was significantly reduced (p=0.039, p=0.0001, respectively, Fig.1). Developmental abnormalities among neonates were observed at most sodium nitrite treatment levels but was low (0 to 9%) and non-significant (p>0.05). This incidence did not conform to a concentration-response relationship. Based upon these results, evaluation of the population-level impacts of sodium nitrite was performed at an exposure concentration of 0.40 mg N/L.

Exposure to sodium nitroprusside concentrations as high as 0.64 mg NO/L had no effect on survival of parental daphnids (Fig. 2). Concentrations as low as 0.13 mg NO/L significantly reduced the number of offspring produced per parental organism (p<0.001, Fig. 2). Exposure concentrations that significantly reduced the number of offspring produced also significantly increased the number of developmentally abnormal offspring (p=0.0001, Fig. 2). Based upon these results, impact of sodium nitroprusside on daphnids populations was evaluated at exposure concentrations of 0.10 and 0.17 mg NO/L.

Population Structure and Growth Assessments

Sodium Nitrite (0.40 mg N/L)

The concentration of nitrite in the exposure media was confirmed (control 0.002±0.002 mg N/L, treatment 0.40±0.01 mg N/L) (Hach Colorimeter DR900, Loveland, CO). The control daphnid populations expanded at a linear rate from approximately days 7 to 21 with the populations growing from approximately 200 organisms to 1200. The microcosm was approaching carrying capacity by day 31 with approximately 1400 individuals. Populations exposed to 0.40 mg N/L sodium nitrite followed the same trend as controls (Fig. 3A).
The control and 0.40 mg N/L sodium nitrite-exposed populations of immature daphnids followed the same trends in growth with a linear rate of increase between approximately days 7 and 18 with a microcosm carrying capacity of approximately 1000 organisms (Fig. 3B).

Juvenile daphnids occupied a smaller proportion of the total population with a linear rate of population growth beginning at approximately day 14 and continuing to the end of the experiment (Fig. 3C). The rate of recruitment into this size class was greater with sodium nitrite treatment (rate increase: 9.7±0.31 individuals/day) as compared to control (7.4±0.28 individuals/day) (p<0.01). The sodium nitrite populations were significantly larger than the controls beyond day 25 (p<0.0001).

Adults within the populations comprised the smallest class size of daphnids (Fig. 3D). Both control and sodium nitrite-exposed populations experienced linear growth rates from approximately days 11 to 25. Carrying capacity of the microcosms was reached by approximately day 28 with approximately 115 adults at capacity with no difference between treatments.

Sodium nitroprusside (0.10 mg NO/L)

Exposure to 0.10 mg NO/L sodium nitroprusside had no effect on total populations (Fig. 4A), immature populations (Fig. 4B), or juvenile populations (Fig. 4C) as compared to the controls, which are the same as described in the sodium nitrite (0.40 mg N/L experiment).

Control adult daphnid populations experienced linear growth from day 11 to day 25 with an increase from 8 to 100 over the fourteen days. Carrying capacity was reached at day 28 with approximately 115 daphnids. Populations of adult daphnids exposed to sodium nitroprusside expanded at a linear rate from days 11 to 25 with the populations increasing from 10 to 75
daphnids. Carrying capacity was reached at day 28 with approximately 75 daphnids (Fig. 4D). The rate of recruitment to the adult class during the linear growth phase was 6.6±0.69 individuals/day in controls versus 4.8±0.41 individuals/day in the sodium nitroprusside treatments; however, this difference was not significant (p=0.17). The control populations were significantly larger than the exposed population beyond day 25 (p<0.0003).

*Sodium nitroprusside* (0.17 mg NO/L)

Total control daphnid populations expanded at a linear rate from approximately days 7 to 21 with the populations growing from approximately 100 organisms to 1600. The maximum capacity of the microcosm was attained at approximately day 21 and the populations declined through the end of the experiment to approximately 1400 individuals. Populations exposed to 0.17 mg NO/L sodium nitroprusside expanded at a linear rate from days 10 to 21 with the populations growing from 100 to 1300. The maximum capacity of the microcosm was attained at approximately day 21 and the populations declined through the end of the experiment to approximately 1200 daphnids (Fig 5A). The rate of growth was greater with sodium nitroprusside treatment (rate increase: 107±4.6 individuals/day) as compared to control (99±3.7 individuals/day), though the difference is not significant (p=.24). The control populations were significantly larger than the exposed populations from days 14 to 24 (p<0.02).

The populations of immature daphnids experienced linear growth from day 7 to 21 with the populations growing from 100 to 1400 individuals. Maximum capacity was attained at day 21 and the populations declined through the end of the experiment to approximately 1200 individuals. Populations exposed to 0.17 mg NO/L sodium nitroprusside expanded at a linear rate from days 10 to 21 with the populations growing from 50 to 1200. The carrying capacity
was attained at day 21 and decreased through the end of the experiment to approximately 1000 (Fig. 5B). The rate of recruitment into the immature size class was greater with sodium nitroprusside treatment (rate increase: 95±6.0 individuals/day) as compared to control (85±4.2 individuals/day), though the difference is not significant (p=.24). The control populations were significantly larger than the exposed populations at days 18 and 24 (p<0.004).

The linear growth rate for control juvenile populations occurred from days 7 to 18 with an increase from 10 to 140 daphnids. The microcosm was approaching carrying capacity at day 31 with 175 daphnids. The linear growth in the juvenile populations exposed to sodium nitroprusside occurred from day 10 to day 21 with an increase from 10 to 120 individuals. Carrying capacity was attained at day 28 at 150 individuals (Fig. 5C). The rate of recruitment into the juvenile size class was greater with control (11±0.44 individuals/day) as compared to sodium nitroprusside treatment (rate increase: 8.7±1.2 individuals/day), though the difference is not significant (p=0.2). The control populations were significantly larger than the exposed populations at day 18 (p<0.0001).

Adult daphnid populations experienced linear growth from days 7 to 31 with an increase from 10 to 125 daphnids. Adults exposed to 0.17 mg NO/L sodium nitroprusside experienced a shorter linear growth phase which occurred from day 7 to day 21 with an increase from 10 to 45 individuals (Fig. 5D). The rate of recruitment into the adult size class was greater with control (5.0±0.38 individuals/day) as compared to sodium nitroprusside treatment (rate increase: 2.7±0.35 individuals/day) (p<0.05). The control populations were significantly larger than the exposed populations beyond day 18 (p<0.0001).
Discussion

In the present study we hypothesized that low concentrations of nitric oxide donors would elicit subtle effects which would combine to alter population dynamics of *D. magna*. Environmental nitrogen oxides can be converted in daphnids to nitric oxide\(^{47}\), a potent signaling molecule, which can bind to the heme moiety of the nuclear receptor E75\(^{30}\). We have shown that E75 regulates the timing of the molt rhythm in *D. magna* (Chapter 2)\(^{39}\) and that its disruption results in an increase in the duration of the molt/reproduction cycle. A lengthening of the molt cycle would lead to a reduced number of molt cycles over the lifetime of the daphnids resulting in a slower growth rate\(^{48}\). Delayed growth would also impact the time to attain reproductive maturation, and fewer molt cycles would result in fewer broods of offspring. Accordingly, lengthening of the molt cycle could have multiple adverse effects on individuals that would negatively impact population growth and sustainability.

The concentrations of nitrogen in the concentration-response experiments were less than the ecoregional criteria set by the US EPA which typically range from 0.10 to 2.2 mg N/L depending on the aquatic environment\(^{16}\). Similar to results observed in previous studies with daphnids, fecundity was the most sensitive endpoint evaluated\(^{49-51}\). Fecundity was compromised in *D. obtusa*, *D. similis*, and *S. similoides* by sodium nitrite concentrations as low as 1.6, 3.1, and 1.6 mg N/L, respectively. Daphnids are able to reduce nitrite to nitric oxide\(^{47}\). Whether the reproductive toxicity to nitrite is due to the parent compound or nitric oxide derived from the parent compound is not known. However, the commonality of endpoints affected by various nitric oxide donors\(^{47}\) suggests that that this metabolite may be the active compound. It is likely that the reproductive effects following exposure to nitrite are a result of nitric oxide targeting E75 to disrupt the molt/reproductive cycle.
The concentration of sodium nitrite used in the population study (0.40 mg N/L) did not adversely impact daphnid population growth, despite predictions of adverse population-level outcomes based upon the organismal experiments. Perhaps most revealing was the increased growth rate observed among juvenile daphnids in these experiments. Nitrite is a recognized source of nitrogen for plants which enhances plant productivity. Algae were used as a food source for daphnids in these experiments. Provision of nitrite resulted in visually discernable increased algae as compared to controls. This increased food source likely increased daphnids population growth rates which may have masked negative effects, or, as in the case of juveniles, increased population growth relative to the rate of increase in controls. These results highlight the complexity of microcosm-type experiments, where effects at one trophic level affect performance of another.

Sodium nitroprusside (0.10 mg NO/L) elicited similar effects to 0.40 mg N/L sodium nitrite. Population growth rates were empirically higher among sodium nitroprusside-treated populations (total population and immature), though these differences were not significant. This concentration of sodium nitroprusside did not significantly reduce population growth rate during the linear growth phase from days 11 to 25; however, this is likely due to the populations having a similar growth rate from days 11 to 18. From day 18 to 25, the growth rate in the sodium nitroprusside-treated group is much slower than the control populations, likely as a result of disruption to the infradian molt/reproductive cycle. This concentration of sodium nitroprusside reduced carrying capacity among the adult class of organisms. As observed with sodium nitrite, the observed indirect positive effect, due to increased algae growth, and the observed negative effect, due to toxicity to daphnids, resulted in no significant difference in total population growth rate. The greater potency of sodium nitroprusside as compared to sodium nitrite observed in this
study is consistent with previous assessments and may represent a greater rate of uptake of sodium nitroprusside or enhanced liberation of nitric oxide as compared to sodium nitrite.

Sodium nitroprusside (0.17 mg NO/L) exhibited negative effects on population growth among all size classes of daphnids. This concentration of sodium nitroprusside elicited multiple effects on population-related parameters including an increase in lag time prior to the linear rate of population growth and a decrease in the proportion of adult class organisms within the population. This latter effect may reflect a shortening of the life span of the organisms by this concentration of sodium nitroprusside.

Results of this study generally support the hypothesis that low levels of nitric oxide donors will significantly alter the population dynamics of daphnids by targeting and disrupting these processes that together are critical to population growth and sustainability. However, as nitrogen sources can serve as stimulants of system productivity, negative effects can be masked. Assessment of size/age classes within a population informs on the response of the population due to unique attributes of the classes. These unique attributes include reduced recruitment of immature daphnids due to reduced fecundity, increased rate of juvenile population growth due to reduced rate of growth to the adult stage, and reduced carrying capacity for adults due to shortening of the life span. Results indicate that typical ecoregional limits placed on nitrogen levels in aquatic environments are appropriate for sodium nitrite. However, these limits may be inadequate for compounds that are more potent donors of nitric oxide.
Acknowledgments

This work was supported by the National Science Foundation grant IOS-1350998 to GAL.

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Contributions

GAL, SAE, and SMS conceived and designed the experiments. SAE and SMS conducted the experiments. EMR and KBF developed and applied the machine learning techniques. SAE and GAL analyzed and interpreted the data and prepared the manuscript.

Competing interests

The authors declare no competing financial interests.

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Figures

**Figure 1. Toxicity of sodium nitrite to individual daphnids.** A: Percentage survival (n=10). B: Offspring production among exposed daphnids. Data are presented as the mean and standard error (n=number of surviving adults). Asterisks denote significance from the control (One-way ANOVA and Dunnet’s Multiple Comparisons Test). C: Abnormally developed neonates. Data are presented as the percentage and standard error of abnormally developed neonates from total offspring born to maternal daphnids (n=number of surviving adults).
Figure 2. Sodium nitroprusside concentration-response endpoints. A: Percent survival (n=10). B: Mean number of neonates. Data are presented as the mean and standard error (n=10 individuals). Asterisks denote significance from the control (One-way ANOVA and Dunnet’s Multiple Comparisons Test). C: Percentage of abnormally developed neonates. Data are presented as the percentage and standard error of abnormally developed neonates from total offspring born to maternal daphnids (n=10 individuals).
Figure 3. *D. magna* population growth during exposure to 0.40 mg N/L nitrite. Presented are number of daphnids in microcosms (mean and standard error, n=3) at indicated times. Error bars are often too small to discern. A: total daphnid population; B: immature (<2.2 mm) daphnid population; C: juvenile (2.2-2.8 mm) daphnid population; D: adult (>2.8 mm) daphnid population. Black data points depict control populations and gray data points depict populations exposed to sodium nitrite. An asterisk denotes a significant difference between the control and sodium nitrite treatment.
Figure 4. *D. magna* population growth during exposure to 0.10 mg NO/L sodium nitroprusside. Presented are number of daphnids in microcosms (mean and standard error, n=3) at indicated times. Error bars are often too small to discern. A: total daphnid population; B: immature (<2.2 mm) daphnid population; C: juvenile (2.2-2.8 mm) daphnid population; D: adult (>2.8 mm) daphnid population. Black data points depict control populations and gray data points depict populations exposed to sodium nitroprusside. An asterisk denotes a significant difference between the control and sodium nitroprusside treatment.
Figure 5. *D. magna* population growth during exposure to 0.17 mg NO/L sodium nitroprusside. Presented are number of daphnids in microcosms (mean and standard error, n=3) at indicated times. Error bars are often too small to discern. A: total daphnid population; B: immature (<2.2 mm) daphnid population; C: juvenile (2.2-2.8 mm) daphnid population; D: adult (>2.8 mm) daphnid population. Black data points depict control populations and gray data points depict populations exposed to sodium nitroprusside. An asterisk denotes a significant difference between the control and sodium nitroprusside treatment.
Tables

Table 1. Feeding schedule for daphnids during the population experiments

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<th>Cells algae</th>
<th>Dry weight fish food</th>
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<td>8 mg</td>
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<tr>
<td>3</td>
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<td>10 mg</td>
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<td>10 mg</td>
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<td>7</td>
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Summary and Conclusions

Levels of atmospheric nitrogen are on the rise do to a variety of anthropogenic sources, such as runoff from CAFOS, use of nitrogen-based fertilizer, and the burning of fossil fuels\(^1\text{-}^3\). Typically, levels of biologically available nitrogen are regulated by the nitrogen cycle which involves the balance of nitrification and denitrification by bacteria\(^4\). Though limits have been set to protect sensitive subpopulations of humans (such as infants) as well as aquatic organisms, concentrations of environmental nitrogen frequently rise above the allowable limits\(^5\text{-}^8\).

Daphnids (\textit{Daphnia magna}) are able to reduce nitrate and nitrite from environmental sources to nitric oxide\(^9\). Nitric oxide is a potent signaling molecule which plays a role in many biological processes such as reproduction, development, and circadian rhythm\(^10\text{-}^{12}\). Nitric oxide can regulate the activity of the nuclear receptor E75 by binding to its heme component resulting in dissociation from its heterodimeric partner HR3\(^13\text{-}^{15}\). E75 may regulate the molt rhythm of arthropods by sequestering HR3 and preventing its activation of genes downstream in the signaling cascade. The molt rhythm begins with a pulse of 20-hydroxyecdysone which stimulates ecdysis and brood release\(^16\). 20-Hydroxyecdysone also induces expression of E75 and HR3\(^17,^{18}\), and we have hypothesized that a pulse of nitric oxide, likely in response to an environmental cue, interacts with the heme moiety of E75 to release HR3 from the dimer. Nitric oxide thus serves to establish the required duration of the intermolt period, based upon environmental conditions, to ensure proper development of offspring. HR3, we propose, induces expression of FTZ which then regulates the expression of cytochrome P450 enzymes responsible for generating the next 20-hydroxyecdysone pulse\(^19\text{-}^{21}\). Thus, we hypothesize that E75 is an early regulator of the infradian molt rhythm and its disruption can adversely impact daphnids, and other arthropod, populations.
In chapter one, we describe the development of a protocol for targeted gene suppression by feeding *E. coli* expressing dsRNA targeting our gene of interest. We evaluated the suppression of hemoglobin mRNA and protein for this development as hemoglobin protein accumulation causes an easily discernible color change within the transparent daphnids. Also, hemoglobin is induced by exposure to the compound pyriproxyfen\textsuperscript{22–26}. When fed *E. coli* expressing dsRNA targeting hemoglobin, hemoglobin mRNA and protein levels were suppressed. Additionally, we hypothesized that high hemoglobin levels would protect daphnids from the toxicity of nitrite while suppressed levels would sensitize the organisms to nitrite. This hypothesis was based upon the premise that nitrite binds to the heme moiety of hemoglobin (REF). High levels of hemoglobin serve as a sink to sequester nitrite without exhausting available hemoglobin for oxygen transport, while low levels of hemoglobin result in the loss of oxygen carrying capacity due to nitrite binding resulting in toxicity. Results supported this hypothesis as induction of hemoglobin by exposure to pyriproxyfen ameliorated toxicity of nitrite whereas suppression of hemoglobin levels by RNAi sensitized the daphnids to the toxicity of nitrite.

Chapter two describes the utilization of this method to investigate the function of E75 in regulating the infradian molt rhythm and the consequences of its disruption. The endpoints we assessed included alterations to mRNA expression profiles for genes proposed to be involved in the ecdysteroid signaling cascade which occurs over the molt rhythm, duration of the molt rhythm, reproduction, and the development of offspring.

Provision of dsRNA targeting E75 elicited the predicted responses on mRNA levels of relevant nuclear receptors. Specifically, dsRNA treatment caused the suppression of E75, no effect on HR3, and an elevation of FTZ. Treatment with dsRNA targeting E75 had no effect on
cytochrome P450s involved in 20-hydroxyecdysone synthesis, but suppressed mRNA levels of CYP18a1 which is responsible for inactivation of 20-hydroxyecdysone.

The suppression of E75 increased the duration of the intermolt period, consistent with our hypothesis. The observed elongation of the molt rhythm is in agreement with previous studies which showed that disruption of E75 function by a pharmaceutical targeting E75 or a nitric oxide-generating compound increased the duration of the intermolt period\textsuperscript{27,28}.

The suppression of E75 also reduced the number of offspring produced by the daphnids. E75 has been shown to regulate oogenesis and vitellogenesis in insects\textsuperscript{29,30}, and suppression of E75 has been linked to a reduction in fecundity\textsuperscript{31}. As such, it is plausible that E75, or some gene product regulated by E75, plays a role in the reproductive processes of daphnids. In conclusion, results demonstrated that E75 is an important regulator of the infradian molt rhythm in daphnids. Disruption of E75 function, as demonstrated herein using RNAi techniques, can adversely affect multiple processes tied to this rhythm including growth and fecundity. These observations lead to the possibility that environmental disruption of E75 function may lead to adverse population-level outcomes. Possible environmental disruptors of E75 include nitrogen oxides.

We next evaluated the premise that exposure to low levels of generators of nitric oxide would elicit adverse effects on population dynamics of daphnids. We investigated the effects of sodium nitrite, which is metabolized to nitric oxide\textsuperscript{32}, and sodium nitroprusside, of which nitric oxide is a product of spontaneous breakdown\textsuperscript{33}.

Daphnids population growth and size/age distribution were evaluated over 31 days exposure to the nitric oxide donors. Exposure concentrations were selected that were predicted to elicit minimal effects on individual daphnids based on preliminary tests. Daphnid population size and class structure was determined periodically during the experiments using machine learning
and convolutional neural networks. Daphnids were classified as: Class 1 immature daphnids, <2.2 mm long; Class 2 juvenile daphnids, 2.2 to 2.8 mm long; Class 3 adult daphnids, >2.8 mm long.

Population experiments revealed the value of evaluating size/age classes within the population to assist in identifying susceptible sub-populations and to inform on the types of effects elicited by the compound under evaluation. Results revealed that nitric oxide donors elicit multiple adverse effects on the population growth rates. However, these compounds also elicit positive effects on daphnid population growth by enhancing the growth of algae, the prime nutrition source for the daphnids. Such opposing effects highlighted the complexities associated with ecological communities that cannot be captured with single species toxicity testing.

Results from this project provide a greater understanding of the role of E75 in *D. magna* as well as consequences of its disruption on individuals and populations. Daphnids are commonly used as a model crustacean in laboratory studies. Understanding the regulation and consequences of the disruption of their molt rhythm likely extends to crustaceans valued commercially. Furthermore, daphnids serve a vital ecological role as converters of bacterial and unicellular algae to a food source for larger aquatic organisms such as fish. Increasing the duration of the molt rhythm would result in fewer molt cycles over the lifetime of the daphnid. Brood release occurs with molting, thus fewer broods would be released over the lifetime of the daphnid and as demonstrated herein, brood sizes would decrease. Evaluating the impact of nitrogen oxide generating compounds on daphnid populations is difficult to predict owing to both positive and negative effects of these compounds. However, as demonstrated with sodium nitroprusside, the adverse effects of these compounds can dominate resulting in compromised populations.
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APPENDIX A: LIGAND-MEDIATED RECEPTOR ASSEMBLY AS AN ENDPOINT FOR HIGH-THROUGHPUT CHEMICAL TOXICITY SCREENING

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This manuscript has been published by the journal Environmental Science and Technology and is in the format of that journal.

My contribution to this work was performing in vivo exposure assessments.
Abstract

The high throughput screening of chemicals for interaction with intracellular targets is gaining prominence in the toxicity evaluation of environmental chemicals. We describe ligand-mediated receptor assembly as an early event in receptor signaling and its application to the screening of chemicals for interaction with targeted receptors. We utilized bioluminescence resonance energy transfer (BRET) to detect and quantify assembly of the methyl farnesoate receptor (MfR) in response to various high-production volume and other chemicals. The hormone methyl farnesoate binds to the MfR to regulate various aspects of reproduction and development in crustaceans. The MfR protein subunits Met and SRC, cloned from Daphnia pulex, were fused to the fluorophore, mAmetrine and the photon generator, Rluc2, respectively. Ligand-mediated receptor assembly was measured by photon transfer from the photon donor to the fluorophore resulting in fluorescence emission. Overall, the BRET assay had comparable or greater sensitivity as compared to a traditional reporter gene assay. Further, chemicals that screened positive in the BRET assay also stimulated phenotypic outcomes in daphnids that result from MfR signaling. We concluded the BRET assay is an accurate, sensitive, and cost/time efficient alternative to traditional screening assays.
Introduction

Endocrine signaling pathways are crucial to survival and reproduction, however these pathways are often susceptible to disruption by environmental chemicals resulting in perturbations in normal physiology \(^1-^4\). Environmental exposure to endocrine disrupting chemicals has been associated with reproductive dysfunction \(^5\), perturbations in reproductive development \(^6,^7\), and population demise \(^8\). As a result, significant effort has gone into the development of screening and testing methods for detecting endocrine-disrupting properties of chemicals, and hazards associated with the use of these chemicals \(^9-^13\). Screening assays used to detect endocrine-disrupting activity of chemicals often consist of hormone-receptor binding assays or reporter gene transcription assays \(^9-^11\). Chemicals that screen positive in one or more of these assays then become a candidate for more definitive testing to assess whole organism consequences of this activity and the exposure concentrations at which effects occur \(^12\).

However, receptor-binding assays are relatively uninformative, because they provide no information on the consequence of binding (e.g., receptor activation, inhibition, or no consequence). Reporter gene transcription assays are more informative, however these assays rely upon reporter gene transcription and translation which increases the time required to provide a measurable endpoint.

Most hormone receptors consist of homo- or hetero-dimers \(^14\). The first step in activation of many of these receptors is ligand-stimulated dimerization \(^14-^16\). This endpoint can serve as an initiating event in the adverse outcome pathway \(^17\) for many endocrine signaling pathways. This study addresses the potential for protein dimerization to serve as an endpoint for the detection of chemical-induced receptor activation using the methyl farnesoate receptor as a model.
Methyl farnesoate has long been recognized as a hormone involved in reproduction and development in crustaceans. Methyl farnesoate stimulates male sex determination, in branchiopod crustaceans and male sex differentiation in some decapod crustaceans. Recently, we and others identified the protein receptor complex that mediates the actions of methyl farnesoate, the methyl farnesoate receptor (MfR). Further, we demonstrated methyl farnesoate stimulates the association of the protein methoprene tolerant (Met) with its partner, steroid receptor coactivator (SRC). These assembled proteins comprise the active MfR.

Bioluminescence resonance energy transfer (BRET) technology has gained prominence as a means of measuring protein-protein interactions in cells and in real time. The method involves the construction of fusion proteins whereby one protein of interest is fused to a luciferase protein. The other protein of interest is fused to a fluorophore. When the proteins associate, photons generated by the luciferase can excite the fluorophore resulting in fluorescence emission. BRET has been extensively used in the study of G-protein coupled receptors, but more recently has been used in the study of homo- and hetero-dimerization of hormone nuclear receptors.

Herein, we describe the construction and optimization of a novel approach to screen chemicals for hormone receptor activation using the MfR cloned from daphnids (Daphnia pulex). We propose that this methodology could be applied to all receptor complexes whose subunit dimerization is agonist driven. Further, we screened several compounds for comparison of specificity and sensitivity to the more traditional luciferase reporter gene transcription assay. Finally, compounds that screened positive in the BRET assay were evaluated in vivo to determine whether results from the cell-based assay accurately predicted phenotypic outcomes in the whole organism.
Material and Methods

Methyl farnesoate (Echelon Biosciences Inc., Salt Lake City, Utah), and all other chemicals (Sigma-Aldrich Corp., St. Louis, MO) screened in BRET and luciferase reporter gene assays, were dissolved in DMSO for delivery to assay solutions. Final DMSO concentrations were 0.001% v/v in the BRET assays and 0.0005% v/v in the reporter gene assays. Hydroprene and diofenolan were dissolved in ethanol for in vivo experiments, where the final concentration of ethanol was 0.0003% v/v.

Fusion Protein Construction

Four fusion proteins were constructed to identify those constructs that provided the optimum BRET signal. The daphnid met open reading frame, described previously, was fused to the fluorophore mAmetrine open reading frame (mAme) (excitation: 510 nm, emission: 535nm) (Addgene, Cambridge MA) at either the 5’ or 3’ end of met. The daphnid SRC open reading frame, described previously, was fused to the Renilla luciferase 2 open reading frame (RLuc2) (substrate: coelenterazine 400A, emission: 410 nm) (Dr. Sanjiv Gambhir, Stanford University, School of Medicine, Stanford, CA) at either the 5’ or 3’ end of SRC.

The met gene was amplified using primers harboring NotHFI (forward containing linker sequence, ATAGCGGAAGTGGTAGCGGAAGTGGT) and ApaI (reverse) restriction enzyme sites, and sub-cloned into the pMT-B vector (ThermoFisher Scientific). mAme was amplified from the pBad cloning vector using primers with KpnI (forward) and NotHFI (reverse) sites, and subcloned at the 5’-terminus of the pMT-met, to create pMT-mAme-linker-met (mAme-Met). A similar procedure was used to construct the pMT-met-linker-mAme (Met-mAme), with some exceptions. Primers harboring KpnI (forward) and NotHFI (reverse) sites were used to amplify
met, while primers with NotHFI (forward containing linker sequence) and BstBI (reverse) sites amplified mAme.

SRC was amplified from the TOPO cloning vector using primers harboring BstB1 (forward) and AgeI (reverse) restriction enzyme sites, and was sub-cloned into the pMT-B vector. Rluc2 was amplified from the pcDNA storage vector using primers harboring XhoI (forward) and BstBI (reverse) sites. The reverse primer also contained a nucleotide “linker” sequence (AGCGGAAGTGGTAGCGGAAGTGGC) to lengthen the distance between the two proteins and decrease probability of incorrect folding. The Rluc2-linker sequence was sub-cloned at the 5’-terminus of the pMT-SRC plasmid, to create pMT- Rluc2-linker-SRC (Rluc2-SRC). A similar procedure was used to construct the pMT-SRC-linker-Rluc2 (SRC-Rluc2), with some exceptions. Primers harboring XhoI (forward) and BstB1 (reverse) sites was used to amplify SRC, while primers with BstB1 (forward containing linker sequence) and AgeI (reverse) sites were used to amplify Rluc2. All four fusion proteins, Rluc2-SRC, SRC-Rluc2, mAme-Met, and Met-mAme, were sequenced (Eton Bioscience, San Diego, CA) to ensure the fluorescent/luminescent proteins were in frame with the respective MfR subunit protein. All fusion constructs were successfully sub-cloned without amino acid substitutions.

**BRET Assay**

BRET assays were performed as we described previously. Assays were performed in *Drosophila* Schneider (S2) cells (Invitrogen). Cells were grown in Schneider’s medium, containing 10% heat inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA), 50 mg/ml penicillin G and 50 mg/ml streptomycin sulfate (Fisher Scientific, Pittsburgh, PA), and incubated...
at 23°C under ambient air atmosphere. Cells were seeded at a density of 3 x 10^6 in 35 mm dishes in 6-well plates and transfected 24 hours after plating.

The relevant plasmids were transiently transfected into cells by calcium phosphate DNA precipitation. Total DNA concentration remained constant across all experiments at 2.8 ng/µL, while the photon donor: protein acceptor ratio was held at an optimized 1: 6 ratio (produced greatest energy transfer). Transcription was induced by exposing cells to 500 µM CuSO₄ for 24 hours. Cells were treated with test chemical or vehicle control for 1 hour in 1x phosphate-buffered saline medium. The Rluc2 substrate, coelenterazine 400A (Biotium, Inc.), was then added to each well (5.0 µM), and light emission was measured immediately at 410 ± 40 nm (emission produced by Rluc2) and 535 ± 15 nm (emission produced by mAme) using a FluoroStar fluorimeter (BMG Labtech). The ratio of light emitted at 535 nm/410 nm (corrected for basal level donor emission of Rluc2 15, 25) is termed the BRET ratio. The BRET ratio provided a quantitative measure of the degree of MfR protein association. All treatments were replicated 3 times.

**Luciferase Reporter Gene Assay**

Luciferase-based transcription reporter gene assays were conducted for comparison to BRET with respect to specificity and sensitivity. S2 cells were transiently transfected with daphnid met full open reading frame fused to the Gal4 DNA binding domain 22, daphnid SRC, Renilla Luciferase (pRL-CMV, internal transfection control, Promega) and the firefly luciferase reporter gene vector (pGL5-Luc, Promega) that contained five upstream GAL4 binding sites. Following transfection, transcription of Met-Gal4 and SRC was induced with CuSO₄ (100 µM for 24 hours). Cells then were treated with test chemical in Ex-cellTM 420 insect serum-free
medium with L-glutamine (SAFC Biosciences, Sigma, St. Louis, MO). Cells were harvested after 24 hours incubation. Emissions from the firefly luciferase and Renilla luciferases were measured using the Dual-Glo® luciferase system (Promega). Firefly luciferase emission was normalized to Renilla luciferase emission, and each chemical treatment group was normalized to DMSO control treated cells. All treatments were replicated 3 times.

**Male Sex Determination**

*D. magna* were cultured under parthenogenetic rearing conditions where all offspring produced are female\(^{29,30}\). Hydroprene and diofenolan were used in *in vivo* exposure assays to determine their potency in stimulating male sex determination. Gravid adult female daphnids of the same age were selected from cultures, and placed individually in 50 mL beakers containing 40 mL daphnid media. The daphnids were exposed to serial dilutions of the evaluated chemicals, where each treatment consisted of ten individual daphnids. Solutions were renewed daily. Animals were fed 0.20 mg (dry wt) fish food and 7 \(\times\) \(10^6\) algae cells (*Pseudokirchneriella subcapitata*), prepared as described elsewhere\(^4\), daily and daphnids were assessed for survival, brood release. Survival of maternal organisms was >90% in all treatments. The percentage of males in the second brood was used to determine chemical potency. Experiments were terminated after the second brood release. Methods used are described in greater detail elsewhere\(^{30,31}\).

**Statistical Analysis**

Comparisons of two means were evaluated for significance (\(p<0.05\)) using Student’s t-test. Equal variance between multiple treatment groups was confirmed with Brown-Forsythe’s
test. One-way analysis of variance (ANOVA), followed by Tukey’s multiple comparison procedure, was used to evaluate significant differences between the control and multiple treatments. All statistics were performed using Origin software (OriginLab Corp., Northhampton, MA).

Results

BRET Assay

Fusion proteins were constructed (Fig. 1) in different configurations and used in BRET assays to determine which configuration provided the strongest BRET ratio. Significant increases in BRET ratio upon the addition of methyl farnesoate occurred with all fusion protein configurations (Fig. 2). However, the greatest increase in BRET ratio between the control and hormone treatment occurred in cells expressing both subunit proteins fused at the N-terminus with the fluorophore or luciferase protein: mAme-Met and Rluc2-SRC (Fig. 2). All subsequent BRET assays were performed with these two protein constructs.

Twenty-nine environmental chemicals (Table S1) were screened at 100 µM for their ability to stimulate MfR subunit association. Chemicals were selected based upon propensity for environmental exposure or structural similarity to methyl farnesoate. All compounds appeared to be non-toxic at 100 µM except for TBBPA which caused a significant (p<0.05, ANOVA) reduction in Rluc2 luciferase. The positive control, methyl farnesoate, and six of the test compounds significantly (p<0.05) stimulated MfR subunit association compared to controls (Fig. 3). These compounds, methoprene, kinoprene, pyriproxyfen, fenoxycarb, diofenoaln, and hydroproene, were all insect growth regulating (IGR) insecticides.
The compounds that screened positive were assayed over a range of chemical concentrations to determine relative potency in stimulating MfR association. Chemical potency or assay sensitivity were judged by the lowest observed effect concentration (LOEC). The positive control, methyl farnesoate, significantly activated subunit association with an LOEC of 3.0 µM (Fig. 4A). Methoprene (LOEC = 10 µM) (Fig. 4B) and kinoprene (LOEC = 100 µM) (Fig. 4C) were both less potent at stimulating subunit assembly compared to methyl farnesoate.

The remaining compounds, pyriproxyfen (LOEC = 1.0 µM) (Fig. 4D), hydroprene (LOEC = 0.3 µM) (Fig. 4E), diofenolan (LOEC = <0.0030 µM) (Fig. 4F), and fenoxycarb (LOEC = 0.0030 µM) (Fig. 4G) were all more potent than the hormone at stimulating MfR subunit association.

**MfR Reporter Gene Activation**

The same suite of twenty-nine environmental chemicals (Table S1) also were screened for their ability to stimulate MfR-initiated gene transcription (Fig. 5). Only the positive control and five of the six compounds that were active in the BRET assay significantly stimulated transcription of the reporter gene at 100 µM. Kinoprene, the weak agonist in the BRET assay (Fig. 3) failed to activate transcription in the reporter gene assay (Fig. 5).

Concentration-response analyses were performed with respect to reporter gene transcriptional activation using methyl farnesoate and the same suite of compounds that were active in the BRET assay. Methyl farnesoate significantly stimulated transcription with an LOEC of 30 µM (Fig. 6A). Methoprene was comparable to the hormone in potency using the reporter gene assay (LOEC = 30 µM, Fig. 6B). Kinoprene, again did not activate transcription in the reporter gene assay (Fig. 6C). Pyriproxyfen (LOEC = 1.0 µM, Fig. 6D), hydroprene (LOEC =
0.30 µM, Fig. 6E), diofenolan (LOEC = 0.30 µM, Fig. 6F), and fenoxycarb (LOEC = 0.010 µM, Fig. 6G) were all more potent than methyl farnesoate in activating gene transcription.

**Male Sex Determination**

Lastly, we evaluated the ability of the BRET MfR subunit association assay to predict male sex determination in *D. magna*. Two of the most potent chemicals, hydroprene and diofenolan, that generated a BRET signal also stimulated the production of male offspring at low exposure concentrations. Daphnids exposed to 0.001 nM diofenolan (Fig. 7A), and 0.03 nM hydroprene (Fig. 7B) produced all male offspring. All other compounds that screened positive in the BRET assay have been previously shown to stimulate male sex determination in vivo \(^{31,32}\), although kinoprene did not stimulate male sex determination in our previous *in vivo* assay \(^{31}\).

**Discussion**

Screening assays that detect the potential for a chemical to elicit toxicity typically utilize an early event in a relevant toxicity pathway\(^{33}\), *e.g.* ligand-mediated gene transcription. In fact, transcriptional activation assays quantified by reporter genes are extensively used to detect nuclear receptor-chemical interactions in toxicity screening formats\(^{34-36}\). We have demonstrated the use of an endpoint upstream of gene transcription that is well-suited for the evaluation of chemical interactions with many different nuclear receptors.

Nuclear receptors typically function as homo- or hetero-dimers, with dimerization initiated by the binding of an activating ligand\(^{37}\). The BRET assay, as described herein, can be exploited to detect this initial receptor assembly response to agonist binding in living cells and in
real-time with no requirement for transcription, translation, and accumulation of a reporter gene product.

We used the BRET assay to screen a battery of 29 chemicals for their ability to activate the methyl farnesoate receptor (MfR). Several of these compounds are designed for environmental use (e.g., pesticides) or designated as high production volume compounds (compounds produced or imported into the United States in quantities equal to or greater than one million pounds per year)\textsuperscript{38, 39} and thus have potential environmental exposure. Others were selected because they are known to stimulate male sex determination in daphnids or are structurally related to such compounds. In comparison to the reporter gene assay, the BRET assay was as or more efficient in detecting MfR agonists with no false negatives detected. There was one positive result from the BRET assay that was not detected using the reporter gene assay, kinoprene. Kinoprene is a member of the insect growth regulating class of insecticides, akin to all other assayed members of this chemical class which yielded positive results in both the BRET and reporter gene assays. This positive result likely represented greater sensitivity of the BRET assay. Furthermore, kinoprene yielded positive results in an \textit{in vivo} assay for activity towards the MfR\textsuperscript{40}. Based upon this limited dataset, the BRET assay appears to have comparable or greater sensitivity than the reporter gene assay (Table S2).

The MfR mediates the action of the hormone methyl farnesoate in crustaceans\textsuperscript{22} and the MfR signaling pathway is involved in larval development, metamorphosis, reproductive maturation, and sex determination of offspring\textsuperscript{21}. The methyl farnesoate hormone is the unepoxided form of an insect hormone ortholog, juvenile hormone III\textsuperscript{41}. Results of the present study suggest that the MfR exhibits a high degree of ligand specificity, as the only compounds that stimulated dimerization, reporter gene activation, and \textit{in vivo} responses were insect growth-
regulating insecticides. These compounds are considered to elicit insecticidal activity by mimicking the action of juvenile hormone\textsuperscript{42}. Further investigation is warranted to establish the extent of this apparent specificity.

All compounds that screened positive in the BRET assay have been shown to stimulate male sex determination in daphnids\textsuperscript{22, 31, 32, 43}. We had not previously evaluated hydroprene and diofenolan \textit{in vivo}, while, others had reported high potency of these compounds with respect to stimulating male sex determination\textsuperscript{40, 44}. Therefore, we evaluated these compounds in the present study. Consistent with previous results, both compounds stimulate male sex determination at low nM concentrations.

A negative aspect of the BRET assay as compared to reporter gene assays is the lower signal intensity. Maximum BRET ratios detected in the present study were less than 10X those measured in controls. While, maximum reporter gene responses approached 50X control values. Despite the lower signal intensity, BRET results were sufficient to derive statistically discernible activation along with concentration-response relationships. Major positive attributes to the BRET assay are its rapidity, its conduciveness to real-time measurements in intact cells, and reduced cost due to reduced time commitment.

The BRET assay has demonstrated utility with other nuclear receptors such as the estrogen receptor dimer\textsuperscript{15}, the peroxisome proliferator-activated receptor: retinoid X receptor heterodimer\textsuperscript{45}, and the thyroid hormone receptor: retinoid X receptor heterodimer\textsuperscript{16}. The assay typically has been used to assess receptor interaction with its endogenous ligand. Clearly, the methodology can be applied to the screening of environmental chemicals for activity with nuclear receptors. With proper validation\textsuperscript{46} such assays could significantly increase the capacity of screening programs\textsuperscript{47} to evaluate receptors and chemicals for interaction.
Supporting Information: Table of the thirty compounds used in screening experiments, along with corresponding IUPAC name, commercial use and/or activity and the screening rationale.

Author Contributions: E.K.M.K and G.A.L contributed equally to the experimental design and manuscript preparation. S.A.E conducted daphnid in vivo exposure experiments. E.K.M.K conducted all other experiments.

Notes: The authors declare no competing financial interest.

Acknowledgments: This research was supported by US EPA grant RD-835165 and NIEHS grant T32 ES007046.

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Phone, (919) 515-7404

Email, Gerald_leblanc@ncsu.edu
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39. USEPA High Production Volume Information System (HPVIS).


Figure 1. The detection of MfR agonists using BRET A) SRC is fused to Rluc2, which metabolizes coelenterazine 400A to emit light at 410 nm. B) In the presence of an agonist (Ag), dimerization with Met is stimulated and the light emitted at 410 nm excites the proximal yellow fluorescent protein, mAmetrine (mAme), that is fused to Met to emit a secondary light at 535 nm.
Figure 2. BRET signaling using MfR subunits with different bioluminescent/fluorescent protein configurations Cells were transfected with four different sets of fusion proteins, where Met was fused with a mAmetrine and SRC was fused with Renilla luciferase 2 at either the N- or C- terminus. White bars indicate cells treated with vehicle (DMSO) control and grey bars indicate cells treated with 100 µM methyl farnesoate. Data are presented as mean with standard deviation (n = 3) and an asterisk denotes a significant increase in Met: SRC binding compared to control (p<0.05).
Figure 3. Chemical screen for MfR activation using BRET assay. White bar indicates DMSO vehicle control, light grey bar indicates the positive control (methyl farnesoate), and dark grey bars indicate screened chemicals. Data are presented as means with standard deviations (n = 3) and an asterisk denotes a significant increase in Met-SRC dimerization compared to DMSO control (p<0.05).
Figure 4. MfR BRET assay: concentration-response analysis for MfR activators identified in the initial BRET screen. Cells containing the MfR BRET fusion proteins were treated with increasing concentrations of compounds that screened positive in the BRET assay: (A) Methyl Farnesoate, (B) Methoprene, (C) Kinoprene, (D) Pyriproxyfen, (E) Hydroprene, (F) Diofenolan, (G) Fenoxy carb. The red circle on the y-axis represents the control group where cells were treated with the DMSO vehicle control. Data are presented as mean with standard deviation (n = 3) and an asterisk denotes significant increase in Met: SRC dimerization compared to control (p<0.01, ANOVA, Tukey’s Multiple Comparison test).
Figure 5. Chemical screen for MfR activation using luciferase reporter gene assay. White bar indicates DMSO vehicle control, light grey bar indicates the positive control (methyl farnesoate), and dark grey bars indicate screened chemicals. Data are presented as means with standard deviations (n = 3) and an asterisk denotes significant increase in Met-SRC dimerization compared to DMSO control (p<0.05).
Identified in the initial BRET screen Cells expressing MfR subunits and a luciferase reporter gene were treated with increasing concentration compounds that screened positive in the BRET assay: (A) Methyl Farnesoate, (B) Methoprene, (C) Kinoprene, (D) Pyriproxyfen, (E) Hydroprene, (F) Diofenolan, (G) Fenoxycarb. The red circle on the y-axis represents the control group where cells were treated with the DMSO vehicle control. Data are presented as means with standard deviations (n = 3) and an asterisk denotes significant increase in transcription of the luciferase reporter gene compared to control (p<0.05, ANOVA, Tukey’s Multiple Comparison test).
Figure 7. Male offspring production in response to diofenolan and hydroprene exposure

Adult female daphnids were exposed to diofenolan (A) and hydroprene (B). Data are presented as the percentage of females that produced a male-containing brood at each treatment concentration (n = 9-10).
Table S1. Chemicals screened in the MfR BRET and reporter gene assays.

<table>
<thead>
<tr>
<th>CHEMICAL NAME</th>
<th>IUPAC NAME</th>
<th>COMMERCIAL USE(S) OR ACTIVITY</th>
<th>SCREENING RATIONALE&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>METHYL FARNESOATE</td>
<td>methyl (2E,6E)-3,7,11-trimethyldodeca-2,6,10-trienoate</td>
<td>crustacean hormone</td>
<td>endogenous hormone</td>
</tr>
<tr>
<td>1,2,4-TRIAZOLE PYRRODIAZOLE DIMETHYLPROPANOIC ACID (DMPA)</td>
<td>1H-1,2,4-triazole</td>
<td>antifungal, fluconazole</td>
<td>1</td>
</tr>
<tr>
<td>TRIZMA BASE</td>
<td>2-amino-2-(hydroxymethyl)-propane-1,3-diol</td>
<td>industrial adhesives, sealant and lubricant</td>
<td>1</td>
</tr>
<tr>
<td>4-VINYL CYCLOHEXENE ISOPHTHALONITRILE</td>
<td>4-ethenylcyclohexene</td>
<td>fuels/additives, solvents</td>
<td>1</td>
</tr>
<tr>
<td>2-NITROPROPANE</td>
<td>2-nitropropane</td>
<td>industrial organic solvent for coatings, dyes, adhesives</td>
<td>1</td>
</tr>
<tr>
<td>RESORCINOL</td>
<td>benzene-1,3-diol</td>
<td>industrial adhesives/sealants, textiles, and plastic</td>
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</tr>
<tr>
<td>ACETYL TRIBUTYL CITRATE</td>
<td>tributyl 2-acetyloxypropane-1,2,3-tricarboxylate</td>
<td>industrial plasticizers, adhesives, and food packaging</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>1</sup> 1) EPA high production volume chemical 2) Chemical is known to stimulate male sex determination in daphnids 3) Chemical has similar structure to known stimulators of male sex determination in daphnids 4) Chemical mimics the action of 20-hydroxyecdysone in Lepidopterous.
<table>
<thead>
<tr>
<th><strong>TABLE S1 CONTINUED</strong></th>
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<tbody>
<tr>
<td><strong>4-HYDROXYANISOLE</strong></td>
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<tr>
<td><strong>2-VINYLPYRIDINE</strong></td>
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<tr>
<td><strong>BENZOYL CHLORIDE</strong></td>
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<td><strong>TRICLOCARBAN</strong></td>
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<td><strong>1,3,5-TRIOXANE</strong></td>
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<tr>
<td><strong>1,5-CYCLOOCTADIENE</strong></td>
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<tr>
<td><strong>ANETHOLE</strong></td>
</tr>
<tr>
<td><strong>3,3',5,5'-TETRABROMOBISPHENOL A (TBBPA)</strong></td>
</tr>
<tr>
<td><strong>TRICLOSAN</strong></td>
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<tr>
<td><strong>METHOPRENE</strong></td>
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<tr>
<td><strong>PYRIPROXYFEN</strong></td>
</tr>
<tr>
<td><strong>FENOXYPAR</strong></td>
</tr>
<tr>
<td>Chemical Name</td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>Dieldrin</td>
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<tr>
<td>Endrin</td>
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<tr>
<td>Atrazine</td>
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<td>Kinoprene</td>
</tr>
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<td>Methyl Geranate</td>
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<tr>
<td>Heptanoic Acid</td>
</tr>
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<td>Diofenolan</td>
</tr>
<tr>
<td>Hydroprene</td>
</tr>
<tr>
<td>Farnesol</td>
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Table S2. Lowest observed effect concentrations (LOEC) derived from concentration-response analysis using the BRET and reporter gene assays.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>LOEC (μM)</th>
<th>BRET</th>
<th>Reporter Gene Assay</th>
</tr>
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<tbody>
<tr>
<td>Diofenolan</td>
<td>≤0.0030</td>
<td></td>
<td>0.30</td>
</tr>
<tr>
<td>Fenoxycarb</td>
<td>0.0030</td>
<td></td>
<td>0.010</td>
</tr>
<tr>
<td>Hydroprene</td>
<td>0.30</td>
<td></td>
<td>0.30</td>
</tr>
<tr>
<td>Pyriproxyfen</td>
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<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Methyl Farnesoate</td>
<td>3.0</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Methoprene</td>
<td>10</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Kinoprene</td>
<td>100</td>
<td></td>
<td>&gt;100</td>
</tr>
</tbody>
</table>
APPENDIX B: STATISTICAL VALIDATION OF STRUCTURED POPULATION MODELS FOR *DAPHNIA MAGNA*

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This manuscript has been published by the journal Mathematical Biosciences and is in the format of that journal.

My contribution to this work was providing technical expertise regarding care of and experimentation with *Daphnia magna*. 
Abstract

In this study we use statistical validation techniques to verify density-dependent mechanisms hypothesized for populations of *Daphnia magna*. We develop structured population models that exemplify specific mechanisms, and use multi-scale experimental data in order to test their importance. We show that fecundity and survival rates are affected by both time-varying density-independent factors, such as age, and density-dependent factors, such as competition. We perform uncertainty analysis and show that our parameters are estimated with a high degree of confidence. Further, we perform a sensitivity analysis to understand how changes in fecundity and survival rates affect population size and age-structure.

Key Words: Sensitivity analysis; structured population model; uncertainty quantification; density-dependence; multi-scale data; *Daphnia magna*
1 Introduction

Structured population models (SPMs) are well characterized for describing aggregate ecological data across a wide variety of species [14, 18]. Numerous studies have exemplified the practical utility of SPMs in conservation biology [16, 21, 46, 47] and hazard assessments [45, 50] by making predictions of population decline or recovery. Importantly, SPMs have been used to analyze factors influencing the imperilment of endangered species populations [17, 26, 28, 29, 51].

The predictive value of a SPM, or of any mathematical model, relies on the degree of fidelity of the model to existing data and in the uncertainty in parameters estimated from that data. Several factors involving data information content can affect the uncertainty in parameters estimated for a structured population model. Beyond the usual issues in optimizing the measurement frequency, variance, and resolution of the structured variable (age/size), a central problem affecting SPM parameter uncertainty is that aggregate data may not support the simultaneous estimation of parameters describing multiple biological scales. This “individual dynamics/aggregate data” problem [10] arises due to the interrelation of individual dynamics and aggregate behavior described by SPMs. For example, the mathematical equations describing a fecundity rate in the model might involve a density-independent rate multiplied by a density-dependent rate. Since a lower density-independent rate can be compensated for by a higher density-dependent rate, the multiplication creates a correlation that contributes to a higher level of uncertainty when these rates are concurrently estimated.

An additional confounding factor in estimating parameters for SPMs is encountered when density-independent demographic rates are time- or age-dependent. For example, the rates describing fecundity and survival are known to vary with age in many species. In addition, these
age-dependent rates may also be affected by exposure of the organism to exogenous chemicals or other stressful environmental conditions. Although SPMs can be easily modified to describe age-dependent demographic parameters, the accurate estimation of those parameters can be prohibited by practical limitations, e.g., computational tractability [2, 52]. Moreover, the individual dynamics/aggregate data problem is exacerbated because time-dependence is mathematically treated by extending a single parameter to a function described by several parameters.

One approach to redressing the “individual dynamics/aggregate data” problem is to collect, when feasible, demographic data from organisms grown in isolation. This data is then used to estimate density-independent parameters comprised in the demographic rates, which are then fixed in the population model. This enables the estimation of the remaining density-dependent parameters in the population model from longitudinal aggregate data. An added advantage to this approach is that age-dependent rates can also be estimated or directly represented by the collected organismal data, removing the rather complex problem of estimating these rates from aggregate data alone.

Here, we present this approach for estimating density- and age-dependent demographic rates in a SPM for *Daphnia magna*. This species of water flea has been characterized by the NIH as a model organism for biomedical research [36]. *D. magna* is also widely used in ecotoxicology to assess the hazard of exogenous chemicals, e.g., pesticides, on ecosystems [31, 32, 48, 49]. These assessments, however, have mainly focused on endpoints below the population level of biological organization, i.e., at the molecular, cellular, or organism levels. SPMs can be used to propagate organismal assessments to the population level, thereby enabling the causal association of organismal responses to ecosystem adversity.
Among the recent literature, several mathematical models were developed to describe the longitudinal dynamics of daphnia populations. Erickson, et al. [22], formulated a SPM to investigate the impact of stochastic fecundity and survival on the ability of their model to describe data from pesticide treated populations. Importantly, the model from this study was calibrated to data that only captured the early population growth phase of daphnids. Thus, it has not been determined whether a SPM with stochastic demographics model can accurately describe the long-term dynamics of daphnia populations, which is qualitatively different from the early growth phase [42]. Preuss, et al. [42], validated an individual based model in order to predict the effect of variable algae concentration levels on daphnia population dynamics. Other recent efforts [19, 20, 23] to develop daphnia SPMs have focused on qualitative analysis of the general population dynamics rather than model validation.

Here, we collected both individual and population level data and developed multiple daphnia SPMs in order to test the importance of several biological assumptions. Specifically, we mathematically tested the validity of assuming a time-delay in density-dependent fecundity. We collected daily reproduction data on thirty daphnids to precisely investigate age-dependent fecundity rates for accurate representation in a SPM. We also validated a mathematical description of density-dependent survival and tested whether density-dependent fecundity and survival could be more accurately modeled as a function of total biomass rather than the total population size. Our investigation of delayed density-dependent fecundity is motivated by previous experimental evidence found in [24, 41]; we note that this assumption has not been tested in the context of SPMs in recent literature and with modern daphnia culture methodology. We also collected precise growth rate data on thirty daphnids (starting at within 2-hours of birth) to calibrate our age-structured observations of juvenile and adult daphnids. We employed
quantitative model comparison techniques to assess the validity of our underlying assumptions.

Finally, we performed quantitative sensitivity and uncertainty analyses on the SPM with the most accurate biologic assumptions among the SPMs we considered.

2 Methods

2.1 Population models

Each model we describe in the sections below is a specification of the following structured population model:

\[
\begin{bmatrix}
p(t+1, 1) \\
p(t+1, 2) \\
p(t+1, 3) \\
p(t+1, i_{\text{max}})
\end{bmatrix}
= 
\begin{bmatrix}
a(t, 1) & a(t, 2) & a(t, 3) & \ldots & a(t, i_{\text{max}}) \\
b(t, 1) & 0 & 0 & \ldots & 0 \\
0 & b(t, 2) & 0 & \ldots & 0 \\
0 & 0 & \ddots & \ddots & \ddots \\
0 & 0 & 0 & \ldots & b(t, i_{\text{max}} - 1)
\end{bmatrix}
\times
\begin{bmatrix}
p(t, 1) \\
p(t, 2) \\
p(t, 3) \\
\vdots \\
p(t, i_{\text{max}})
\end{bmatrix}.
\]

The population is divided into one-day age classes, ranging from neonates at age \( i = 1 \) to a maximum lifespan at age \( i = i_{\text{max}} \), where the number of daphnids of age \( i \) at a time \( t \) is \( p(t, i) \).

Here, we assume \( i_{\text{max}} = 74 \) based on our individual level experiments, and based on simulations of our models fit to experimental data, i.e., the maximum life span observed in the simulations was always less than 74 days. The fecundity of each age class \( i \) is given by \( a(t, i) \) and the survival rate is given by \( b(t, i) \).

We generated several models to investigate the importance of several density-dependent mechanisms in modeling \textit{D. magna} populations. Significance of the different mechanisms was assessed by using statistical comparison tests between different models fit to the same structured
population data. We specified the functional forms for $a(t,i)$ and $b(t,i)$ to generate four different structured population models for this assessment, which we refer to as models A through D (Table 1). The four models we consider are organized by the sequential generalization of the functional forms for fecundity and survival, i.e., models A and D have the least and most number of parameters, respectively.

### 2.1.1 Delayed density-dependent fecundity

To evaluate the importance of delayed density-dependency fecundity, we generated models A and B (Table 1). In model A, we assume density-dependent fecundity for all daphnia age classes. We used a functional form for fecundity that decreases with total population size $N(t)$ [27](see $a(t, i)$ in Table 1a). The strength of the density-dependent effect on fecundity is represented by the parameter $q$; the fecundity is density-independent when $q = 0$. Model A assumes a density- and age-independent survival rate, i.e., the constant rate $\mu$. We did not consider age-dependent survival here, thus the rate $\mu$ is the same for each age class. We will consider generalizations of $\mu$ in future work and note that constant survival rate has been used previously for structured population modeling of daphnia [24, 41].

Model B generalizes model A by considering a delayed effect of density on fecundity. This generalization is based on previous studies which showed that number of offspring produced by a gravid female daphnia in their current cohort was unaffected by increases in population density. Instead, increased population density had an effect on subsequent cohorts [24, 41]. Since daphnids in their reproductive stage produce neonates approximately every 3 days, we bounded the time-delayed fecundity effect, $\tau$, between 0 and 6 days.

### 2.1.2 Density- and age-dependent survival

We next evaluated whether density and age were important factors for modeling survival
in daphnid populations. To test this, we created model C, which generalizes model B by including a reduced fitness for daphnids classified as juveniles in our data, i.e., less than 5 days old (see Figure 1). This generalization is based on the observation that larger daphnids consume more algae than smaller daphnids [44]. The restriction of density-dependent survival to juvenile daphnids is in agreement with previous studies which suggested that the survival of adult daphnids is not affected by competition [37]. This competitive effect is likely an important consideration for the daphnia in our population experiments, since our populations were fed a constant amount of algae each day. Indeed, previous modeling studies have suggested that daphnid survival rates would best be modeled as an age- or size-dependent function rather than as a constant [15, 25, 40, 42].

2.1.3 A density-dependent model with biomass

Lastly, we evaluated whether total biomass could more accurately capture the density-dependence of fecundity and survival than the total number of daphnids in our daphnia populations. This consideration is in concordance with the generalization in model C, which relies on the observation that larger daphnids contribute more heavily to competition through resource depletion than smaller daphnids [44]. To test our hypothesis about biomass dependency, we generated Model D by replacing the total population size, \( N(t) \), in model C by total biomass, \( M(t) \) (see Table 1). To model total biomass, we calculated a weighted population value using a function that relates age to size. Specifically, we found that the logistic function accurately models the average size of daphnids as a function of age based on fits to individual-level experimental data (Figure 2). Consequently, we used the logistic function to weight the daphnid size in the model for the total biomass \( M(t) \) (see Table 1b).
2.2 Laboratory studies

We conducted two studies in the laboratory to generate data for refining and parameterizing our mathematical model. The first study was performed at the individual daphnid level to track the baseline fecundity and growth rates in isolation, i.e., density-independent rates. The second study was performed at the population level, in duplicate, for 102 days. The individual level data was used to estimate the density-independent parameters used in our population model. The population data was then used to estimate the remaining density-dependent parameters. Cultured daphnids were maintained using previously described protocols and conditions [48]. Cultured daphnids were kept in media reconstituted from deionized water [1]. Cultured daphnids for both studies were maintained in an incubator maintained at 20 degrees Celsius with a 16-h light, 8-h dark cycle. The daphnids used in our study came from a colony that was maintained at North Carolina State University for over 20 years (clone NCSU1 [43]).

2.2.1 Individual study

Thirty daphnids were longitudinally observed to estimate population average rates of fecundity and growth. Less than 2-h old neonates were placed individually into 50mL beakers containing 40mL of media each. Media was changed daily. Daphnids were fed daily with $7.0 \times 10^6$ cells of algae (Pseudokirchneriella subcapitata) and 0.2 mg (dry weight) Tetrafin™ fish food suspension prepared as described previously [38]. The number of neonates produced by each individual daphnid was recorded and then removed daily. Fecundity measurements were performed until no daphnids remained (74 days). The size of each individual daphnid was measured with a digital microscope (Celestron, Torrance, CA, USA) at periodic intervals until they died, starting at less than two hours old. The major axis was used to determine size, since the maximum possible length was used to classify daphnids into different size classes, i.e.,
juveniles and adults (see below).

2.2.2 Population study

A 102-day population study was conducted, in replicate, using *D. magna*. Two beakers containing 1L of media each were both seeded with five 6-day-old female daphnids. We note that these daphnids did not reproduce prior to the beginning of the population study. Each 1L beaker was fed twice daily (at approximately 10 a.m. and 3 p.m.) with $1.4 \times 10^8$ cells of algae (*P. subcapitata*) and 4 mg dry weight of fish food suspension. The media was changed and the number of daphnids was counted every Monday, Wednesday, and Friday. During counting, daphnids were separated into two size classes (which we call the juvenile class and adult class) using a fine mesh net with a 1.62-mm pore size. The total number of daphnids was then counted for each size class. Importantly, we note that classification into the juvenile or adult group only defines the size of the daphnid, and does not define whether the daphnid had reached a reproductive stage.

2.3 Estimation of density-independent rates

We used data from our individual level study to estimate the density-independent fecundity rate, which we call $\alpha(i)$. We parameterized the function $\alpha(i)$ defined at each age by directly using the number of neonates produced per daphnid per day observed in our individual level study (Figure 3). We used the individual study growth (size) data to estimate the relationship between age and size. We considered several functional forms for $f(i)$, the average size of a female daphnid at age i, within a nonlinear mixed effects model framework and found that the logistic equation most accurately fit the data for individual daphnid growth (Figure 2, Table 2). Based on the mean parameter values estimated with the nonlinear mixed effects model, we inferred that the daphnids classified as juveniles in our population experiments were less than
or equal to 4 days old, and that adults were greater than 5 days old (Figure 1). The function \( f(i) \) was also used to replace total population size with a model for total population biomass in one of the population models we described above.

### 2.4 Parameter Estimation

Parameters were estimated from the population data using a vector ordinary least squares (OLS) framework [10, 12]. For each model, we consider a vector of parameters \( \theta \) to estimate.

Based on our individual level modeling, the number of juveniles and adults are given by 
\[
J(t, \theta) = \sum_{i=1}^{4} p(t, i) \quad \text{and} \quad A(t, \theta) = \sum_{i=5}^{\text{max}} p(t, i),
\]
respectively. The corresponding observation vector is given by 
\[
f(t, \theta) = [J(t, \theta), A(t, \theta)]^T.
\]
We assumed a constant statistical error model of the form
\[
Y_j = f(t_j, \theta_0) + E_j, \quad j=1,2,...,n
\]
where \( Y_j \) is a random variable with realizations \( y_j \) (i.e., the data) and \( f(t_j, \theta_0) \) is the model observation with the hypothesized “true” parameter vector \( \theta_0 \). The error terms \( E_j \) are independent and identically distributed (i.i.d) random variables with mean \( E[ E_j ] = 0 \) and
\[
\text{var}(E_j) = \text{diag}(\sigma_1^2, \sigma_2, 0, \sigma_2, 0). \quad \text{An estimate, } \hat{\theta}, \text{ for the true parameter vector } \theta_0 \text{ is obtained by implementing an iterative algorithm (see [10] for details).}
\]

### 2.5 Model Comparisons

#### 2.5.1 Model Hypothesis Testing

We used a statistical model comparison test [7, 12] to evaluate the significance in considering various components, e.g., delayed density-dependence, for models A through C.

Briefly, this methodology evaluates the significance of a \( \chi^2 \) statistic generated by the residual sum of squares to test the null hypothesis, \( H_0 \), that a certain parameter or set of parameters is not
needed to describe the system. We note that this method requires nested models. For example, model A is “nested” in model B because model B reduces to model A when \( \tau = 0 \). If we can reject the null hypothesis \( H_0 \) then we conclude that the parameters in question cannot be taken equal to zero and infer that they are needed to accurately describe the data. For further details and previous applied examples of this methodology see [7, 9, 12].

2.5.2 Akaike Information Criteria

The Akaike Information Criterion (AIC) score gives an approximately unbiased form of the Kullback-Leibler Distance, or a measure of the distance between a model and the corresponding data [10]. The AIC score is used to compare the accuracy of different models to the same data set; a lower AIC score indicates higher accuracy. We note that the AIC score is applicable to more model comparisons than the \( \chi^2 \) based test described above, since it does not require the compared models to be nested. The AIC score corrected for small sample size (\( n/p < 40 \), \( n = \) number of data points, \( p = \) number of parameters) is given by as \( \text{AIC}_C = \) (See PDF version of manuscript), where RSS is the residual sum of squares [10, 13]. We used the \( \text{AIC}_C \) score to compare the non-nested population models we considered, e.g., model C and model D.

2.6 Parameter Uncertainty Quantification

We calculated standard errors and 95% confidence intervals for the estimated parameters \( \hat{\theta} \) using asymptotic theory, and used bootstrapping for verification. We provide a brief description of the application of these two methods here, but for more details see [10, 12].

2.6.1 Asymptotic Theory

The observation variance \( V_0 \) in the vector OLS framework using a constant statistical error model is, given estimates \( \hat{\theta} \), approximated by
The resulting approximation of the covariance matrix is given by

\[
\hat{\Sigma}^n = \left( \sum_{j=1}^{n} D_j^T(\hat{\theta}) \hat{V}^{-1} D_j(\hat{\theta}) \right)^{-1},
\]

where the 2 x p matrix \( D_j(\hat{\theta}) \) is given by

\[
D_j(\hat{\theta}) = \begin{pmatrix}
\frac{\partial J(t_j, \hat{\theta})}{\partial \theta_1} & \cdots & \frac{\partial J(t_j, \hat{\theta})}{\partial \theta_p} \\
\frac{\partial A(t_j, \hat{\theta})}{\partial \theta_1} & \cdots & \frac{\partial A(t_j, \hat{\theta})}{\partial \theta_p}
\end{pmatrix},
\]

where \( p=2 \) in Models A and B and \( p=3 \) in Models C and D. Then asymptotic theory [9,11] yields that the OLS estimator has a limiting distribution given approximately by an \((\theta, \Sigma^n)\) distribution.

We calculated standard errors and 95% confidence intervals [9,11] in order to quantify the uncertainty in estimating each element of the parameter estimate \( \theta \) for our best model with vector observation \( f(t, \theta) \). The standard error and 95% confidence interval of the \( k \)th parameter \( \theta_k \) are given by \( \text{SE}(\theta_k) = \sqrt{\sum_{k,k}^{n \Sigma}} \) and \([\theta_k - 1.96 \text{SE}(\theta_k), \theta_k + 1.96 \text{SE}(\theta_k)]\), respectively [11].

### 2.6.2 Bootstrapping

Bootstrapping is implemented for an estimated parameter vector \( \theta \) by first calculating standardized residuals

\[
\bar{r}_j = \sqrt{\frac{n}{n-p}} (y_j - f_i(t_j, \hat{\theta})), \quad j = 1, \ldots, n,
\]

where \( n \) is the number of data points, \( p \) is the number of parameters, \( i=J \) or \( A \) represents either
the juvenile or adult observation, Here, \( f_J(t_j, \theta^*) = J(t_j, \theta^*) \) and \( f_A(t_j, \theta^*) = A(t_j, \theta^*) \). Bootstrap sample points are created by sampling the standardized residuals for each observation (J or A) and adding them to the respective model solutions, either \( J(t_j, \theta^*) \) or \( A(t_j, \theta^*) \). We created \( M=1000 \) simulated bootstrap data sets in this fashion and then conducted \( M \) inverse problems to fit the model to each of these simulated data sets. For the \( m \)th simulated bootstrap data set, we then find the corresponding parameter estimate \( \theta^m \). The mean, variance, and standard errors for \( \theta^* \) are approximated by the following formulas [9]:

\[
\hat{\theta}_{\text{BOOT}} = \frac{1}{M} \sum_{m=1}^{M} \hat{\theta}^m,
\]
\[
\text{Var}(\hat{\theta}_{\text{BOOT}}) = \frac{1}{M-1} \sum_{m=1}^{M} (\hat{\theta}^m - \hat{\theta}_{\text{BOOT}})(\hat{\theta}^m - \hat{\theta}_{\text{BOOT}})^T,
\]
\[
\text{SE}_k(\hat{\theta}_{\text{BOOT}}) = \sqrt{\text{Var}(\hat{\theta}_{\text{BOOT}})_{kk}}.
\]

The 95% confidence interval for each \( \theta^*_k \) is calculated as the range between the 25\(^{\text{th}}\) and 975\(^{\text{th}}\) entries in the ordered set of \( M \) parameter estimates from bootstrapping.

3 Results

3.1 Model Selection

When comparing models A and B we found that a 6 day time-delay on the fecundity effect provided a significantly improved fit to the daphnid population data versus the non-delayed model for both population data sets (\( P = 5.029\times10^{-4}, \) Replicate 1; \( P = 3.219\times10^{-3}, \) Replicate 2, \( \chi^2 \)-test, Figure 4). We note that we also tested whether larger \( \tau \) values could provide a more accurate fit to population data but found no significant differences in fits to the population data when using \( \tau = 6 \) (\( P = .3071, \) Replicate 1; \( P = .1139, \) Replicate 2, \( \chi^2 \)-test). We found that the
inclusion of both density and age dependence in survival provided significantly improved fits to population data for one of the two replicates ($P = 1.615 \times 10^{-1}$, Replicate 1; $P = 3.96 \times 10^{-2}$, Replicate 2, $\chi^2$-test). Overall, these results suggest that model C is more appropriate for modeling our daphnia populations than model B, since it describes a wider range of observed biological dynamics. We note that we also considered other models that did not significantly increase the accuracy of the model to experimental population data (results not shown). For example, we considered models in which the density-dependent effects were of different functional forms. In addition, models in which all age classes (beyond 4 days old) had density-dependent survival rates did not result in significantly better fits to the population data.

Using the AIC$_C$ score, we found that model D better described the population data from both replicates than model C. For replicate 1, the AIC$_C$ for models C and D were 276.15 and 266.23, respectively. For replicate 2, the AIC$_C$ for models C and D were 379.43 and 280.19, respectively. The evidence ratio, based on the calculation of Akaike weights [13] (p. 74-79), for model D versus model C was 141.85 for the replicate 1 data set. The evidence ratio for model D versus model C was $3.57 \times 10^{21}$ for the replicate 2 data set. These results highly suggest that model D is better than model C at representing the population data from both replicates. Hence, dependence of birth and death demographics on population density is most likely a function a total biomass rather than the absolute number of daphnids counted regardless of size or age. See Figure 5 for fits of model D to the population data. Moreover, the parameter estimates for both replicates were strikingly similar, indicating that our validation of model D is repeatable despite the possibility of biological variability between population experiments.

3.2 Uncertainty Analysis

We quantified uncertainty in our parameter estimates for model D. Uncertainty
quantification provides an estimation of the statistical confidence in each parameter for a given data set, where confidence is determined by estimating a distribution for each parameter. We calculated standard errors and 95% confidence intervals for each parameter using asymptotic theory and bootstrapping (Table 3, Figure 6). Both the results from asymptotic theory and bootstrapping support that the standard errors were low and the 95% confidence intervals were narrow for the parameter estimates in both replicates. These results indicate a high confidence that our model validation results are repeatable.

To investigate how parameter uncertainty propagates through the model solution over time, and to quantitatively assess the performance of model D, we generated a 95% confidence region for the model using Monte Carlo (MC) simulations. We sampled 1000 parameter vectors from the 95% confidence interval (C.I.) of the joint parameter distribution estimated with asymptotic theory for model D. We then generated 95% confidence regions for the number of juveniles, the number of adults, and the total population size by simulating the corresponding 1000 model solutions and plotted these along with the data and their error bars (Figure 5). We then calculated the percentage of data points with error bars that overlapped with the 95% confidence region simulations to assess the overall model performance and account for uncertainty. We found that model D matched the population data from both replicates with high accuracy for both juveniles and adults, as well as for the total population size (Table 4).

3.3 Parameter Sensitivities

We applied a sensitivity analysis to our best validated model (model D) to understand how changes in estimated parameters governing fecundity and survival affect population size and structure. We calculated the relative time-dependent sensitivity functions for juvenile, adults, and
total population size (Figure 7). Interestingly, we observed that the maximum total population size for our two replicates was achieved on day 19, dividing the population dynamics into two phases, which we call the “early phase” and the “late phase” (Figure 8). In the early phase (≤ 19 days) of the population experiments, the population grows rapidly and exceeds its carrying capacity. In the late phase of the population experiments (> 19 days), the total population size converges towards steady state levels as an excess juvenile population rapidly dies off or progresses to the adult stage. Dividing our sensitivity analysis between these two phases revealed that the effect of increasing fecundity or survival is both temporally and life-stage dependent (Figure 9).

We found that the juvenile, adult, and total population sizes were most sensitive to changes in μ in both the early and late phase as compared to the other estimated parameters q and c. The sensitivity analysis indicates that increasing the survival rate μ will increase the juvenile population in the early phase and decrease it in the late phase, whereas an increased survival rate increases the adult population size in both the early and late phase. Although increased survival increases the total population size in the early phase, the late phase is much less sensitive. These findings suggest that changes in the survival rate will cause a shift in the population distribution towards the adult stage and that this shift mainly occurs during the early phase of population growth.

Our sensitivity analysis indicates that increasing q, the effect of density on fecundity, has a greater effect in the late phase of the population experiment than in the early phase for the juveniles, adults, and total population size. This result is expected, since a lower fecundity rate should lead to lower population sizes overall and within specific life stages. We hypothesize that the late phase is more heavily influenced by a decreased density-dependent fecundity rate than
the early phase because of the time delayed effect. If so, this would imply that most of the offspring in the early phase are produced by female daphnia whose fecundity has not yet been effected by density.

Lastly, our sensitivity analysis indicates that increasing c, the effect of density on the survival of juveniles, leads to lower numbers of juveniles and adults, and a lower total population size in the early phase. This relationship is more pronounced in the late phase for both the number of adults and total population size. Unexpectedly, our sensitivity analysis indicates that increasing the parameter c can cause the number of juveniles to increase in the late phase of the population experiments.

Taken together, these findings suggest that a higher density-dependent juvenile survival rate can cause a shift towards juveniles in the equilibrium age distribution of daphnia populations, even though the total population size decreases overall. Our results highlight the importance of mathematical modeling to understand non-intuitive temporal shifts in the age distribution of daphnia populations that may occur under environmental conditions that increase competition, e.g., if the amount of algae decreases.

4 Conclusions and Discussion

We tested several hypotheses concerning the significance of several biological assumptions in describing daphnia populations with a structured population model. One assumption we evaluated, delayed density dependent fecundity, had been suggested previously [24, 41]. Importantly, this hypothesized mechanism was not quantitatively verified due to a lack of statistical comparison tools at the time they were proposed. We applied a $\chi^2$ based model comparison test and found strong statistical evidence for a time delay in density dependent fecundity. We also found statistical evidence for the assumption that intraspecific competition
mainly affects juvenile daphnids, previously suggested in [15, 25, 40, 42]. Lastly, we determined that the effect of density on daphnia demographics is more accurately modeled as a function of total biomass, rather than total population size [24, 44]. Our findings indicate that the assumptions we investigated can improve the accuracy of future daphnia population modeling efforts and may provide increased accuracy in other daphnia models which may not have considered all of these assumptions [19, 20, 22, 23, 24, 42].

We found that parameterizing the density-independent components of demographic rates with individual level data enabled the estimation of density-dependent parameters from aggregate structured population data. The most complex density-independent component that we discovered was for daphnid fecundity (Figure 3). Our data revealed a clear periodic pattern in the timing of offspring production in which daphnids begin releasing neonates at 9-days-old. Notably, the maximum offspring production rate is significantly higher in the first 4 broods than in subsequent broods (P = 0.0011, Mann-Whitney U-test). To the best of our knowledge, fecundity oscillations with a consistent frequency and time-dependent amplitude has not previously been observed for daphnids. We note that without employing individual level time-dependent fecundity data, our attempts to fit daphnia population data gave extremely poor results (data not shown). We suspect that the collection of similarly precise individual-level data will be necessary to parameterize structured models from field data of daphnia populations. For example, daphnids could be sampled in the field and cultured/observed under experimental conditions similar to their natural environment. Alternatively, one may be able to employ computational methods designed to estimate time-dependent rates from aggregate data alone [3, 2, 4, 5, 6, 8, 11, 12]. However, these methods have only been previously applied to density-independent structured population models, and thus they remain largely untested and
underdeveloped in density-dependent scenarios.

An underlying challenge in performing hazard assessments is to generate a highly repeatable baseline control for comparison. For our best validated model (Model D), the parameter estimates, uncertainty quantification, temporal variations in sensitivity patterns, and overall degree of accuracy to the data were all extremely similar between replicates (Figures 5 and 9, Tables 3 and 4). These results highlight the need for comprehensively evaluating biological assumptions about daphnid populations grown under non-stressed environmental conditions, i.e., the control case. Our results also suggest the need for further improvement, since Model D underestimated the early phase (≤ 19 days) growth rate and the time at which the peak size was reached for the juvenile population in the second replicate. One possible adjustment that may increase the accuracy of model D is to incorporate a time-dependent daphnid survival rate. From our sensitivity analysis, we infer that increasing the juvenile survival rate will likely remedy the underestimation of the early phase growth rate (Figure 9). For simplicity, we assumed a constant survival rate in the modeling efforts reported here, however, this assumption is a current focus of our ongoing investigations.

Acknowledgements

This research was supported in part by the National Institute of Allergy and Infectious Diseases under grant number NIAID R01AI071915-10, in part by the Air Force Office of Scientific Research under grant number AFOSR FA9550-12-1-0188, in part by the National Science Foundation under Research Training Grant (RTG) DMS-1246991, Undergraduate Biomathematics grant number DBI-1129214, NSF grant number DMS-0946431, in part by the EPA under US EPA STAR grant RD-835165, and in part by the NIEHS under training grant ES7046.
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Figure 1: Calibration of the maximum size for classification of juveniles. We determined the maximum juvenile daphnid size by simulating the logistic growth curve with mean parameter values from the nonlinear mixed effects model (Figure 2, Table 2). The pore size of the mesh we used to separate juveniles from adults was 1.62 mm, and this value is plotted as a horizontal line. The vertical line gives the average daphnid age at which their major axis length is equal to the mesh pore size. Based on this calculation, we inferred that the maximum age at which daphnids can fit through the mesh was 4 days old. Thus, we chose to classify juveniles in our models as ≤ 4 days old.
Figure 2: Results for nonlinear regression performed on individual level growth data using a logistic equation within a nonlinear mixed effects model (NLMEM). Growth data are represented by stars symbols. Best model fits are drawn as lines for each individual. We collected data for thirty daphnids, but these plots show results for twenty four daphnids for which an adequate number of data was collected to fit a NLMEM. Nonlinear regression was performed using the nlmefit function in Matlab. We tested several models for growth, including logistic, gompertz, constant, and linear equations. Based on AIC values, it was determined that the logistic model provided the most accurate fit to the data. See Table 2 for estimated parameters and variances, including fixed effects and random effects.
Figure 3: The number of neonates produced per female daphnia per day. Data were collected from thirty female daphnia whose birth was known to within two hours of accuracy. Daily data are represented by star symbols and connecting lines are drawn to show general trends. This data was used to parameterize the age-dependent function α(i) (see Table 1).
Figure 4: The ordinary least squares (OLS) cost from the inverse problem performed on model B with $\tau \in [0, 6]$ for each replicate. These results suggest that the optimal value for $\tau$ is 6 days, since it results in the smallest OLS cost.
Figure 5: Results from fitting model D to juvenile and adult longitudinal population data for *D. magna*. Results are shown for juveniles (top), adults, (middle), and total population size (bottom), for replicate number 1 (left) and 2 (right). Black lines: Simulation results using the estimated parameter vector for model D. Gray lines: Simulated population model results with parameters sampled from the 95% confidence interval of the joint asymptotic distribution. The data are plotted as open circles with error bars for the estimated observation variance. The parameter values used for these plots can be found in Table 3 below.
Figure 6: The parameter distributions obtained from bootstrapping for each estimated parameter $(\mu, q, c)$ and each replicate for model D.
Figure 7: The relative time-dependent sensitivities for juveniles, adults, and the total population with respect to each of the estimated parameters (μ, q, c) for model D.

Sensitivities were calculated for the number of juveniles J(t) (top row), the number of adults A(t) (middle row), and the total population size N(t) (bottom row). The left column corresponds to μ, the middle row to q, and the right column to c. Solid lines: Replicate 1. Dashed lines: Replicate 2.
Figure 8: Longitudinal data for the total population size for two population replicates. The vertical dashed line is at 19 days, and shows the division between the early phase and late phase dynamics.
Figure 9: Average sensitivities of juveniles, J(t), adults, A(t), and total population size, N(t), with respect to the survival rate, $\mu$, the effect of density on fecundity, $q$, and the effect of density on survival, $c$. Sensitivities are divided between the early phase of the population experiments (before the peak size is reached on day 19) and the late phase (after day 19).
Tables

Table 1: Descriptions of models, parameters, and variables.

<table>
<thead>
<tr>
<th>Model</th>
<th>(a(t, i))</th>
<th>(b(t, i))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>(\alpha(i)(1 - q)^{N(t)})</td>
<td>(\mu)</td>
</tr>
<tr>
<td>B</td>
<td>(\alpha(i)(1 - q)^{N(t)}, \mu)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>(\alpha(i)(1 - q)^{N(t)}, \mu) if (i \leq 4), (\mu) if (i \geq 5)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>(\alpha(i)(1 - q)^{M(t)}, \mu) if (i \leq 4), (\mu) if (i \geq 5)</td>
<td></td>
</tr>
</tbody>
</table>

(a) Age-dependent rates of fecundity, \(a(t, i)\), and survival, \(b(t, i)\).

<table>
<thead>
<tr>
<th>Parameter/Variable</th>
<th>Description</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>(p(t, i))</td>
<td>Number of daphnids of age (i)</td>
<td># of daphnids</td>
</tr>
<tr>
<td>(N(t))</td>
<td>Total population size at time (t := \sum_{i=1}^{t_{max}} p(t, i))</td>
<td># of daphnids</td>
</tr>
<tr>
<td>(q)</td>
<td>Density-dependent fecundity constant</td>
<td>dimensionless</td>
</tr>
<tr>
<td>(\alpha(i))</td>
<td>Density-independent fecundity rates</td>
<td># neonates-daphnid(^{-1})-day(^{-1})</td>
</tr>
<tr>
<td>(\mu)</td>
<td>Density-independent survival rate</td>
<td>day(^{-1})</td>
</tr>
<tr>
<td>(\tau)</td>
<td>Delay for density-dependent fecundity</td>
<td>days</td>
</tr>
<tr>
<td>(c)</td>
<td>Density-dependent survival constant</td>
<td>dimensionless</td>
</tr>
<tr>
<td>(M(t))</td>
<td>Total biomass at time (t := \sum_{i=1}^{t_{max}} p(t, i), KZ_0c^{2i})</td>
<td>mm</td>
</tr>
<tr>
<td>(K)</td>
<td>Average maximum daphnid size (major axis)</td>
<td>mm</td>
</tr>
<tr>
<td>(\tau)</td>
<td>Average daphnid growth rate</td>
<td>mm/hour</td>
</tr>
<tr>
<td>(Z_0)</td>
<td>Average neonate size (major axis)</td>
<td>mm</td>
</tr>
</tbody>
</table>

(b) Parameter/Variable descriptions.
Table 2: Mean parameter estimates and variances along with individual daphnid parameter estimates for the logistic equation using a nonlinear mixed effects model (see Figure 2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$K$</th>
<th>$r$</th>
<th>$M_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed Effect Mean Value</td>
<td>3.7346</td>
<td>0.0157</td>
<td>0.7333</td>
</tr>
<tr>
<td>Random Effect Variance</td>
<td>0.0010533</td>
<td>0.0048239</td>
<td>6.8978 x 10^{-7}</td>
</tr>
</tbody>
</table>

(a) Mean values and variances (random effects) estimated for the logistic equation with a nonlinear mixed effects model.

<table>
<thead>
<tr>
<th>Daphnid</th>
<th>$K$</th>
<th>$r$</th>
<th>$M_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.6148</td>
<td>0.0157</td>
<td>0.7333</td>
</tr>
<tr>
<td>2</td>
<td>3.7342</td>
<td>0.0160</td>
<td>0.7333</td>
</tr>
<tr>
<td>3</td>
<td>3.6834</td>
<td>0.0156</td>
<td>0.7333</td>
</tr>
<tr>
<td>4</td>
<td>3.8267</td>
<td>0.0156</td>
<td>0.7333</td>
</tr>
<tr>
<td>5</td>
<td>3.6262</td>
<td>0.0162</td>
<td>0.7333</td>
</tr>
<tr>
<td>6</td>
<td>3.6340</td>
<td>0.0157</td>
<td>0.7333</td>
</tr>
<tr>
<td>7</td>
<td>3.8957</td>
<td>0.0169</td>
<td>0.7334</td>
</tr>
<tr>
<td>8</td>
<td>3.8895</td>
<td>0.0154</td>
<td>0.7333</td>
</tr>
<tr>
<td>9</td>
<td>3.8556</td>
<td>0.0152</td>
<td>0.7333</td>
</tr>
<tr>
<td>10</td>
<td>3.9009</td>
<td>0.0145</td>
<td>0.7333</td>
</tr>
<tr>
<td>11</td>
<td>3.8718</td>
<td>0.0170</td>
<td>0.7334</td>
</tr>
<tr>
<td>12</td>
<td>3.8482</td>
<td>0.0148</td>
<td>0.7333</td>
</tr>
<tr>
<td>13</td>
<td>3.6902</td>
<td>0.0140</td>
<td>0.7333</td>
</tr>
<tr>
<td>14</td>
<td>3.4604</td>
<td>0.0138</td>
<td>0.7333</td>
</tr>
<tr>
<td>15</td>
<td>3.7969</td>
<td>0.0150</td>
<td>0.7333</td>
</tr>
<tr>
<td>16</td>
<td>3.7530</td>
<td>0.0158</td>
<td>0.7333</td>
</tr>
<tr>
<td>17</td>
<td>3.7092</td>
<td>0.0162</td>
<td>0.7333</td>
</tr>
<tr>
<td>18</td>
<td>3.7758</td>
<td>0.0164</td>
<td>0.7333</td>
</tr>
<tr>
<td>19</td>
<td>3.7397</td>
<td>0.0159</td>
<td>0.7333</td>
</tr>
<tr>
<td>20</td>
<td>3.6688</td>
<td>0.0178</td>
<td>0.7334</td>
</tr>
<tr>
<td>21</td>
<td>3.6662</td>
<td>0.0153</td>
<td>0.7333</td>
</tr>
<tr>
<td>22</td>
<td>3.6387</td>
<td>0.0163</td>
<td>0.7334</td>
</tr>
<tr>
<td>23</td>
<td>3.7070</td>
<td>0.0166</td>
<td>0.7333</td>
</tr>
<tr>
<td>24</td>
<td>3.6806</td>
<td>0.0147</td>
<td>0.7333</td>
</tr>
</tbody>
</table>

(b) Individual parameter estimates for each daphnid.
Table 3: Results from uncertainty quantification with asymptotic theory and bootstrapping for model D.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Parameter</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$\mu$</td>
<td>$9.5051 \times 10^{-1}$</td>
<td>$1.0428 \times 10^{-2}$</td>
<td>$(9.2889 \times 10^{-1}, 9.7214 \times 10^{-1})$</td>
</tr>
<tr>
<td>1</td>
<td>$q$</td>
<td>$1.7206 \times 10^{-3}$</td>
<td>$1.5426 \times 10^{-4}$</td>
<td>$(1.4007 \times 10^{-3}, 2.0405 \times 10^{-3})$</td>
</tr>
<tr>
<td>1</td>
<td>$c$</td>
<td>$1.5153 \times 10^{-4}$</td>
<td>$2.9689 \times 10^{-5}$</td>
<td>$(8.9972 \times 10^{-5}, 2.1310 \times 10^{-4})$</td>
</tr>
<tr>
<td>2</td>
<td>$\mu$</td>
<td>$9.8559 \times 10^{-1}$</td>
<td>$8.1785 \times 10^{-3}$</td>
<td>$(9.6863 \times 10^{-1}, 1.0025)$</td>
</tr>
<tr>
<td>2</td>
<td>$q$</td>
<td>$1.3542 \times 10^{-3}$</td>
<td>$1.7762 \times 10^{-4}$</td>
<td>$(9.8590 \times 10^{-4}, 1.7225 \times 10^{-3})$</td>
</tr>
<tr>
<td>2</td>
<td>$c$</td>
<td>$2.8005 \times 10^{-4}$</td>
<td>$4.1701 \times 10^{-5}$</td>
<td>$(1.9358 \times 10^{-4}, 3.6652 \times 10^{-4})$</td>
</tr>
</tbody>
</table>

(a) Parameter estimates, asymptotic standard errors, and asymptotic 95% confidence intervals (C.I.) for model D.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Parameter</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$\mu$</td>
<td>$9.5051 \times 10^{-1}$</td>
<td>$8.7505 \times 10^{-3}$</td>
<td>$(8.8922 \times 10^{-1}, 9.2551 \times 10^{-1})$</td>
</tr>
<tr>
<td>1</td>
<td>$q$</td>
<td>$1.7206 \times 10^{-3}$</td>
<td>$2.3202 \times 10^{-4}$</td>
<td>$(2.0358 \times 10^{-3}, 2.9980 \times 10^{-3})$</td>
</tr>
<tr>
<td>1</td>
<td>$c$</td>
<td>$1.5153 \times 10^{-4}$</td>
<td>$2.3608 \times 10^{-5}$</td>
<td>$(-4.8952 \times 10^{-5}, 4.8953 \times 10^{-5})$</td>
</tr>
<tr>
<td>2</td>
<td>$\mu$</td>
<td>$9.8559 \times 10^{-1}$</td>
<td>$2.3660 \times 10^{-2}$</td>
<td>$(9.3715 \times 10^{-1}, 1.0355)$</td>
</tr>
<tr>
<td>2</td>
<td>$q$</td>
<td>$1.3542 \times 10^{-3}$</td>
<td>$3.2867 \times 10^{-4}$</td>
<td>$(6.8486 \times 10^{-4}, 2.0517 \times 10^{-3})$</td>
</tr>
<tr>
<td>2</td>
<td>$c$</td>
<td>$2.8005 \times 10^{-4}$</td>
<td>$9.7547 \times 10^{-5}$</td>
<td>$(8.3218 \times 10^{-5}, 4.8888 \times 10^{-4})$</td>
</tr>
</tbody>
</table>

(b) Parameter estimates, bootstrap standard errors, and bootstrap 95% confidence intervals (C.I.) for model D.
Table 4: The percentage (% accuracy) and fraction of observed data points with error bars that overlapped with the 95 % confidence region for model D. Results are shown for the number of juveniles, the number of adults, and the total population size.

<table>
<thead>
<tr>
<th>Daphnid Classification</th>
<th>Replicate</th>
<th>% Accuracy</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Juvenile</td>
<td>1</td>
<td>100 %</td>
<td>25/25</td>
</tr>
<tr>
<td>Adult</td>
<td>1</td>
<td>96 %</td>
<td>24/25</td>
</tr>
<tr>
<td>Total</td>
<td>1</td>
<td>96%</td>
<td>24/25</td>
</tr>
<tr>
<td>Juvenile</td>
<td>2</td>
<td>92 %</td>
<td>23/25</td>
</tr>
<tr>
<td>Adult</td>
<td>2</td>
<td>96 %</td>
<td>24/25</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>96 %</td>
<td>24/25</td>
</tr>
</tbody>
</table>
APPENDIX C: VALIDATION OF A TWO-GENERATIONAL REPRODUCTION TEST

IN *DAPHNIA MAGNA*: AN INTERLABORATORY EXERCISE

Carlos Barata ¹*, Bruno Campos ¹, Claudia Rivetti ¹, Gerald A. LeBlanc ², Stephanie Eytheson ², Stephanie Street ², Marysia Tobor-Kaplon ³, Selinda de Vries Buitenweg ³, Suhyon Choi ⁴, Jinhee Choi ⁴, Elena I Sarapultseva ⁵, Marie-Agnès Coutellec ⁶, Maïra Coke ⁷, Pascal Pandard ⁸, Arnaud Chaumot ⁹, Hervé Quéau ⁹, Nicolas Delorme ⁹, Olivier Geffard ⁹, Fernando Martínez-Jerónimo ¹⁰, Haruna Watanabe ¹¹, Norihisa Tatarazako ¹¹, Isabel Lopes ¹², João Pestana ¹², Amadeu M.V.M. Soares ¹², Cecilia Manuela Pereira ¹³, Karel De Schamphelaere ¹³

This manuscript has been published by the journal Science of the Total Environment and is in the format of that journal.

My contribution to this work was performing a two-general reproduction test in *Daphnia magna.*

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Abstract

Effects observed within one generation disregard potential detrimental effects that may appear across generations. Previously we have developed a two generation *Daphnia magna* reproduction test using the OECD TG 211 protocol with a few amendments, including initiating the second generation with third brood neonates produced from first generation individuals. Here we showed the results of an inter-laboratory calibration exercise among 12 partners that aimed to test the robustness and consistency of a two generation *Daphnia magna* reproduction test. Pyperonyl butoxide (PBO) was used as a test compound. Following experiments, PBO residues were determined by TQD-LC/MS/MS. Chemical analysis denoted minor deviations of measured PBO concentrations in freshly prepared and old test solutions and between real and nominal concentrations in all labs. Other test conditions (water, food, *D. magna* clone, type of test vessel) varied across partners as allowed in the OECD test guidelines. Cumulative fecundity and intrinsic population growth rates ($r$) were used to estimate “No observed effect concentrations “NOEC using the solvent control as the control treatment. EC$_{10}$ and EC$_{-50}$ values were obtained regression analyses. Eleven of the twelve labs succeeded in meeting the OECD criteria of producing more than 60 offspring per female in control treatments during 21 days in each of the two consecutive generations. Analysis of variance partitioning of cumulative fecundity indicated a relatively good performance of most labs with most of the variance accounted for by PBO (56.4%) and PBO by interlaboratory interactions (20.2%), with multigenerational effects within and across PBO concentrations explaining about 6% of the variance. EC$_{50}$ values for reproduction and population growth rates were on average 16.6 and 20.8% lower among second generation individuals, respectively. In summary these results suggest that the proposed assay is
reproducible but cumulative toxicity in the second generation cannot reliably be detected with this assay.

**Keywords:** Daphnia reproduction, multigeneration assay, interlaboratory, OECD211, contaminants, life-history, offspring quality
1. Introduction

Most eco-toxicological test guidelines only evaluate the effects observed within one generation, thus disregarding those potential detrimental effects that may appear across generations. The toxicity of chemicals may decrease, increase, or remain unchanged across generations. Toxicity can also “emerge” across generations, with modes of action which do not operate in the first generation of exposure (e.g. epigenetics)(Marczylo et al., 2016). However, in many cases pollutants affect both exposed organisms and their progeny, hence adverse effects may become more severe in subsequent generations (Campos et al., 2016). This is the case for some endocrine disruptors that by altering hormonal levels in the mother can have detrimental effects in their offspring (Colborn et al., 1996). In this regard OECD Work Related to Endocrine Disrupters http://www.oecd.org/env/ehs/testing/oecdworkrelatedtoendocrinedisrupters.htm recommends that in vivo assays be used that provide more comprehensive data on adverse effects on endocrine relevant endpoints over extensive parts of the life cycle of the organisms. Reproductive toxicity assays in mammals typically assess detrimental effects on the parental and offspring generations (Janer et al., 2007). Indeed there are standardized test procedures designed to assess multigenerational effects in mammals and fish (Janer et al., 2007; Nakamura et al., 2015), but there are no equivalent tests for invertebrates, despite their short life-cycles, which allow for cost-effective assessments of toxicity over multiple generations (OECD, 2006; Oliveira-Filho et al., 2009a; Oliveira-Filho et al., 2009b; Verslycke et al., 2007).

Among existing ecotoxicological assays, the *Daphnia magna* reproduction test is probably the most employed standardized life cycle test in aquatic toxicology. Indeed the "Daphnia Multi-generation Assay" is already mentioned in Level 5 of the OECD Conceptual Framework for endocrine disrupters. From the available literature on multi-generational effects of chemical
contaminants and other stressors (UV radiation and radioactivity) in *D. magna* or related species, it was not always possible to evaluate whether concentration effect levels increased, decreased or remained unchanged across generations as it is established by OECD; (Baldwin et al., 2001; Brennan et al., 2006; Campos et al., 2016; Dalla Bona et al., 2015; Dietrich et al., 2010; Faassen et al., 2015; Hammers-Wirtz and Ratte, 2000; Huebner et al., 2009; Jacobasch et al., 2014; Jeong et al., 2015; Kim et al., 2012; Kim et al., 2014; LeBlanc et al., 2013; Li et al., 2016; Li et al., 2014; Massarin et al., 2010; Muyssen and Janssen, 2004; Papchenkova et al., 2009; Parisot et al., 2015; Plaire et al., 2013; Sarapultzseva and Dubrova, 2016; Völker et al., 2013; Ward and Robinson, 2005; Yang et al., 2013). About half (13 out of 25) of the above mentioned studies followed OECD TG211 or related protocols, which allowed to compare effect concentration levels across generations and hence demonstrating the potential of this protocol for evaluating toxicity beyond a single generation.

In summary there is experimental evidence indicating that it is possible to study multigenerational effects by just extending the OECD TG 211 protocol (OECD, 2012) to an additional generation, but there is a need to harmonize testing protocols and to assess interlaboratory variability. In a previous study Campos et al. (2016) showed that selection of the clutch size to initiate the second generation is important and that third brood neonates allowed to obtain more consistent results across generations and contaminants. Campos et al. (2016) also reported that piperonyl butoxide (PBO) was one compound showing significant multigenerational effects on reproduction. Effects of this compound were mostly related with embryonic development arrest. PBO is a well-known and widely used insecticide synergist, known to inhibit the activity of the insect cytochrome P450 detoxification system (Hardstone et al., 2015). This compound inhibits steroid hydroxylases in daphnid (Baldwin and LeBlanc,
1994), which are necessary for embryo development (LeBlanc et al., 2000). Furthermore, as a broad inhibitor of cytochrome P450 enzymes, this compound can alter metabolic pathways in exposed embryos. In zebrafish embryos PBO is embryotoxic (Wang et al., 2012). Therefore, exposure of second-generation organisms to PBO during their embryo development may adversely influence their reproductive competence.

This study aimed on assessment of test validity using interlaboratory variability in assessing multigenerational effects on reproductive responses in *D. magna*. More specifically it was studied the consistency in measuring multigenerational cumulative toxic effects of PBO across 12 different labs using a two-generation *D. magna* reproduction protocol developed previously. The experiment was designed in accordance with TG211 requirements. This provided a way to test the robustness of the guideline without testing all possible combinations of parameters that are allowed to vary in this guideline. For example, all laboratories did not use the same clone and culture conditions, because TG211 does not recommend the use of a particular clone or culture conditions, i.e., the test is considered reliable irrespective of the clone or culture conditions used.”

2. Material and methods

2.1 Chemicals

Piperonyl butoxide (PBO, CAS 51-03-6) was purchased from Sigma-Aldrich. All other chemicals were analytical grade and were obtained from Merck.

2.2 Participating laboratories

Up to 12 different laboratories from 10 countries from Europe (6), Asia (3) and America (2) participated in this inter-laboratory calibration exercise (detailed addressed are in authors affiliations). 1. Department of Environmental chemistry, (IDAIA, CSIC), Spain; 2. Toxicology

For confidentiality purposes partner number depicted in results do not correspond to the order of participating labs.

2.3 Experimental animals and culture conditions

Following OECD TG 211 guidelines, different clones of D. magna were used and animals were maintained individually in artificial reconstituted water (ASTM, M7, M4, COMBO) or in de-chlorinated tap water at 20°C. The photoperiod regime was 16:8 h or 12:12 h light: dark cycle. Animals were cultured in 50 or 100 mL of media and fed with different algae species using different food rations and food additives. Culture and test media were changed every other day. A brief summary of cultured conditions across labs is depicted in Table 1.

2.4 Experimental design

Experiments were conducted following the D. magna reproduction test OECD guidelines (OECD, 2012) with minor modifications (Campos et al., 2016). Treatments included five concentrations of PBO (50, 100, 200, 400, 800 µg/L) although some labs included an additional lower concentrations (25 µg/L). Selected chemical concentrations allowed to fully define concentration-response curves for cumulative offspring production and to estimate low
concentration effects. Stocks of PBO (x 20, 000) were prepared by the study coordinator in pure ethanol and sent to each partner in sealed chromatographic vials. All PBO treatments and the solvent control received 0.05 ml/L of ethanol. A control without solvent was also included.

Once first generation females, hereafter referred as parental generation (F0), released the third clutch of offspring, we initiated the second generation (F1). Individuals from the F1 generation were maintained as those of the parental one for 21 days, following also the OECD guidelines (OECD, 2012). Measured life-history traits were: juvenile and adult survival; age at first reproduction; clutch size and intrinsic population growth rates (r), computed from the age specific survival and reproduction rates according to the Lotka equation (Barata et al., 2002b). Most partners provided detailed information of number of dead and aborted embryos/neonates releases in each brood. These data was used to estimate the percentage of non-viable offspring.

2.5 Chemical analyses

pH, oxygen levels and temperature were measured on freshly and old (24 or 48 h) test solutions using each partner’s electrode devices. Upon termination of the test, each partner sent test samples to the coordinator for analysis of PBO levels. Chemical analyses of PBO were limited to the freshly and old test solutions of 50, 100, 200 and 400 µg/L of PBO. Samples of 25 µg/L solution were not analyzed because this concentration was not studied by all laboratories. Actual concentrations of PBO were measured by Ultra Performance Liquid Chromatography coupled with Mass Spectrometry (UPLC-MS) following previous methods (Campos et al., 2016; Mayer-Helm et al., 2008). In short, PBO was measured using an Acquity Ultra Performance LC system (Waters, Mildford, MA, USA) connected to a Triple Quadruple Detector Acquity, using a Luna C18 (150 mm×2 mm ID, particle size 5 µm, Phenomenex, Torrance, USA) equipped with a SecurityGuard pre-column. The mobile phase composition consisted of binary mixtures with
0.1% formic acid in ACN (A) and 0.1% formic acid in water (B). The gradient of elution started at 5% A, then increased to 40% A in 4 min, 60% A in 7 min, reaching 100% A in 11 min and then return to initial conditions within 4 min. The system was operated at room temperature, the flow rate was set at 200 µL/ min and 10 µL were injected. Acquisition was performed in SRM mode under positive electrospray ionization (ESI+) using two transitions from [M+H]+ precursor ion to daughter ions. The transitions used as well as the cone voltages and collision energies were in accordance with [27]. Quantification was based on external calibration standard 8 point curve (r^2 > 0.98, range between 10-1000 µg/L). Limits of detection and quantification defined as the minimum detectable amount of analyte with a signal to noise ratio of 3:1 and 10:1, respectively, were determined from the spiked water samples. The data were acquired and processed using the MassLynx v4.1 software package.

2.6 Data analyses

Within (E) and between lab (L) variation in cumulative offspring production and population growth rates across PBO treatments (PBO) and generations (G) were assessed by determining the components of variance of a three way ANOVA, which was conducted using General Linear Models and IV sum of squares, which account for unbalanced designs (since PBO treatments varied across partners and generations). Not all labs used the same clones, which may have affected interlab sensitivity variation across PBO concentrations (V_{LxPBO}). Thus to account for the potential influence of clone, two distinct models were used, including either laboratory or clone as factor crossed with chemical treatment and generation. Indeed, laboratory and clone could not be considered in the same model, because they were not totally independent (some labs used the same clone and some others did not). The two models for variance partitioning were:

(1) \( VT = V_L + V_{PBO} + V_G + V_{LxPBO} + V_{LxG} + V_{PBOxG} + V_{LxPBOxG} + V_E, \)
(2) $VT = V_c + V_{PBO} + V_{G} + V_{C \times PBO} + V_{C \times G} + V_{PBO \times G} + V_{C \times PBO \times G} + V_E,$

in which “x” terms in sub-indices indicate two and three factor interaction terms for L, PBO and G.

One-way ANOVA and Student’s T test analyses were performed to compare exposure treatments and controls. NOECs (No observed effect concentrations) were determined using one side Dunnett’s post hoc tests and using the solvent control as the reference treatment. Regression analyses were used to determine EC10 (10% effect concentration) and median effect concentrations (EC50) of the tested chemical. Regression analyses were limited to cumulative fecundity and population growth rate and determined from fitting responses to the allosteric decay regression model following previous procedures (Barata et al., 2000b). Statistical comparison of population growth rate was based on a jack-knife procedure (Barata et al., 2001). Prior to ANOVAs, assumptions of normality and variance homoscedasticity were assessed and data was log transformed when required. Percentage survival and age at first reproduction were analysed by non-parametric Kruskal-Wallis and Wilcoxon tests (Zar, 1996). LOECs (Lowest observed effect concentration) and NOECs were obtained using one side Dunnett’s test or the equivalent test for non-parametric analyse (Zar, 1996). ANOVA and non-linear regression analyses were conducted using IBM SPSS v26 and Sigma plot v13.0, respectively.

3. Results

3.1 Water physical-chemical parameters and PBO concentrations

Measured water quality parameters during the test (pH, oxygen levels and temperature) of participating labs were quite similar and met OECD 211 quality criteria. pH and Oxygen levels
(Mean ± SD) were 7.9 ± 0.5, 8.6 ± 0.3 mg/L, respectively, and temperature was kept at 20 ± 0.5 °C.

Measured concentrations of PBO in freshly prepared (Tf) and old test solutions (Told) varied little across labs and generations, thus samples were considered together in Table 2. Freshly prepared solutions were from 2 to 20% lower than nominal concentrations. Stability of PBO varied little during the tests and across labs, since measured levels in old test solutions (Told in Table 2) were from 7 to 20% lower than those of freshly prepared ones (Tf in Table 2). For the sake of clarity, hereafter, our results are referred to nominal concentrations.

3.2 Effects over two generations

A total of 14 different trials were performed since partners 2 and 5 each conducted the two-generation assay twice. In all but one of the assays, non-solvent control treatments were also included. In 13 out of 14 trials, cumulative fecundity in control treatments over 21 days were ≥ 60, thus OECD validity of test criteria was met in most trials (Table 3).

The coefficient of variation was <25% in 91% of the control treatments (control and solvent controls) (Table 3). Results of a three way ANOVA indicated that cumulative fecundity varied significantly (P<0.05) between controls and solvent controls (F 1,390 = 11.4), among labs (F 11,390 = 115.9), and between generations (F 1,390 =105.8). In general cumulative fecundity of controls (Mean ± SD, 105.8 ± 47.5) was higher than those of solvent controls (100.3 ± 33.6) and decreased from first (110.8 ± 41.7) to the second generation (94.6 ± 39.4). Significant interaction terms involving lab, however, denoted heterogeneity in the performance of controls between generations. Indeed significant differences (P<0.05) were found between control and solvent controls in 2 out of 13 trials in the first generation and in 10 out of 13 trials in the second generation (bold values in Table 3). Nevertheless, only 4 of these differences exceeded 25%
(C/CS or CS/C * 100, bold and underlined values, in Table 3), which is the recommended maxima for the OECD TG 211 guideline.

Individual replicated values for cumulative offspring production and population growth rate across labs, PBO concentrations, and generations are depicted in Figs. 1 and 2, respectively, together with fitted regression lines obtained for each lab. The range of variation of individual values for cumulative fecundity was quite large exceeding 3 to 4 fold in most PBO treatments (Fig 1). The same trend was observed for population growth rate responses, despite that r values were less variable (Fig 2). Depicted individual regression lines indicated that an important part of this variability was related to inter-lab variation within and across PBO treatments, whereas variation between generations within labs was low. Variance partitioning of cumulative fecundity and population growth rate responses across PBO treatments, labs or clones and generations are depicted in Fig 3. For both responses all main factors and their interactions were significant (P<0.05) and PBO treatment alone (PBO) and across labs (PBO x L) accounted for most variation (76 and 68% for fecundity and population growth rate, respectively). For cumulative fecundity, generational effects within and across PBO treatments accounted for 6.3% of variability (Fig 3A), whereas for population growth rate such effects only accounted for 2.6 % of variation (Fig 3B). When clone instead of lab was considered, variance partitioning across most factors remained similar except for the contribution of the environment by clone interaction (PBO x L), which decreased by half (from 20 to 10%) (Fig 3A1).

Reported effect levels for cumulative fecundity and population growth rate are shown in Tables 4 and 5, respectively. Significant nonlinear regression curves (P<0.05) were obtained in 27 and 24 out of 28 cases for cumulative fecundity and population growth rate, respectively. Regression models explained more than 60% of the total variance (r^2 > 0.6) and in most cases
allowed to get feasible EC10 and EC50 estimates. The lower success obtained for population growth rate (24 out of 28 cases) was related to the fact that this parameter is less variable than cumulative fecundity and hence it was not always possible to fit regression curves. For cumulative fecundity, second generation individuals (F1) had greater sensitivity to PBO than first generation ones in only 46, 54 and 28% of the cases for effect levels EC10, EC50 and NOEC, respectively (ratios > 1 in Table 4). However, effect concentration endpoints of F0 (EC10, EC50 and NOEC) measured across the 14 trials were not significantly (P<0.05) different than those of F1 (based on paired sample Student’s T tests). For population growth rate and EC50, F1 individuals were significantly more sensitive than F0 ones in 70% of the cases (paired sample Student’s T tests, t_{10} =2.34, P<0.05; Table 5).

Besides fecundity, the life-history traits that most affect population growth rate, namely juvenile and adult survival, age at first reproduction and embryo survival, showed large variation (error bars in Fig 5). These variations, however, were consistent across PBO treatments and generations showing the greatest effects on the percentage of viable embryos, which was significantly affected at 200 µg/L (based on non-parametric ANOVA tests), followed by age at first reproduction and adult and juvenile survival, which were affected, respectively, at 400, 400 and 800 µg/L of PBO.

4. Discussion

The aim of this inter-laboratory calibration exercise was to test for feasibility and consistency for evidence of adverse effects in the second generation individuals using a 21 days reproduction test protocol similar to the OECD 211. This exercise was successfully performed by 12 different labs across two generations using the pesticide synergist PBO, which was selected as the compound having greatest effects in F1 from a previous study (Campos et al., 2016). The
reproduction test performed by these labs met OECD quality criteria in most cases in terms of having acceptable levels of water quality (pH, oxygen levels), reproduction (production of more than 60 neonates per female in 21 days in control treatments) and for intra-lab variability (coefficient of variation for reproductive responses in controls not exceeding 25%). The stability of the tested chemical was also within acceptable values (20% of nominal concentration), which facilitated interlab and multigenerational comparisons. Differences in reproductive performances between control and solvent controls, however, were quite frequent in second generation trials relative to first generation ones. Differences in cumulative fecundity between solvent and non-solvent controls, however, rarely exceeded 20%. Interestingly cumulative fecundity in solvent controls was higher than that of controls in 9 out of the 12 significant trials, which indicates that other factors than cumulative toxicity of solvent accounted for such differences (Hallare et al., 2006; Hutchinson et al., 2006). To minimize false positives due to carrier effects, thus, effect concentrations were always estimated using the solvent control treatment.

The analysis of sources of variance in cumulative fecundity, which is the target endpoint for the OECD211 *D. magna* assay, showed that treatment effects alone and across labs accounted for most variation, which indicates that PBO was the factor affecting most reproduction responses followed by the variability of response to PBO between labs. A large variation observed in the response of participating labs across PBO concentrations (about 20%) has also been reported for other intercalibration exercises. According to Baird et al. (Baird et al., 1989; Baird et al., 1991) in clonal organisms such as *D. magna* the use of different clones may inflate treatment x interlab variability (PBO x L) since different clones may have different sensitivities. Indeed several studies indicated that clonal by environmental variation is one of the factors affecting most *D. magna* performances across chemical pollutants (Barata et al., 2000a; Barata et al., 2002a). In
our study, several labs used the same clone, thus it was possible to recalculate variance components using clone instead of participating lab. Obtained results showed a 50% reduction of the variation accounted for by participating labs across PBO concentrations (i.e., 10% variation), which is in line with previous studies (Barata et al., 2000a; Barata et al., 2002a). Thus, taking together the previous percentage and the variation accounted for by labs alone (i.e. 3% variation), makes about 13% of variation across labs explained by other factors than clone. The use of different culture conditions across participating labs is likely to explain observed differences in sensitivities and reproductive outputs. In line with the previous argument, several studies have reported that the use of different algal diets, food ratios, water types and culture volumes may affect reproduction in *Daphnia* and its life cycle performance across pollutants (Barry et al., 1995; Ginjupalli et al., 2015; Hansen et al., 2008; Heugens et al., 2006; Martínez-Jerónimo et al., 2000; Pavlaki et al., 2014; Samel et al., 1999). About 8% of the variation was not explained by any of the studied factors and thus it was associated to intra-lab variability.

Having an intra-lab variability < 10% is acceptable in environmental toxicology (OECD, 1997).

Population growth rate responses, which allow integration of other important life history traits such as juvenile and adult survival and the timing of reproduction (Barata et al., 2000a; Barata et al., 2002a) behaved similarly to cumulative fecundity responses. Indeed we found that PBO affected mostly embryo viability and hence cumulative fecundity (which in this study refers to alive offspring production) rather than juvenile or adult survival and age at first reproduction. Piperonyl butoxide (PBO) is a cytochrome P450 (CYP) inhibitor (Hardstone et al., 2015) that inhibits steroid hydroxylases in *Daphnia* (Baldwin and LeBlanc, 1994), and hence is likely to alter the biosynthesis and metabolism of ecdysteroids, which are crucial for embryo development (LeBlanc et al., 2000). This is in line with a previous study that found that effects of PBO on *D*.
magna population growth rate was mostly related to PBO effects on embryo survival and hence on cumulative fecundity. Accordingly, variance partitioning across the studied sources were equivalent to those reported for cumulative fecundity.

Multigenerational effects within and across PBO treatments were significant and accounted for about 6% and 2.6% of total variation of cumulative fecundity and population growth rate, respectively. Note that 8.5 and 11.2% of total variance (for cumulative fecundity and population growth rate, respectively) was accounted for by the three interaction terms that involved multigenerational effects across PBO treatments and labs. The occurrence of greater effects of PBO in second generation individuals was heterogeneous and varied across effect levels, endpoints and labs. In some cases toxic effects aggravated in F1 individuals, whereas in others were unchanged or decreased. The greatest differences between F0 and F1 were observed when comparing EC50, probably due to the observed intra-lab variation, which was close to 10%, thus making median effect concentration estimates more accurate and reliable than low effect levels (EC10 or NOEC). In 54 and 70% of cases, second generation individuals (F1) were more sensitive (had lower EC50) than first generation ones for cumulative fecundity and population growth rate responses, respectively. However, on average EC50s in second generation individuals were only 16.5 and 20.8 % lower than those of first generation for cumulative fecundity and population growth rate, respectively. Note, however, that the coordinator lab (i.e. lab1 in Figures and Tables) succeed in obtaining aggravation effects of PBO in F1, which is in line with his previous results (Campos et al., 2016). This means that the proposed protocol is reproducible and consistent within the same lab. Amongst the 13 studies reported in Table 6, 10 out of 19 compounds and/or environmental stressors (52%) had greater toxic effects in second generation individuals than in first generation ones. The occurrence of multigenerational effects
increased to 55% when the analysis was restricted to hormones or endocrine disruptors. There were also discrepancies across results from different labs for a given contaminant (i.e. 4-nonylphenol, (Brennan et al., 2006; Campos et al., 2016). The two previous mentioned experiments differed in many respects: Daphnia clone, solvent (ethanol vs acetone). It is also not clear in Brennan et al. (2006) how the second generation was set up and if the brood rank was considered. All these differences are potential sources of discrepancy between the two studies. Contrary to the studies reported in Table 6, ours was performed with a contaminant (PBO), which a priori was previously found to aggravate toxic effects on cumulative fecundity in second generation individuals (Campos et al., 2016). This means that our success in detecting aggravated effects in second generation individuals using the OECD TG211 testing protocol was moderate (54-70%), and not different to the reported occurrence of multigenerational detrimental effects (52-55%; Table 6).

5. Conclusions

The results reported in the present study showed that the proposed two generation *D. magna* reproduction assay was reproducible across the tested labs. Variability in cumulative fecundity among labs across PBO treatments was quite large (20%) but within the range that is often reported in other inter-calibration tests (Baird et al., 1989; Bradley et al., 1993; Samel et al., 1999). About half of this variability was due to the different sensitivities of clones as stated in early work (Baird and Barata, 1998). Nevertheless, differences in cumulative fecundity between control and solvent control treatments varied across labs and across generations. In first generation (F0) cumulative fecundity in controls (Mean ± SD, 119 ± 43) was higher than those of solvent controls (106 ± 27) and decreased in generation F1 (controls; 97 ± 40; solvent controls; 96 ± 25). Decreased fecundity in generation F1 was probably related to the fact that
distinct culture conditions preceding the initiation of test were used for F0 and F1. The OECD TG 211 guideline and our multigeneration test protocol do not strictly regulate the pre-feeding conditions of the mothers used to generate the experimental progeny to initiate F0. However, during the test, feeding conditions of F0, (i.e. mothers that generated the progeny from which F1 individuals were collected), are highly regulated. *D. magna* reproductive females regulate the quality and quantity of their offspring depending on the feeding conditions that they encounter and require about three generations to adapt their progeny to a given new feeding condition (Barata and Baird, 1998). This feature is known as maternal effects, which is often an important source of unexplained variance difficult to control and highly neglected in ecotoxicological investigations (Barata and Baird, 1998). Further development of this test will require to minimize maternal effects and setting up density and feeding conditions in daphnid cultures before initiating multigenerational assays. The proposed assay had little success in assessing multigenerational effects across labs. Note, however, that the coordinator lab (i.e. lab1 in Figures and Tables) succeed in obtaining aggravation effects of PBO in F1, which is in line with his previous results (Campos et al., 2016). ANOVA results indicated a small contribution of generation within and across PBO treatments on the total variance of cumulative fecundity (6.3%). This was in contrast with the high contribution of treatments within and across labs (76.6 %). Indeed aggravation of toxic effects of PBO in second generation individuals only occurred in 54% of cases when EC50 and cumulative fecundity was considered and in 70% of cases when EC50 and population growth rate response were accounted for. This low occurrence was in line with the rather small increase of sensitivity to PBO measured in second generation individuals: EC50s in second generation individuals were for cumulative fecundity and population growth rate, respectively, only 16.5 and 20.8 % lower than those of first generation. PBO affected
embryo viability and hence the number of live offspring produced at the highest tested doses, which prevented from initiating F1 experiments. This resulted in fewer PBO treatments in F1, which may have diminished the power of ANOVA analyses to detect effect concentrations in F1. The lower magnitude of aggravation accounted by the NOEC (28%) relative to the EC50 (54%) for cumulative fecundity supports this argument. This means that further developments of the OECD TG 211 are needed to better detect cumulative toxicity across generations.

Acknowledgments

This work was supported by the Spanish MEC grant CTM2014-51985-R, and by the SETAC working group EVOGENERATE.
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Figure 1. Cumulative fecundity of *D. magna* individuals exposed to piperonyl butoxide (PBO) during two successive generations across 14 different assays performed by 12 different labs. Each symbol corresponds to a single observation. Curves are fits to the allosteric decay regression model. White and black symbols and black and grey lines correspond to F0 and F1 generations, respectively. Axis X is in log scale.
Figure 2. Population growth rate responses of *D. magna* individuals exposed to piperonyl butoxide (PBO) by two generations across 14 different assays performed by 12 different labs. Each symbol corresponds to a single observation. Lines are fits to the allosteric decay regression model. White and black symbols and black and grey lines correspond to F0 and F1 generations, respectively. Axis X is in log scale. SC, solvent control.
Figure 3. Variance explained by treatment (PBO), Laboratory (L), Generation (G),
treatment by laboratory (PBO x L), treatment by generation (PBO x G), laboratory by
generation (L x G), and the three factor interaction (PBO x L x G) terms. Unexplained
residual variance is also included. Results for cumulative fecundity and population growth rate
using ANOVA model 1 are presented in graphs A and B, respectively. Graph A1 shows the
results obtained for cumulative fecundity when lab was substituted by clone and model 2 was
used (see methods, data analysis for further details). In graph A1 green, purple, grey and pink
areas corresponds to clone, PBO x clone, clone x Generation and PBO x clone x Generation,
respectively.
Figure 4. Survival probabilities (juvenile: Sj; adult: Sa), age at first reproduction, and percentage of non-viable offspring (Mean ±SD, N = 9-138) as function of PBO concentration across all trials. White and black bars correspond to F0 and F1 generations, respectively. Within each generation, * indicates significant differences from solvent control (SC) following Kruskal-Wallis and multi-comparison Wilcoxon and Wilcox tests (Zar, 1996). Death or the absence of reproduction make sample size (N) to differ largely across treatments.
### Tables

**Table 1.**
Distinctive culture conditions of each participant lab. FDTW, filtered de-chlorinated tap water

<table>
<thead>
<tr>
<th>Lab</th>
<th>Photoperiod</th>
<th>Clone</th>
<th>Water</th>
<th>Medium renewal</th>
<th>Food regime</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16 h:8 h L:D</td>
<td>F</td>
<td>100 ml ASTM</td>
<td>2 d</td>
<td><em>Chlorella vulgaris</em>, 0.1 mg C/animal/day (25x10^6 cells/animal/day)</td>
</tr>
<tr>
<td>2</td>
<td>16 h:8 h L:D</td>
<td>own</td>
<td>40 mL ASTM</td>
<td>2 d</td>
<td>Raphidocelis subcapitata (7x10^6 cells/daphnia/day), Tetrafin (0.2 mg dry wt.)</td>
</tr>
<tr>
<td>3</td>
<td>16 h:8 h L:D</td>
<td>own</td>
<td>50 ml M7</td>
<td>2 d</td>
<td><em>C. pyrenoidosa</em> daily (0.2 mg C/daphnia/day)</td>
</tr>
<tr>
<td>4</td>
<td>16 h:8 h L:D</td>
<td>own</td>
<td>100 ml M4</td>
<td>2 d</td>
<td><em>C. vulgaris</em>, daily (0.15 mg C/daphnia/day)</td>
</tr>
<tr>
<td>5</td>
<td>12 h:12 h L:D</td>
<td>own</td>
<td>50 ml FDTW</td>
<td>2 d</td>
<td><em>C. vulgaris</em>, 0.09-0.1 C/ daphnia/day (1,87 mg C/ml, 4x10^5 cells/ml)</td>
</tr>
<tr>
<td>6</td>
<td>16 h:8 h L:D</td>
<td>F</td>
<td>50 mL M4</td>
<td>2 d</td>
<td><em>C. vulgaris</em> (0.1 - 0.2 mg C/daphnia/working day)</td>
</tr>
<tr>
<td>7</td>
<td>16 h:8 h L:D</td>
<td>F</td>
<td>100 mL M4</td>
<td>2 d</td>
<td><em>R. subcapitata</em> (29.6 x 10^6 cells/daphnia/day)</td>
</tr>
<tr>
<td>8</td>
<td>16 h:8 h L:D</td>
<td>own</td>
<td>100 ml ASTM</td>
<td>2 d</td>
<td><em>R. subcapitata</em> (40x10^6 cells/daphnia/day)</td>
</tr>
<tr>
<td>9</td>
<td>16 h:8 h L:D</td>
<td>F</td>
<td>50 ml FDTW</td>
<td>2 d</td>
<td><em>C. vulgaris</em> (10 x 10^6 cells/daphnia), <em>Desmodesmus subspicatus</em> (7x10^6 cells/daphnia)</td>
</tr>
<tr>
<td>10</td>
<td>16 h:8 h L:D</td>
<td>NIES</td>
<td>50 ml M4</td>
<td>2 d</td>
<td><em>C. vulgaris</em>, 0.2 mg C/animal/day (50x10^6 cells/animal/day)+yeast, Cerophile, Trout chow additive</td>
</tr>
<tr>
<td>11</td>
<td>16 h:8 h L:D</td>
<td>F</td>
<td>50 ml ASTM</td>
<td>2 d</td>
<td><em>R. subcapitata</em> (30x10^6 cells/daphnia/day)</td>
</tr>
<tr>
<td>12</td>
<td>16 h:8 h L:D</td>
<td>F</td>
<td>40 ml COMBO</td>
<td>2 d</td>
<td><em>R. subcapitata</em> (2.5-5 mg C/L/daphnia/day)</td>
</tr>
</tbody>
</table>
Table 2.

Nominal and measured PBO concentrations of freshly prepared (Tf) and old (Told) test solutions

<table>
<thead>
<tr>
<th>Nominal</th>
<th>Tf Mean</th>
<th>Tf SD</th>
<th>Tf N</th>
<th>Told Mean</th>
<th>Told SD</th>
<th>Told N</th>
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<tbody>
<tr>
<td>50</td>
<td>40.2</td>
<td>11.8</td>
<td>56</td>
<td>37.3</td>
<td>6.6</td>
<td>55</td>
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<td>100</td>
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<td>9.7</td>
<td>53</td>
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<tr>
<td>200</td>
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<td>23.2</td>
<td>54</td>
<td>322.4</td>
<td>47.3</td>
<td>52</td>
</tr>
</tbody>
</table>
Table 3.
Cumulative offspring production in first (F0) and second (F1) generation in control (C) and solvent control (SC) treatments and associated coefficient of variation (CV). Sample size varied between 8-10. CV values exceeding 25% and significant (P<0.05) distinct mean values of C and SC are in bold. Mean values for C and SC differing more than 25% are also underlined. Empty spaces are missing values.

<table>
<thead>
<tr>
<th>F0</th>
<th>SC</th>
<th>F1</th>
<th>SC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean± SD</td>
<td>CV</td>
<td>Mean± SD</td>
</tr>
<tr>
<td>lab</td>
<td></td>
<td></td>
<td></td>
</tr>
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Table 4.  
Low (EC10, NOEC) and median (EC50) effect concentrations of PBO for cumulative fecundity across participant labs and generations (F0, F1). Ratios between F0/F1 endpoints are also reported. Values are in µg/L. *, ANOVA analyses were not significant (P<0.05) so NOEC was the highest tested concentration. <, effects were observed at the lowest tested concentration, thus it was not possible to estimate a proper NOEC.

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<th>F0 EC50 ± SE</th>
<th>F1 EC50 ± SE</th>
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<th>F1 NOEC</th>
<th>F0 EC10</th>
<th>F1 EC50</th>
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<th>F1 NOEC</th>
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Table 5.
Low (EC10, NOEC) and median (EC50) effect concentrations of PBO for population growth rates across participant labs and generations (F0, F1). Ratios between F0/F1 endpoints are also reported. Values are in µg/L. *, ANOVA analyses were not significant (P<0.05) so NOEC was the highest tested concentration. <, effects were observed at the lowest tested concentration, thus it was not possible to estimate a proper NOEC.

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