ABSTRACT

HANNAS, BETHANY REEVES. Mechanistic Evaluation of the Reproductive and Developmental Toxicity of Nitrate and Nitrite. (Under the direction of Gerald A. LeBlanc.)

The purpose of this research was to investigate the mechanism for reproductive and developmental toxicity associated with the ubiquitous aquatic contaminants nitrate and nitrite, in the crustacean *Daphnia magna*. Two hypotheses were tested: 1) Nitrate and nitrite are converted to the signaling molecule NO, resulting in disruption of endocrine-related processes; and 2) NO interferes with endocrine signaling by lowering steroid hormone levels or by binding a heme-containing nuclear receptor involved in steroid signaling.

In the first study, the toxicity of nitrate and nitrite to daphnids was evaluated. Both compounds increased incidence of developmental abnormalities and reduced fecundity in a concentration-dependent manner. Toxicity was consistent with toxicity elicited by the NO donor sodium nitroprusside. Developmental toxicity of nitrite and NO was ameliorated by the NO-scavenger ß-carotene. Since toxicity of nitrate and nitrite mimicked that of NO, experiments were performed to determine if arthropod cells could convert nitrate and nitrite to NO. *Drosophila* S2 cells converted both nitrate and nitrite to NO in a substrate and cell concentration-dependent manner. Together, these results are consistent with nitrate and nitrite eliciting toxicity via their intracellular conversion to NO. Although the observed toxicity was indicative of an anti-ecdysteroid mechanism of action, we were unable to detect significant, consistent decreases in ecdysteroid levels in daphnids exposed to the NO donor. These results suggest that an alternative mechanism was responsible for the observed NO-induced toxicity.

The next study focused on characterizing a potential target of NO toxicity in the ecdysteroid signaling pathway. In this study, the nuclear receptors E75 (group NR1D) and

HR3 (NR1F) were cloned and sequenced from *Daphnia magna*. Both receptors shared identity with the insect and human orthologs. E75 possessed conserved histidine and cysteine amino acid residues in the ligand binding domain that likely bind heme. NO potentially binds E75-heme as a ligand. HR3 was significantly induced by 20-hydroxyecdysone, whereas E75 was minimally responsive to the hormone. The results of this study implicate both E75 and HR3 of daphnids in the ecdysteroid signaling pathway as potential targets of the action of exogenous NO.

Finally, E75 and HR3 were functionally characterized in regards to regulating gene transcription, to determine if NO alters this regulatory activity. HR3 cloned from *Daphnia pulex* activated transcription of a retinoid orphan receptor element (RORE)-driven luciferase reporter. E75 did not activate the reporter, but served to repress HR3 activation. Experiments revealed no evidence that NO interferes with E75 repression of HR3. Therefore, the mechanism by which nitrate and nitrite-derived NO elicits developmental and reproductive toxicity remains unknown.

Overall, this research highlights the potential threat posed to the reproductive and developmental success of aquatic organisms exposed to nitrate and nitrite. Additionally, this work advances understanding of crustacean endocrinology, while demonstrating the need for further information to identify the mechanism of action for interfering environmental contaminants.

Mechanistic Evaluation of the Reproductive and Developmental Toxicity of Nitrate and Nitrite

by
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DEDICATION

I dedicate this work to my husband for bringing me through everyday with lots of love, support and happiness.

BIOGRAPHY

Bethany Reeves Hannas was born on March 31, 1981 in Bridgeport, Connecticut. She was welcomed to the world by her parents Wendy Anderson and Robert Reeves and her older brother, Chris. Her family later expanded to include her step-father Jeffery Anderson, step-mother Deborah Reeves and younger brother Clarke. Growing up, Bethany was exposed to science and "how things work" from a young age. Her older brother, Chris and her father, Robert were always "fiddling around" with one thing or another. And of course, as the little sister, Bethany's curiosity was peaked in the process of trying to be just like her big brother. She has fond memories of watching Mr. Wizard's World, building science projects such as "Why do earthquakes occur?" and mounting and identifying insects with her mother, Wendy, for her 9th grade project. Bethany lived in CT through the end of high school until she attended Marist College in Poughkeepsie, NY where she studied Environmental Science. It was at Marist that Bethany discovered the field of toxicology and was intrigued by a field that applies several different facets of the basic sciences. Outside of research, class-work and preparing for graduate school, Bethany participated in intramural volleyball and the college choir, and was also a member of the Marist College Ski Team. Upon graduating from Marist, Bethany decided that although she knew graduate school was in her future, she wasn't ready to begin yet. Instead, she obtained a visa and moved to England where she worked in a food research company as a lab technician. Upon returning from this year abroad, Bethany joined the Toxicology Department at North Carolina State University and

began working in Dr. Leblanc's lab at the end of 2004. Two years later, with one written preliminary exam down, Bethany met her husband and personal cheerleader-to-be, Ben. The two were married in December 2008 and are enjoying life in Raleigh along with their little dog, Matics. Bethany thoroughly enjoyed the opportunities afforded to her while in grad school. She hopes to apply all that she's learned and experienced to a career related to the continued advancement of scientific knowledge in the field of endocrine disruption.

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INTRODUCTION

Endocrine disrupting chemicals

The endocrine system, in both vertebrate and invertebrate species, is responsible for delivering signals from one cell type to another by way of messenger molecules, or hormones. This signaling system plays an essential role in regulating numerous physiological functions including, but not limited to metabolism, growth, reproduction and development.

Over the past two decades, several chemicals have been classified as endocrine disrupting contaminants (EDCs), deeming them capable of interfering with endocrine processes, which can lead to adverse biological effects in organisms. Endocrine signaling drives reproductive and developmental processes and therefore susceptibility to the effects of EDCs varies with the timing of exposure during an organism's life cycle. The mechanisms by which EDCs disrupt normal endocrine signaling also vary. A chemical can bind to and either activate a receptor (as an agonist) or inactivate a receptor (as an antagonist), or alter hormone synthesis/secretion, or inactivation/clearance.

In 1998 the United States Environmental Protection Agency (USEPA) established the Endocrine Disruptor Screening Program (EDSP) in response to both the Food Quality Protection Act of 1996 and the Safe Drinking Water Act of 1996. These regulations were enacted by Congress to improve the efficacy of drug/chemical screening for endocrine

disrupting activity (USEPA 1998). The initial emphasis for research by the EDSP was on compounds effecting estrogen, androgen and thyroid hormone-dependent processes. Consequently, a large body of endocrine disruption research followed, which focused mostly on these three systems. Less is known about chemicals that may interact with other hormones. However, there is emerging evidence of contaminant-inflicted endocrine disruption in wildlife populations that is beyond the scope of classically defined estrogen, androgen or thyroid hormone endocrine disruption. These findings document additional impacted hormone systems and many of the associated contaminants come from classes not previously recognized as endocrine disruptors. In particular, nitrates and nitrites have historically been regulated for toxicity associated with acute exposures to high levels. However, more recent findings indicate that further consideration is needed with regards to the endocrine disrupting ability of these chemicals at chronic, lower-dose exposure levels.

History of nitrates and nitrites as environmental pollutants

Nitrates and nitrites are major environmental pollutants in the aquatic compartment (Edwards and Guillette 2007; Kaiser 2001). Nitrate (NO_3^-) is a component of the nitrogen cycle and is naturally produced by nitrogen fixing bacteria through oxygenation of atmospheric nitrogen (N2)(Vitousek *et al.* 1997). Normally, natural nitrogen cycling does not contribute to excessive accumulation of NO_3^- or NO_2^- in the environment. However, over the past two centuries, humans have stimulated global nitrogen fixation to about double that of

natural nitrogen fixation on land resulting in major alterations in the bioavailability of nitrate throughout the world (Camargo *et al.* 2005; Galloway and Cowling 2002; Vitousek *et al.* 1997). Activities responsible for this human-driven nitrogen fixation include production of nitrogen-containing fertilizers for agriculture, and the release of fixed nitrogen through mining for and burning fossil fuels, clearing land and draining wetlands. Concentrated livestock and poultry farming produce millions of tons of nitrate-containing manure each year (Camargo and Alonso 2006; Galloway and Cowling 2002; Jenkinson 2001; Vitousek *et al.* 1997). In addition, cultivation of seed legume crops (including peas and soy beans) increases production of bioavailable nitrogen because these crops symbiotically host N_2-fixing bacteria (Galloway *et al.* 2004; Vitousek *et al.* 1997).

The majority of artificially-fixed nitrogen is produced for agricultural uses, with 83 million tons of nitrogen fixed annually for fertilizer (Jenkinson 2001). This figure is likely to increase with worldwide increasing demands for food production and is predicted to exceed 135 million metric tons of nitrogen by 2030 (Vitousek *et al.* 1997). Nitrogen is applied to soils in the form of inorganic ammonium which bacteria convert to nitrates that are then either: assimilated directly by plants, immobilized as nitrate in organic matter, or converted to atmospheric gases by the process of denitrification (Rao and Puttanna 2000). Excess nitrate that is not sequestered is readily available to leach out of the soil root zone and invade water bodies as runoff. Therefore, anthropogenic production of excessive amounts of water-soluble nitrates and nitrites (NO_2^-; an intermediate compound produced during denitrification of ammonium) has led to their accumulation in the aquatic environment through rain runoff

into surface waters or leaching into groundwaters (Edwards and Guillette 2007; Guillette and Edwards 2005; Sampat 2000).

Regulations exist that limit levels of nitrates in drinking water to protect human health. However, levels above these established limits are commonly detected in water supplies, rivers, ponds, and aquifers (Guillette and Edwards 2005). The United States Geological Survey (USGS) implemented the National Water-Quality Assessment (NAWQA) Program in 1991 to investigate the water-quality of 60 study sites (large river basins and aquifer systems) throughout the country (Nolan *et al.* 2001). Amongst the findings, they concluded that nitrate concentrations exceeded the U.S. Environmental Protection Agency's drinking-water standard (10 milligrams N per liter) in about 12 percent of domestic-supply wells and these wells were generally located in agricultural areas. Runoff of agricultural nitrogen has increased by 20-fold within the U.S. alone over the last 50 years (Barbeau and Gullette 2007; Puckett 1995) and will only continue to rise without regulation.

Water supplies are also threatened by nitrate pollution on a global scale. A substantial portion of water supplies in the United Kingdom contain nitrate concentrations above the Environmental Commission (EC) limit of 11.5 mg N/L, as established by the EC Drinking Water Directive in 1985 (Skinner *et al.* 1997). The percentage of Nitrate Vulnerable Zones (NVZs), defined as land that produces run-off draining to water containing nitrate above or approaching the designated safe limit, was extended across England from 55% to 70%, following a 2006 review of water quality data (ADAS 2007). During the 1990's, drinking water wells in the Indian states of Punjab and Haryana typically contained

nitrate levels that were five to fifteen times higher than acceptable levels (Sampat 2000). Major rivers in the Taihu region of China typically have nitrogen levels as high as 8 mg/l due to fertilizer use (Ju *et al.* 2009). In developing countries, where three quarters of the world's population currently resides, significant increases in food production has increased nitrate pollution of groundwater at disconcerting rates (Rao and Puttanna 2000). As the human population continues to grow and the need for agriculture increases, we are likely to see even greater global increases in the levels of nitrate pollution.

Classical toxicity associated with nitrate exposure

Aquatic organisms can take up nitrates and nitrites from the aquatic environment through both passive and active mechanisms and terrestrial species are exposed largely through consumption of contaminated water. Humans are additionally exposed to nitrates and nitrites through their use in pharmaceuticals and as food additives. Once an organism consumes nitrate, microorganisms that reside in the digestive tract can reduce the compound to nitrite. Nitrite can bind to hemoglobin in red blood cells to cause methemoglobinemia. This condition occurs when nitrite oxidizes divalent or reduced iron, within the heme moiety of the hemoglobin, to the trivalent form, forming methemoglobin (Avery 1999). The trivalent iron group of methemoglobin is incapable of binding oxygen, thus decreasing the overall oxygen carrying capacity of the blood. When nitrate/nitrite exposure levels are sufficiently high, the resulting increased concentrations of methemoglobin will reduce

oxygen transport to tissues and can lead to asphyxiation and death (Fan *et al.* 1987; Fewtrell 2004). For decades, the occurrence of methemoglobinemia in humans and wildlife species has been recognized as the primary mode of toxicity associated with nitrates and nitrites (Camargo and Alonso 2006).

An additional concern related to nitrate input in the environment is the indirect toxicity associated with eutrophication of surface waters. Eutrophication occurs when nitrogen input to surface waters leads to massive algal blooms. Death and subsequent decomposition of the algae by oxygen-consuming microbes then depletes the dissolved oxygen level in the water. The decrease in water quality can cause declines in populations of fish and other organisms. This process has major consequences on aquatic ecosystems (Smith *et al.* 1999). In particular, eutrophication of coastal waters can lead to the formation of dead zones, which are areas in which the dissolved oxygen levels are too low to support ocean life. The number of dead zones identified worldwide has increased from just 49 in the 1960s to 405 in 2008 (Diaz and Rosenberg 2008).

Nitrate contamination of aquatic systems can also lead to direct acute toxicity. Several studies demonstrate toxicity at environmentally-relevant nitrate and nitrite concentrations. For example, mortality of cutthroat trout (*Salmo clarki*), Chinook salmon (*Oncorhynchus tshawytsha*) and rainbow trout (*Oncorhynchus mykiss*) larvae was documented at nitrate concentrations as low as 2.3-7.6 mg N/L (Kincheloe *et al.* 1979). The 96 h LC50 values for nitrate fell between 13.6-39.3 mg N/L in anuran species including the American toad (*Bufo americanus*), the Chorus frog (*Rana pipiens*) and the Green frog (*Rana*

clamitans) tadpoles (Camargo *et al.* 2005; Hecnar 1995). Additionally, nitrate concentrations as low as 8.11 mg N/l were lethal to tadpoles of the Cuban tree frog, *Osteopilus septentrionalis* (Punzo and Law 2006). These examples demonstrate that current regulations for nitrate and nitrite limits in surface waters do not ensure the protection of aquatic organisms.

Evidence that nitrates and nitrites may be acting as EDCs in wildlife

Historically, acceptable levels of nitrates and nitrites in water have been based on the toxicity associated with methemoglobinemia. However, more recent studies demonstrate that exposure to these compounds can also cause endocrine toxicity and therefore, necessitate reevaluation of these regulations. In their 2005 commentary, Guillette and Edwards review studies that support a direct relationship between disruption of the reproductive endocrinology of organisms (usually wildlife) and exposure to nitrates. These authors (2005) provide a retrospective analysis of their previously obtained data concerning sex steroid concentrations in juvenile alligators from contaminated lakes in Florida. They revealed an inverse relationship between total nitrogenous contaminant levels in the lakes and plasma testosterone concentrations in the alligators.

Various laboratory studies corroborate the theory that nitrates cause endocrine disruption in exposed organisms. For example, exposure to nitrates caused a decrease in testosterone synthesis in cultured mouse Leydig cells (Panesar 1999) and the inhibition of

androgen synthesis in Sprague-Dawley rats given water containing sodium nitrite ($NaNO_2$) at a concentration of roughly10 mg N/l (Panesar and Chan 2000). Similar effects were seen in Leydig cells of bulls that were orally administered nitrates (Zraly *et al.* 1997).

Several studies have shown effects of nitrates and nitrites on endocrine-controlled processes in aquatic organisms including: development, reproduction and thyroid function. Chronic reproductive toxicity was reported for the crustacean *Ceriodaphnia dubia*, with EC50s for nitrate and nitrite of 21 mg N/L and 0.22 mg N/L, respectively (Dave and Nilsson 2005). These are levels commonly detected in aquatic systems receiving fertilizer contaminated runoff (Bogardi 1991; Camargo *et al.* 2005). In this study, the reproduction during chronic exposure was more sensitive to nitrate and nitrite toxicity than was survival during acute exposure, with acute-chronic ratios of 39 for nitrate and 170 for nitrite. Additionally, a strong correlation was reported between exposure to increasing concentrations of nitrate and reproductive changes in the mosquitofish *Gambusia holbrooki*, (Edwards and Guillette 2007).

Studies on effects of nitrate exposure on amphibian species link nitrates to thyroid hormone disruption. Exposure related alterations in growth and metamorphosis have been observed in several different frog species including: toad tadpoles (*Bufo bufo*) (Xu and Oldham 1997), southern toad tadpoles (*Bufo terrestris*) (Edwards *et al.* 2006), cascade frogs (*Rana cascadae*)(Marco and Blaustein 1999) and the northern leopard frog (*Rana pipien*) (Orton *et al.* 2006). In amphibians, thyroid hormones influence aspects of development and metamorphosis, suggesting that thyroid function may be compromised in nitrate-exposed

frogs. Endocrine toxicity was documented in the silver sea bream (*Sparus sarba*) following exposure to levels 25 mg N/L and 50 mgN/L NO_2 , In this marine teleost species, nitrite exposure caused a decrease in the serum level of the thyroid hormone, thyroxine (Deane and Woo 2007).

Possible mechanisms of endocrine toxicity

Several mechanisms have been proposed for the endocrine toxicity of nitrates and nitrites. One proposed mechanism is that these compounds are converted *in vivo* to the potent signaling molecule nitric oxide (NO), which then disrupts normal endocrine functions (Panesar and Chan 2000). NO is normally produced endogenously from L-arginine and molecular oxygen by the enzyme nitric oxide synthase (NOS) (Bredt *et al.* 1990). At appropriate levels, NO regulates immunity, vasodilation and many other physiological processes. NO can mediate its biological effects by binding to a variety of targets, including heme groups, cysteine residues, and iron and zinc clusters. Since there are numerous targets for NO-mediated signaling, tight regulation of NO production is required to maintain normal biological activity. When NO levels are too high or sustained for too long, as may occur following exposure to nitrates or nitrites, a number of important biological processes can be disrupted.

Under anoxic or low pH conditions, nitrite is spontaneously converted to NO through disproportionation. Zweier *et al* (1995) reported increasing NO generation from nitrite with

decreasing pH in ischemic heart tissue. They demonstrated that this synthesis was independent of NOS-mediated production. Therefore, nitrite could be spontaneously converted to NO in eutrophic environments, which are often approaching hypoxic conditions, or in environments impacted by acid precipitation. An intracellular pH shift to acidic values was measured during development in the nematode, *Caenorhabditis elegans* (Wadsworth and Riddle 1988). Intracellular pH values were as low as 5.8, which is sufficient for the conversion of nitrite to NO. Therefore, organisms exposed to nitrates/nitrites during development may experience periods of intracellular acidic pH which could cause aberrant production of NO resulting in toxicity.

Nitrite is also enzymatically converted to nitric oxide. Castello et al (2006) demonstrated that cytochrome *c* oxidase is responsible for conversion of NO_2^- to NO in both yeast and rat liver cell mitochondria. Also, the enzyme xanthine oxidase (XO) reduces NO_3^- to NO_2^- and then to NO (Li *et al.* 2003). Various mechanisms for *in vivo* conversion of NO_2^- to NO exist. All of these mechanisms support the concept that organisms may produce excess amounts of NO when exposed to nitrate and nitrite.

Nitric oxide could interfere with steroidogenic pathways by either decreasing synthesis or interfering with regulation of receptor signaling. NO decreased steroidogenesis in mouse and rat Leydig cell lines by inhibiting the conversion of cholesterol to pregnenolone (Del Punta *et al.* 1996). This finding suggests that NO binds to the cholesterol side chain cleaving enzyme ($P450_{scc}$), which is responsible for this step in the pathway. NO is capable of binding to the heme group of this enzyme and thereby inhibiting its activity. Several

heme-containing cytochrome P450 enzymes contribute to both the anabolic and catabolic metabolism of steroid hormones and all are potential targets for the action of NO. Some nuclear receptors, such as rev-erb and its orthologs, contain heme and are regulated by the interaction of NO with the heme (Reinking *et al.* 2005). These receptors function along endocrine signaling pathways (Segraves and Hogness 1990; Triqueneaux *et al.* 2004) and their function could be altered by nitrate/nitrite-derived NO.

Another possible mechanism by which nitrates and nitrites may influence reproductive or developmental endocrinology is by altering chloride ions in steroidogenic cells (Guillette and Edwards 2005). Nitrate can substitute for chloride in some chloride transporters across cellular membranes (Guillette and Edwards 2005; Guinamard *et al.* 1995). Additionally, Panesar (1999) showed decreased androgen synthesis when mouse MLTC-1 tumor Leydig cells were grown in inorganic nitrate supplemented media, compared to those treated with chloride supplemented media. The roles of chloride ions and choride channels in steroidogenesis remain unclear (Skoblina 2002), however it is apparent that chloride conductance is a component of steroidogenesis activation in Leydig cells (Choi and Cooke 1990). Therefore, nitrate exposure potentially affects synthesis by altering intracellular chloride ion concentrations (Guillette and Edwards 2005).

Daphnia sp. as model species

Endocrine disrupting properties of compounds are generally characterized in vertebrate species. However, invertebrate species must also be considered due to their critical role in ecosystem structure and function. The endocrine systems of both invertebrate and vertebrate species regulate developmental and reproductive processes. However, the hormone signaling pathways behind these processes are often distinctly different between vertebrates and invertebrates. Therefore, endocrine disruption in vertebrates does not necessarily infer endocrine disruption in invertebrates and vise versa.

This work was primarily conducted with two freshwater crustacean species within the genus *Daphnia*, specifically *Daphnia magna* and *Daphnia pulex*. Daphnids are small (3-10 mm) inhabitants of freshwater ecosystems. Their small size contrasts greatly with the large role that these keystone species play in their environment. They are primary consumers of algae and a food source for fish (Manca *et al.* 2008; Tessier *et al.* 2000) and are therefore critical players in aquatic food webs. They also serve as excellent sentinels of exposure in freshwater lakes. For these reasons, it is imperative to monitor contaminant exposure and understand effects on daphnids.

Daphnids are incredibly amenable to lab culture conditions and therefore, are widely used as a standard model for research in a variety of fields, including toxicology, ecology, population genetics, and physiology. Their life cycling time is relatively rapid and reproduction can be manipulated in the laboratory. Daphnids typically reach maturity in 5-

10 days. Under favorable conditions, including abundant food, low population density, and appropriate photoperiod, daphids reproduce by parthenogenesis, or asexual reproduction (Hobaek and Larsson 1990; Kleiven *et al.* 1992). Under these conditions, females release broods of female offspring every 2-3 days that are genetically identical to the mother. Prior to release, these broods of embryos are readily amenable to removal and *ex vivo* manipulations in the laboratory. Changes in food abundance, population density, or photoperiod signal the daphnids to produce males and haploid eggs that must be fertilized to develop. The cycle of sexual reproduction provides for genetic recombination within the population, purging deleterious mutations, and culminating in the production of diapause eggs. Diapause eggs can withstand desiccation and freezing, are subject to dispersal, and have been hatched from sediments decades after being released (Wolf and Carvalho 1989). Together, these characteristics make daphnids a favorable species to use for the assessment of reproductive and developmental toxicity.

Daphnids were selected as the model species for this study because they are an excellent model for assessing endocrine disruption. Two major hormones have been described in daphnids as related to reproduction and development, namely methyl farnesoate and 20-hydroxyecdysone. Methyl farnesoate, the crustacean juvenoid hormone, regulates many aspects of reproduction including sex determination and results in male offspring when administered during oocyte ovarian maturation (Olmstead and LeBlanc 2002). 20-Hydroxyecdysone is a steroid hormone that regulates molting or ecdysis and intiate signaling between various genes involved in development, growth and reproduction (LeBlanc and

McLachlan 1999; Subramoniam 2000). These hormone pathways are susceptible to perturbation by environmental contaminants, which in some cases can be detected by analysis of the organism's gross appearance. Exposure of daphnids to juvenoid-like compounds can be detected by production of male offspring (Wang *et al.* 2005). Altered development of the shell spine and second antennae is indicative of exposure to chemicals possessing anti-ecdysteroid activity (Mu and LeBlanc 2002). These phenotypic indicators of endocrine disruption can serve as useful tools in determining the mechanism of toxicity for a particular compound.

Additionally, daphnid species are currently on the cusp of serving as an even more sensitive means for screening endocrine disrupting activity of chemicals. The complete *Daphnia pulex* genome became available in 2007 through the Daphnia Genomics Consortium (http://daphnia.cgb.indiana.edu/). Currently, the *Daphnia magna* genome sequencing project is on schedule for completion in 2009. These resources will greatly contribute to the characterization of daphnid endocrine signaling pathways and should thereby facilitate more detailed mechanistic studies.

Research Outline

This study tested two hypotheses aimed at investigating the mechanism for NO_x endocrine toxicity: 1) NO_x are converted to the signaling molecule NO, resulting in disruption of endocrine-related processes; and 2) NO interferes with endocrine signaling by

lowering steroid hormone levels or by binding a heme-containing nuclear receptor involved in steroid signaling. Arthropod embryonic cells and the crustaceans *Daphnia magna* and *Daphnia pulex* were used as model systems for these investigations.

The first chapter addresses the hypothesis that NO_x cause toxicity through their conversion to NO. The first objective of this study was to determine if arthropods are capable of converting NO_x to NO. The second objective was to determine if NO_x elicit endocrine toxicity consistent with that elicited by NO. Results indicated that all three compounds were targeting ecdysteroid signaling. Therefore, the third objective of the study was to determine if NO alters ecdysteroid signaling by lowering ecdysteroid hormone levels in daphnids.

Results provided no evidence to suggest that NO was eliciting anti-ecdysteroid toxicity through a decrease in ecdysteroid levels. Therefore, further research focused on an alternate target for NO within the ecdysteroid signaling pathway. Chapter two takes a closer look at the crustacean ecdysteroid signaling pathway to gain further understanding of the steps involved in this endocrine cascade which culminate in regulation of reproductive and developmental endpoints. This chapter addresses the hypothesis that crustaceans express the nuclear receptors HR3 (ortholog to vertebrate ROR) and E75 (ortholog to vertebrate rev-erb) in response to ecdysteroid signaling. These receptors function coordinately in insects to mediate ecdysteroid signals and we viewed E75 as a potential target of NO_x. The primary objective of the study was to clone and sequence these receptors from *D. magna* and evaluate their expression under different conditions or at different time points including: embryonic

developmental stages, following ecdysteroid treatment, and throughout an entire molting cycle.

Finally, chapter three explores the likelihood that E75 is a target for NO toxicity. This chapter addresses the hypothesis that E75 negatively regulates HR3 and NO_x-derived NO interferes with this regulation. The primary objectives of this study were to (1) develop a luciferase-based transcription reporter assay driven by an HR3 response element; (2) demonstrate that HR3 activates transcription in a ligand-independent fashion and cotransfected E75 inhibits this transcriptional activation; and (3) determine if NO/NO_x reverses E75 suppression of HR3.

Overall, the results from this research demonstrate that NO_x elicit endocrine disrupting toxicity potentially by interfering with ecdysteroid signaling. The mechanism for this toxicity likely involves intracellular conversion of NO_x to NO. However, following the pursuit of E75 as a likely target for NO interaction, the specific target for NO within the ecdysteroid signaling pathway remains unknown. These results advance our understanding of the mechanism for endocrine disruption induced by aquatic NO_x and will help guide future research with regards to further elucidating this mechanism. At the very least, this research program will undoubtedly serve as a starting point for reevaluating the impact of environmental NO_x exposures on the development, growth and reproduction of aquatic organisms.

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CHAPTER ONE: INTRACELLULAR CONVERSION OF NITRATES AND NITRITES TO NITRIC OXIDE: AN ENVIRONMENTAL HEALTH THREAT?

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Abstract

Nitrates and nitrites (jointly referred to as NO_x) are ubiquitous environmental contaminants to which aquatic organisms are at particularly high risk of exposure. We tested the hypothesis that NO_x are intracellularly converted to the potent signaling molecule nitric oxide (NO) resulting in the disruption of endocrine-regulated processes. These experiments were performed with insect cells (*Drosophila* S2) and whole organisms *Daphnia magna*. We first evaluated the ability of cells to convert nitrate (NO_3^-) and nitrite (NO_2^-) to NO using amperometric real-time NO detection. Both NO_3^- and NO_2^- were converted to NO in a substrate concentration-dependent manner. Next, daphnids were continuously exposed to the NO-donor sodium nitroprusside (positive control) and to environmentally-relevant concentrations of NO_3^- and NO_2^-. All three compounds interfered with normal embryo development and reduced daphnid fecundity. NO_2^- was more potent than was NO_3^-. The NO-scavenger β-carotene provided significant protection to daphnids against the developmental toxicity of NO_2^-, further indicating that NO_2^--derived NO was responsible for the developmental toxicity. Developmental abnormalities were characteristic of those elicited by compounds that interfere with ecdysteroid signaling. However, no compelling evidence was generated to indicate that NO reduced ecdysteroids titers. Results demonstrate that nitrite elicits developmental and reproductive toxicity at environmentally relevant concentration due likely to its intracellular conversion to nitric oxide.

Keywords: nitric oxide, ecdysteroids, arthropods, nitrite, nitrate, endocrine disruption

Introduction

Nitrogen makes up 78% of the earth's atmosphere and is also the fourth most abundant element in organisms. Atmospheric nitrogen (N_2, N_2O, NO) is not biologically available and must be transformed or "fixed" for use in biological processes. Under natural nitrogen cycling conditions, bacterial nitrogen fixation produces nitrate (NO_3^-) or ammonium (NH_4^+) ions. Nitrifying bacteria oxidize NH_4^+ first to nitrite (NO_2^-) and then to NO_3^- [1, 2]. Normally, levels of nitrite and nitrate (jointly referred to as NO_x) do not accumulate to excessive levels in the environment because they are assimilated as sources of nitrogen by plants or ultimately converted back to atmospheric nitrogen through bacterial denitrification. As such, biologically available nitrogen is typically a major limiting factor for life on the planet and nitrogen cycling serves as a major regulator of the structure, function, and integrity of ecosystems.

However, human activities have led to drastic increases in the amount of biologically available NO_x found in the environment [3, 4]. Anthropogenic sources of NO_x pollution include both non-point sources such as runoff from agricultural areas containing manufactured nitrogen fertilizer or nitrate-containing manure generated from concentrated animal feeding operations (CAFOs), and point sources such as municipal wastewater effluents and industrial discharges [1]. NO_x are highly water soluble, and thus can readily enter surface and ground

waters through rain events. Consequently, aquatic organisms are at high risk for exposure to these compounds.

Human-driven contributions of NO_x to the environment are on the rise globally, and therefore contamination of aquatic systems is not only ubiquitous, but also increasing. Concentrations of NO_x in surface waters often exceed 10 mg N/L (e.g., [5] [6]), the United States drinking water standard for nitrate [7]. Drinking water standards set for NO_x are directed largely towards the protection against methemoglobinemia in infants [8]. Relatively little attention has been directed towards potential adverse impacts from chronic, low-level exposure to these compounds in humans or wildlife. Reproduction was significantly reduced during continuous exposure of the water flea (*Ceriodaphnia dubia*) to nitrate concentrations as low as 2 mg N/L [9]. This value stands in contrast to the criterion of 40 mg N/L nitrate established by the Canadian Ministry of Environment for the protection of aquatic life against chronic toxicity [10].

Guillette and Edwards [11] provided compelling evidence that levels of NO_x in the environment may be disrupting reproductive physiology of wildlife. They proposed that chronic toxicity of NO_x is not due to methemoglobinemia, but rather, may be due to 1) competitive displacement of chloride ions from membrane transporters, 2) binding to the heme groups of steroidogenic enzymes and inhibiting hormone synthesis, or 3) conversion to the potent signaling molecule nitric oxide. In support of the later two proposed mechanisms,

Panesar and Chan [12] reported that NO_x inhibited steroidogenesis in exposed Leydig cells. These investigators proposed that NO_x is converted in the cell to NO which then binds to the heme group of steroidogenic enzymes and suppresses their catalytic activity. The conversion of environmental NO_x to NO could therefore be a major cause of endocrine disruption in aquatic species where exposure is potentially high. Conversion of NO_x to NO may occur through numerous mechanisms including: non-enzymatic acidic disproportionation [13], mitochondrial cytochrome *c* reductase activity under hypoxic conditions [14, 15], reduction by cytochrome P450s [16], enzymatic conversion by xanthine oxidoreductase [17, 18], reduction by deoxyhemoglobin [19, 20], or conversion by nitrate/nitrite reductase enzymes associated with oral or gastric bacteria [21]. Several of these NO_x reduction mechanisms are relevant to aquatic organisms that possess the required biochemical machinery, or live under environmental conditions (hypoxia, low pH) that are conducive to the generation of NO.

NO is a potent, short-lived signaling molecule that regulates a variety of physiological processes. NO is normally produced endogenously from L-arginine and molecular oxygen by the enzyme nitric oxide synthase [22]. NO can mediate its biological effects by binding to many targets, including heme groups, cysteine residues, and iron and zinc clusters. Since there are numerous targets for NO-mediated signaling, tight regulation of NO production is required to maintain normal biological activity. When NO levels are too high or sustained for too long, as may occur following exposure to nitrates or nitrites, toxicity and disease may occur.

We hypothesize that environmental NO_x pose risk of chronic toxicity through their conversion to NO. We further hypothesize that NO interferes with endocrine signaling by lowering steroid hormone levels. We evaluated the ability of arthropod cells (*Drosophila* S2) to convert nitrate and nitrite to NO. Furthermore, we evaluated whole organism effects of NO_x during continuous exposure of the arthropod species *Daphnia magna*. Finally, we evaluated ecdysteroid levels in daphnids following NO exposure. Arthropods serve as keystone species in many ecosystems and are commonly used as sentinel species for contaminant exposures. Arthropod cells and organisms used in the present study serve as a model for evaluating biological interactions with NO_x and provide direct information for making decisions regarding the health threat posed to aquatic ecosystems by these compounds.

Experimental Section

Measurement of NO_x conversion to NO

Drosophila Schneider S2 cells were used to evaluate the cellular conversion of NO_x to nitric oxide. Cells were cultured in Schneider's medium + 10% heat inactivated fetal bovine serum. NO production by cells provided NO_x was measured using an ISO-NO Mark II meter, equipped with a Clark-type electrode, and DUO-18 data acquisition system (World Precision Instruments, Sarasota, FL). During experiments, the cell suspension was housed inside a sealed chamber (World Precision Instruments) with minimal airspace. The electrode

was immersed in the cell suspension through a port in the chamber. The potential NO liberators $NaNO_2$ and $NaNO_3$ (Sigma-Aldrich, St. Louis, MI, USA) were injected into the chamber with a Hamilton syringe fitted through an injection port on the chamber. All experiments were conducted at 23°C. NO levels in the chamber were continuously measured from time of injection of NO_x until a state of equilibrium was reached. Equilibrium occurred when the rate of NO production was equal to the rate of NO loss. NO-generated millivolts, at equilibrium, were converted to the concentration of NO (nM) in solution using a standard curve. Standards curves were generated according the instrument manufacturer's recommendations using KNO_2 as a substrate under acidic conditions. Negative controls consisted of injections of deionized water or NaCl (Sigma-Aldrich), assayed at Na concentrations comparable to those used in the experiments with $NaNO_2$ and $NaNO_3$. Each experiment was replicated at least three times.

Model organism

The crustacean *Daphnia magna* was used in all toxicological experiments. Daphnids were derived from cultures maintained in our laboratory for more than 15 years. The original stock was acquired from the US Environmental Protection Agency, Mid-Continent Ecology Division (Duluth, MN). Daphnid media consisted of reconstituted deionized water (192 mg/L $CaSO_4 \cdot H_2O$, 192 mg/L $NaHCO_3$, 120 mg/L $MgSO_4$, 8.0 mg/L KCl, 1.0 µg/L selenium and 1.0 µg/L vitamin B_{12}). Cultures were maintained at a density of 50 daphnids/L media. Medium was changed and the oldest adults discarded weekly. Culture daphnids were fed

twice a day with 1.0 mL (4 mg dry weight) of Tetrafin® fish-food suspension (Pet International, Blacksburg, VA, USA) and 2.0 mL (1.4 x10^8 cells) of a unicellular green algae *Pseudokirchneriella subcapitata* suspension. The algae were cultured in Bold's basal medium. All daphnid cultures and experiments were housed at 20°C with a 16-hr photoperiod. These culture conditions maintained the daphnids in the parthenogenetic reproductive phase with production of all-female broods.

Reproductive and developmental toxicity

Developmental and reproductive toxicity of sodium nitroprusside (Sigma-Aldrich), sodium nitrate, and sodium nitrite were evaluated. Daphnids were individually exposed to 10% serial dilutions of each material for a total of 20-30 concentrations. A control was provided for each experiment that consisted of 10 daphnids individually exposed to assay media. The compounds were delivered as an aqueous stock to the daphnid media. Daphnids were individually exposed in 50 mL beakers containing 40 mL solution. Solutions were changed every other day. The concentration ranges of the materials were based upon preliminary experiments and concentrations of NO_3^- and NO_2^- spanned environmentally-relevant levels. Exposures began with daphnids < 24 hours old and continued through 4 brood cycles (about 21 days). Each daphnid was fed 100 μL algae suspension and 50 μL of fish-food suspension daily until they reached 7 days old, after which they were fed 200 μL algae suspension and 100 μL fish-food suspension, daily. Daphnids were evaluated daily for survival and release of offspring. Following the release of each brood, the number of

neonates in the brood was determined and individuals were evaluated a light microscope (Nikon) for the presence of gross developmental abnormalities.

NO scavenger rescue

Adult daphnids (7-8 days old) were treated with sodium nitroprusside or NO_2^- in the presence or absence of the NO scavenger β-carotene [23] to determine if NO scavenging protects neonates against developmental toxicity. β-carotene (Sigma-Aldrich) stock was prepared with DMSO. Treatments consisted of DMSO (0.0001%), β-carotene (1.0 mg/l), sodium nitroprusside (1.0 mg N/L), $NaNO_2$ (2.0 mg N/L), sodium nitroprusside (1.0 mg N/L) + β-carotene (1.0 mg/L), and $NaNO_2$ (2.0 mg N/L) + β-carotene (1.0 mg/L). One daphnid was housed in a single 50 mL beaker containing 40 mL treatment solution and each treatment included 20 daphnids. Solutions were changed every other day and each beaker received 200 μL algae suspension and 100 μL fish-food suspension daily. The first brood of offspring released from each adult was discarded and the second brood was assessed for developmental abnormalities as above.

Ecdysteroid measurements

Daphnids of various life stages were exposed to sodium nitroprusside (1.0 mg N/L) as described above. For daphnid *ex vivo* embryo exposures, developmental stage 1 eggs were isolated from untreated daphnids as described previously[24]. Entire broods isolated from 2 maternal organisms were randomly assigned to wells of a 12-well tissue culture plate and

treated in media with 1.0 mg N/L sodium nitroprusside, dissolved directly into the media or media only in 2 mL treatment solution/well. Exposure solutions were renewed every 24 hours with fresh solution. Embryos were incubated at 20°C under 16 hour photoperiod and were exposed for 72 hours. To measure ecdysteroid concentrations in embryos following maternal exposure, embryos were removed from the brood chamber of three maternal organisms per treatment and processed for analysis in groups. Daphnid ecdysteroid levels were measured by radioimmunoassay with a standard curve of 20-hydroxyecdysone as described previously[25].

Statistical Analysis

Data generated from the reproductive and developmental toxicity assessments were analyzed using Origin 7.5 software and regression lines fitted using the sigmoidal fit function. The threshold concentration for each compound was derived from the point at which the regression line that defined the concentration-response relationship crossed within one standard deviation for the mean control value. Significant difference in ecdysteroid levels between a single treatment group and respective control was evaluated by Student's *t*-test using JMP software (SAS Institute, Cary, NC). Significant reductions in toxicity from co-exposure to β-carotene were evaluated using Fisher's exact test.

Results

Cellular conversion of NO_x to NO

Drosophila S2 cells were exposed to increasing concentrations of NO_3^- or NO_2^- to determine if these compounds are converted to NO by arthropod cells. NO was generated from NO_x in a substrate concentration-dependent manner (Fig. 1). NO_2^- was also converted to NO in a cell density-dependent manner (Fig. 2). NO_2^- was more efficiently converted to NO relative to the conversion of NO_3^- due likely to its increased cellular uptake or requirement for a single reduction reaction as compared to the dual reduction required of NO_3^-. Results clearly demonstrate that arthropod cells can convert nitrate and nitrite to nitric oxide.

Reproductive and developmental toxicity

Having established that NO_x is susceptible to intracellular conversion to NO, we set out to identify reproductive and developmental toxicity associated with NO, using sodium nitroprusside as the NO donor, and determine whether nitrate and nitrite elicit toxicity that is comparable to that caused by NO. Sodium nitroprusside reduced the number of offspring produced per maternal daphnid in a concentration-dependent manner with a threshold effect concentration of 0.34 mg N/L (Fig. 3A). Sodium nitroprusside also caused the production of abnormally developed offspring (Fig. 4A). Abnormal neonates presented with under-developed second antennae, an un-extended or poorly-extended shell spine, or overall underdeveloped body form. Developmental abnormalities occurred with a threshold

concentration of 0.23 mg N/L over the same approximate sodium nitroprusside concentration range as observed for reproductive toxicity.

Both NO_2^- and NO_3^- elicited reproductive and developmental toxicity that qualitatively mimicked that of sodium nitroprusside. NO_2^- decreased the reproductive capacity of the daphnids (Fig. 3B) and increased the incidence of developmental abnormalities (Fig. 4B) with threshold effect concentrations of 0.64 and 0.33 mg N/L, respectively. Developmental abnormalities were the same as described with sodium nitroprusside. NO_3^- was a significantly less potent in eliciting reproductive and developmental toxicity. NO_3^- reduced the number of offspring produced with a threshold effect concentration of 123 mg N/L (Fig. 3C) and increased the incidence of developmental abnormalities with a threshold effect concentration of 140 mg N/L (Fig. 4C). Consistency in the effects observed between sodium nitroprusside and NO_x supports the premise that reproductive and developmental toxicity of NO_x are due to the generation of NO. As noted above, lower potency of NO_3^- as compared to NO_2^- is likely due to its reduced uptake by the organisms and requirement for more extensive metabolism to generate NO.

In addition to the reproductive and development toxicity that was characteristic of all three NO generators, NO_2^- was also lethal to maternal organisms at concentrations >1.2 mg N/L. This lethality limited the assessment of the concentration-response relationship for

developmental toxicity. This toxicity unique to NO_2 may have been due to methemoglobinemia (see Discussion).

β-carotene protection

The anti-oxidant β-carotene acts as a NO scavenger, preventing free NO from binding other targets and causing toxicity [23]. Maternal daphnids were exposed to sodium nitroprusside or NO_2^- in the presence and absence of β-carotene to determine if developmental toxicity could be attenuated by this scavenger. Exposure of maternal daphnids to 1.0 mg N/L sodium nitroprusside resulted in a 50% incidence of broods containing developmentally abnormal neonates (Fig. 5). This incidence was reduced to 35% upon co-exposure to β-carotene. Similarly, exposure of maternal daphnids to 1.0 mg N/L NO_2^- resulted in a 71% incidence of developmentally abnormal broods which was lowered to 35% with β-carotene co-exposure (Fig. 5). Significant mortality of maternal organisms occurred with exposure to NO_2. This mortality was completely ameliorated by co-treatment with β-carotene.

Ecdysteroid measurements

Various sodium nitroprusside exposure scenarios were executed with embryonic, adult, or juvenile daphnids to determine if developmental and reproductive toxicity of nitric oxide was due to the lowering of ecdysteroid levels (Table 1). Ecdysteroid levels detected by

RIA were not significantly different between untreated (control) and sodium nitroprusside-exposed daphnids following most exposure scenarios tested. Ecdysteroid levels were significantly decreased in embryos following 11-13 days of maternal exposure (Table 1). However, this observation could not be replicated. Therefore, these experiments provided no strong evidence that nitric oxide reduces ecdysteroids levels in daphnids.

Discussion

The objectives of this study were: a) to demonstrate the ability of arthropods to convert NO_x to NO; b) to determine whether NO_x elicit toxicity consistent with that of NO; and c) to determine whether NO elicits reproductive and developmental toxicity by lowering ecdysteroids titers. Results revealed that NO_x are indeed reduced to NO by arthropod cells and that NO_x elicit reproductive and developmental toxicity that is consistent with toxicity elicited by NO. However, no strong evidence was provided to indicate that NO elicits reproductive and developmental toxicity by lowering ecdysteroids titers.

Until recently, NO_3^- was generally accepted to be biologically inert in animals and only converted to NO_2^- within the body by commensal bacterial nitrate reductase [26]. However, enzymatic serial reduction of NO_3^- to NO_2^- to NO was detected in mammalian tissues under normoxic conditions by xanthine oxidoreductase [27]. Therefore, detection of NO production following NO_3^- administration to arthropod cells suggests that similar reductase activity may be present. Preliminary experiments demonstrated that NO_2^--dependent NO

production in these cells is inhibited by potassium cyanide. This finding suggests that mitochondrial cytochrome *c* reductase plays a role in NO_2^- reduction, as reported previously in other cell types [14, 28]. Additional mechanistic evaluation is required however to identify the source of nitrate reductase activity in arthropod cells.

The cellular conversion of NO_x to NO is of great significance for aquatic species living in NO_x-contaminated environments. Nitrates and nitrites are the most ubiquitous and abundant contaminants in freshwater and coastal ecosystems [29]. Nitrogen pollution has been associated with various environmental impacts including the alteration of food webs and loss of biodiversity [30]. These consequences of nitrogen pollution have been largely attributed to eutrophication. However, nitric oxide contributes substantially to the regulation of numerous reproductive functions [31] and the conversion of nitrates and nitrites to nitric oxide may contribute to the adverse consequences of nitrogen pollution.

Sodium nitroprusside elicited developmental toxicity to neonatal daphnids that was ameliorated with the NO scavenger β-carotene. These experiments provide good evidence that nitric oxide was responsible for the developmental toxicity. NO_x elicited developmental toxicity that was consistent with that caused by sodium nitroprusside and this toxicity also was attenuated by β-carotene. These results suggest that NO_2^- is converted to nitric oxide at sufficient levels to disrupt normal development. The developmental abnormalities elicited by NO_x were reminiscent of effects elicited by compounds that interfere with normal

ecdysteroids signaling [25, 32, 33]. In arthropods, ecdysteroids function to regulate molting, reproduction, and embryonic development [34]. Anti-ecdysteroidal compounds may act by reducing ecdysteroids titers, competing antagonistically for the ecdysteroids receptor, or modulating the availability or activity of downstream contributors to the signaling cascade. We viewed binding of NO to the heme groups of cytochrome P450 enzymes involved in ecdysteroid synthesis as a particularly attractive mechanism of anti-ecdysteroidal activity as precedence exists for NO_x reducing steroidogenesis [12]. However, we found no strong evidence that NO decreased ecdysteroids titers. Thus, other mechanisms by which NO may disrupt reproduction and development should be considered.

The ecdysone-inducible nuclear receptor E75 is a heme-binding transcription factor that functions downstream in the ecdysteroids signaling pathway in *Drosophila melanogaster* [35, 36]. E75 negatively regulates the activity of another transcription factor HR3 [37-39]. Nitric oxide is capable of binding to the heme group of E75 which relieves it of its suppressive activity towards HR3 [36]. Thus, pulses of endogenous nitric oxide may serve as an on/off switch to precisely regulate the activity of E75 during critical periods of development. Nitric oxide derived from exogenous NO_x may maintain E75 in its "off" position for extended periods of time resulting in aberrant signaling by HR3. Evaluation of E75 sensitivity to NO warrants further investigation within the context of environmental NO_x exposure and the effect on regulation of the ecdysteroid signaling cascade.

In addition to the anti-ecysteroid phenotype associated with neonates, NO_2^- was lethal to a portion of maternal organisms. This toxicity was unique to NO_2^-, indicating that it was not due to nitric oxide, but was ameliorated by β-carotene. β-carotene also can sequester NO_2^- [40] which suggests that this toxicity may have been due to the parent compound. One plausible explanation for this toxicity is that the higher exposure levels of NO_2^- caused methemoglobinemia in the maternal daphnids. This condition occurs when levels of methemoglobin, defined as an oxidized form of hemoglobin that is unable to bind and transport oxygen, are at levels high enough to interfere with sufficient oxygenation. At the higher concentration, NO_2^- may have asphyxiated the organisms.

NO_2^- was clearly more toxicologically significant than was NO_3^-, due to its reproductive and developmental toxicity at low exposure levels and acute lethality at higher concentrations. NO_2^- is considered to be a short-lived intermediate molecule in the aquatic environment [41]. However, it is readily taken up by aquatic organisms through the Cl^-/HCO_3^- exchanger, an active uptake mechanism [42]. NO_2^- is produced in the environment as the result of ammonium (NH_4^+) oxidation[43], or bacterial nitrate reduction in low-oxygen environments [44]. NO_2^- levels in surface waters are not typically measured. Rather, NO_3^- or total NO_x concentrations are more commonly reported. In multiple samplings along the Neuse River, North Carolina USA, NO_2^- and NO_3^- levels averaged 16 and 84%, respectively, of the total NO_x load in the river [45]. Turner et al. reported total NO_x levels in the Mississippi River, USA as high as 12 mg N/L with average yearly levels being about half that level [46]. Applying the

NO_2^-:NO_3^- ratio derived from the Neuse River data, average to peak levels of NO_2^- in the Mississippi River are expected to be 1-2 mg N/L. Consistent with these expectations, Harris and Smith [47] reported NO_2^- concentrations as high as 1.63 mg N/L in the Powder River Basin tributary of Wyoming, USA. Assuming that daphnids serve as a model for other crustaceans, this concentration of NO_2^- would be likely to cause significant developmental and reproductive alterations in indigenous crustacean populations.

Toxicity of chronic NO_3^- exposure occurs at higher concentrations than levels typically detected in aquatic environments. However, this compound can undergo bacterial reduction in the environment to the much more potent NO_2^-. Often, aquatic systems with elevated nitrogen levels have depleted dissolved oxygen levels as a result of eutrophication [48]. Bacteria are capable of reducing NO_3^- to NO_2^- in low oxygen conditions [49, 50] under which NO_3^- is used as an electron acceptor during respiration. Therefore, NO_3^- may serve as a reservoir for NO_2^- in eutrophic environments.

Numerous examples of unexplained population declines currently exist within aquatic environments [11]. Toxicological and mechanistic information obtained in this study may provide support for the hypothesis that environmental NO_x could potentially play a role in those instances. Further investigation of the mechanism behind the reproductive and developmental toxicity observed in this study will strengthen our ability to explain the effects

seen with environmental NO_x exposures. These results support a movement to improve efficiency and reduce waste/runoff associated with nitrogen use.

Acknowledgements

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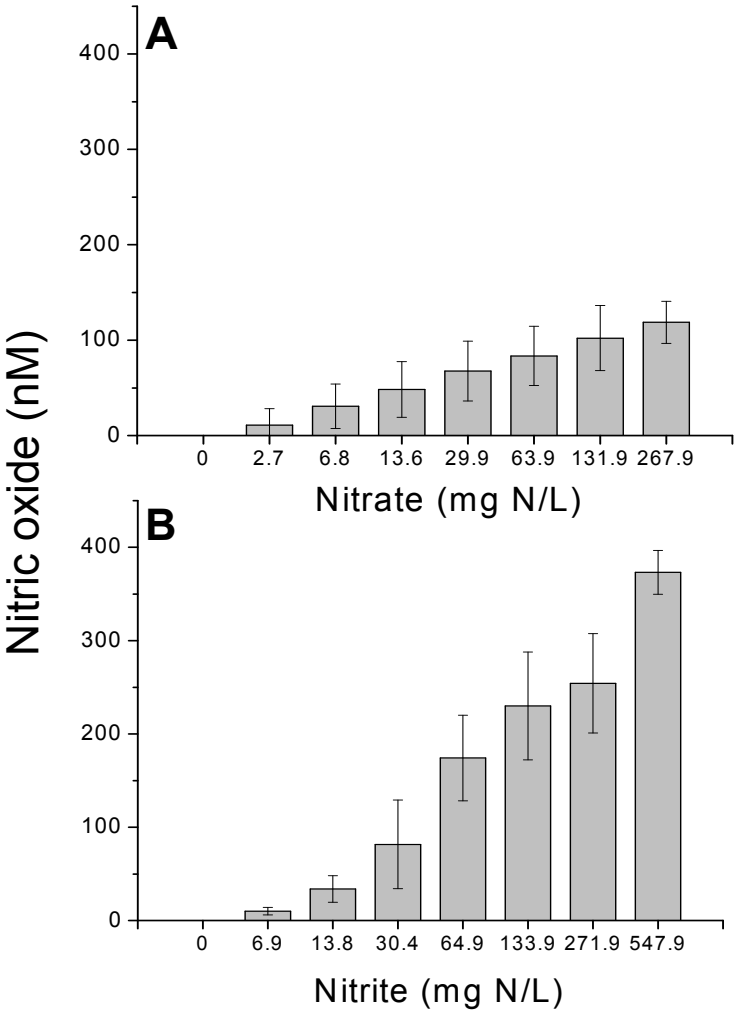


Figure 1. Concentration-dependent conversion of NO_3^- (**A**) or NO_2^- (**B**) to NO by *Drosophila* S2 cells. Each column represents the mean ± SD of 4-7 experiments with different cell batches.

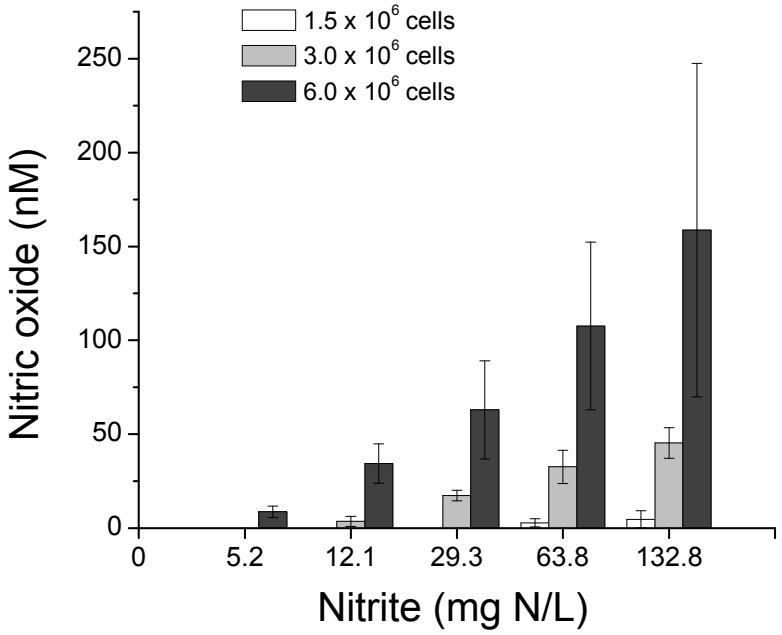


Figure 2. NO_2^--dependent NO production at increasing cell densities. Each bar represents the mean ± SD of 2-3 experiments with different batches of cells.

Figure 3. Total offspring released from daphnids chronically exposed to increasing concentration of (**A**) sodium nitroprusside, (**B**) $NaNO_2$, or (**C**) $NaNO_3$. Each data point represents the total number of offspring released from a single exposed daphnid over the entire exposure period. Mean $\pm$ SD control performance is depicted by the solid line bracketed by dotted lines.

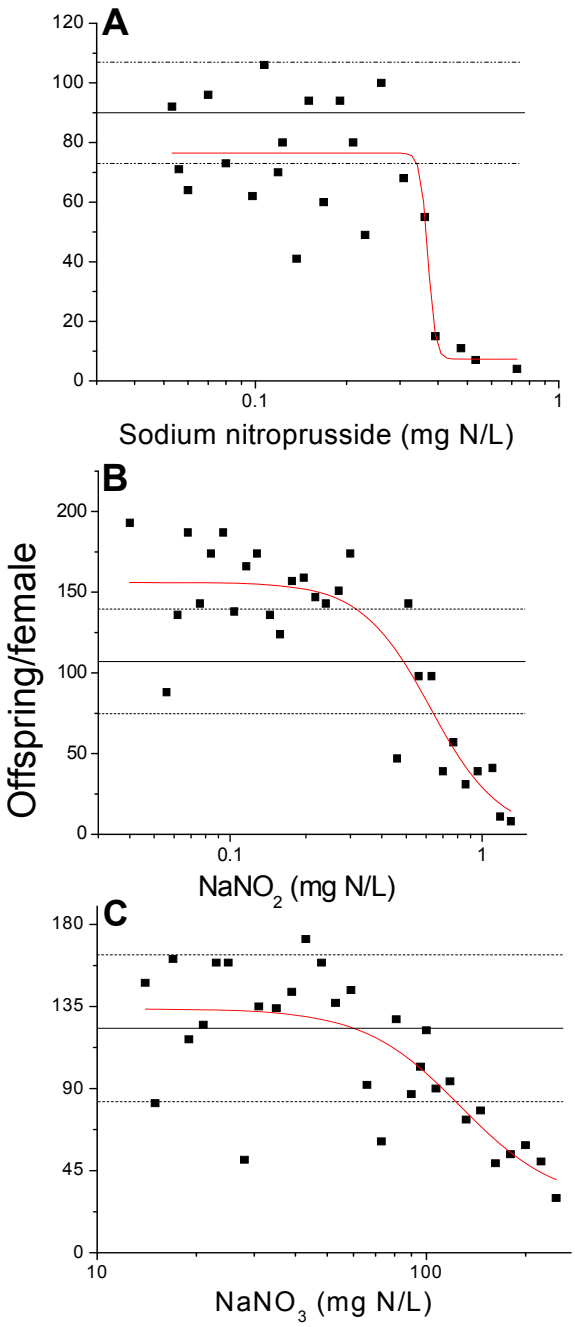
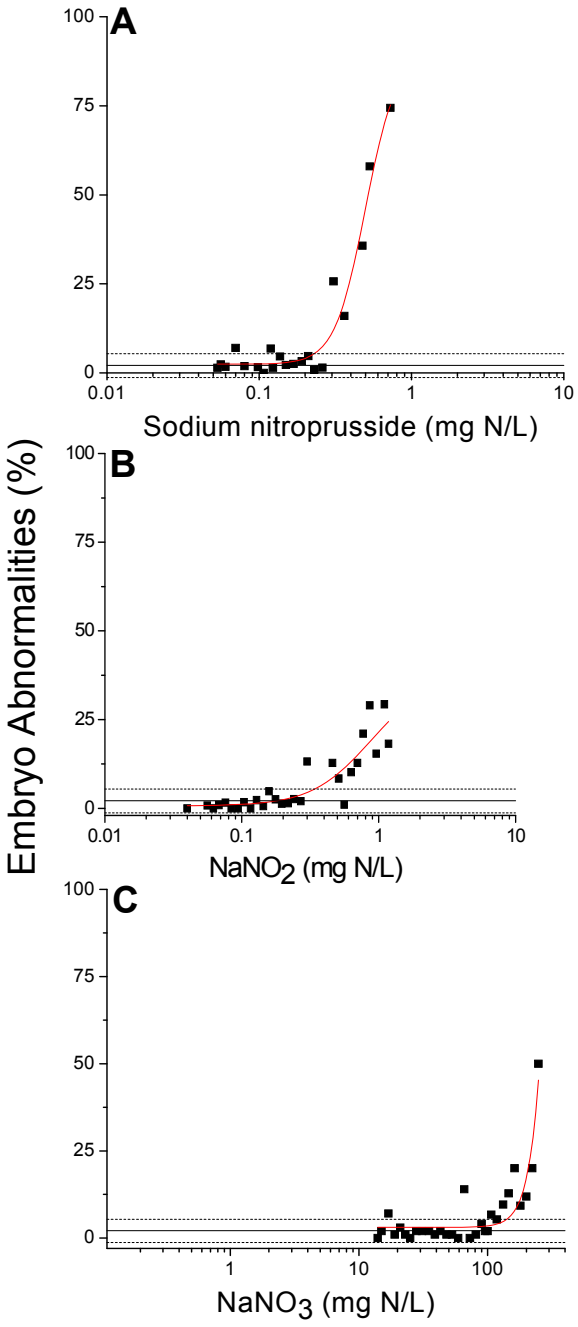

A
120
100
80
60
40
20
0
0.1
1
Sodium nitroprusside (mg N/L)
B
Offspring/female
200
150
100
50
0
0.1
1
NaNO2 (mg N/L)
C
180
135
90
45
0
10
100
NaNO3 (mg N/L)

Figure 4. Frequency of neonatal abnormalities among daphnids exposed to increasing concentrations of (**A**) sodium nitroprusside, (**B**) $NaNO_2$, or (**C**) $NaNO_3$. Each data point represents the percentage of abnormal neonates produced by a single maternal daphnid. Mean $\pm$ SD control performance is depicted by the solid line bracketed by dotted lines.


A
B
C
Embryo Abnormalities (%)
Sodium nitroprusside (mg N/L)
NaNO2 (mg N/L)
NaNO3 (mg N/L)
100
75
50
25
0
0.01
0.1
1
10
100

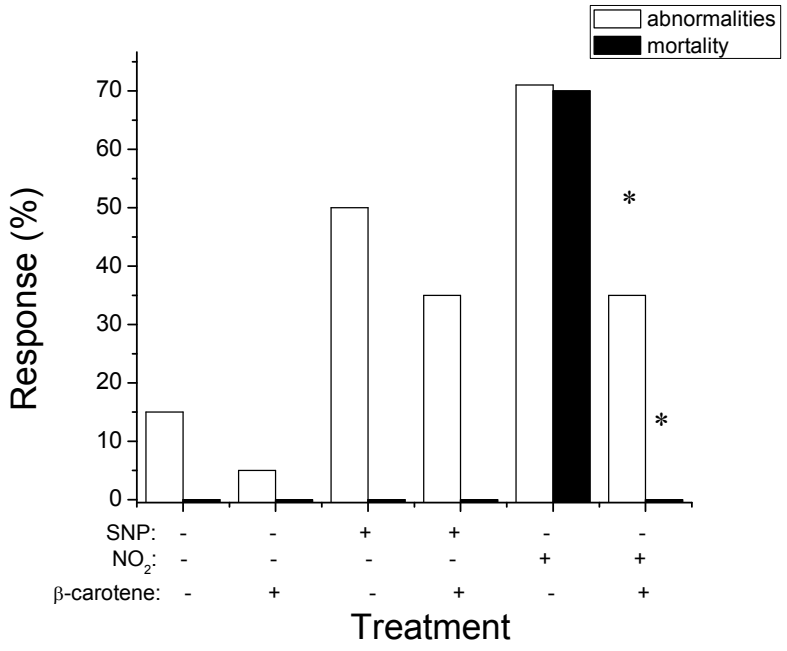


Figure 5. Protection by β-carotene (1.0 mg/L) against developmental abnormalities and mortality caused by sodium nitroprusside (1.0 mg N/L) or $NaNO_2$ (2 mg N/L). Each bar represents the percentage of broods containing abnormally developed neonates (n = 20 broods from 20 maternally organisms; n < 20 if maternal lethality was observed). Significant ($p < 0.05$) protection by β-carotene was evaluated by Fisher's exact test. Results presented are representative of the results of six different experiments.

Table 1. Ecdysteroid levels in daphnids exposed to 1.0 mg N/L sodium nitroprusside (SNP). Ecdysteroid (pg/individual) values represent the mean ± SD (n= 3-5 treatment groups, with numbers of individuals per group as indicated).

Age at Start of Exposure	Life stage at analysis	Exposure duration (days)	Exposure group	Individuals per group	Ecdysteroid (pg/individual)
isolated embryos	Exposed embryos (*ex vivo*)	3	Control SNP	39-55 44-56	1.6±0.3 1.5±0.2
8 days	embryos (isolated from exposed daphnids)	8	Control SNP	65-77 53-78	1.9±2.3 1.1±1.0
<24 hours	embryos (isolated from exposed daphnids)	11-13	Control SNP	30-35 7-29	2.9±0.1 0.9±0.1*
<24 hours	embryos (isolated from exposed daphnids)	11-13	Control SNP	66-68 65-75	10.1±12.9 40.0±34.5
3-4 days	Exposed daphnids	3	Control SNP	10 10	106.6±4.7 131.4±14.7
<24 hours	Exposed daphnids	6	Control SNP	10 10	125.3±14.4 129.4±10.9
<24 hours	Exposed daphnids	9	Control SNP	7-10 7-10	138.1±33.5 115.1±22.8

* Denotes a significant difference from the respective control at $P \leq 0.05$ (Student's *t*-test).

CHAPTER TWO: EXPRESSION AND ECDYSTEROID RESPONSIVENESS OF NUCLEAR RECEPTORS HR3 AND E75 IN THE CRUSTACEAN *DAPHNIA MAGNA*

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Abstract

Ecdysteroids initiate signaling along multiple pathways that regulate various aspects of development, maturation, and reproduction in arthropods. Signaling often involves the induction of downstream transcription factors that either positively or negatively regulate aspects of the pathway. We tested the hypothesis that crustaceans express the nuclear receptors HR3 (ortholog to vertebrate ROR) and E75 (ortholog to vertebrate rev-erb) in response to ecdysteroid signaling. HR3 and E75 cDNAs were cloned from the crustacean *Daphnia magna*. The DNA-binding domain and ligand-binding domain of the daphnid HR3 was 95% and 61% identical to those of *Drosophila melanogaster*. The DNA-binding domain and ligand-binding domain of the daphnid E75 were 100% and 71% identical to those of *D. melanogaster*. Both receptors exhibited structural characteristics of binding to DNA as a monomer. The expression of these receptor mRNAs was evaluated through the adult molt cycle and during embryo development. E75 levels were relatively constant throughout the adult molt cycle and through embryo development. HR3 levels were comparable to those of E75 during the initial phases of the adult molt cycle but were elevated ~30-fold at a time in the cycle co-incident with the pre-molt surge in ecdysteroid levels. HR3 mRNA levels in embryos also varied co-incident with ecdysteroids levels. To substantiate a role of ecdysteroids in the expression of HR3, daphnids were continuously exposed to 20-hydroxyecdysone and changes in gene expression were measured. HR3 levels were significantly induced by 20-hydroxyecdysone; while E75 levels were minimally affected.

These results are consistent with the premise that transcription of HR3 is regulated by ecdysteroids in the crustacean *Daphnia magna* and that HR3 likely serves as a mediator of ecdysteroid regulatory action in crustaceans. The marginal induction of E75 by 20-hydroxyecdysone may represent limited, tissue or cell-type-specific induction of this transcription factor.

Introduction

The nuclear receptors comprise an ancient family of transcription factors whose origins predate the emergence of the Cnidaria (Laudet et al., 1992; Thornton, 2003; Bertrand et al., 2004). Seven nuclear receptor subfamilies are currently recognized (0 through VI) and members of all subfamilies have been identified in both deuterostomes and protostomes. Among the fully sequenced insect genomes, the mosquitoes (*Aedes aegypti, Anopheles gambiae*) possess 20 nuclear receptor genes (Holt et al., 2002; Cruz et al., 2009), the fruitfly (*Drosophila melanogaster*) possesses 21 nuclear receptor genes (Adams et al., 2000), and the honeybee (*Apis mellifera*) possesses 22 nuclear receptor genes (Velarde et al., 2006). Recently, the water flea (*Daphnia pulex)* genome was sequenced (http://wFleaBase.org) representing the first fully sequenced crustacean genome. We identified 25 nuclear receptor genes in the *D. pulex* genome (Thomson et al., 2009).

Among the approximately two dozen nuclear receptors of arthropods, only one, the ecdysteroid receptor (EcR), has been clearly established to be ligand-activated. Ecdysteroids (e.g., 20-hydroxyecdysone, ponasterone A) bind to and activate the EcR which in heterodimeric association with the nuclear receptor RXR/USP activates transcription of responsive genes (Riddiford et al., 2000). We recently demonstrated that the daphnid RXR is activated by the ligand tributyltin; however, a physiologically relevant ligand to this receptor was not identified (Wang and LeBlanc, 2009). With the exception of EcR and RXR, little is

known of the function of the nuclear receptors of crustaceans, despite the tremendous economic value of these organisms.

In insects, ecdysteroid signaling results in the induction of downstream nuclear receptors which expand the breadth of gene networks regulated by the hormone (Thummel, 1995; Thummel, 1996). These downstream receptors are recognized as orphans and may function as ligand-independent transcription factors. Two such downstream receptors HR3 (NR1F) and E75 (NR1D) tend to function reciprocally in mediating ecdysteroid-initiated responses. Both genes are induced in response to ecdysteroids in insects (Palli et al., 1995; Jindra and Riddiford, 1996); however, E75 serves as a repressor of HR3-mediated gene regulation (Swevers et al., 2002). Repression occurs both through heterodimerization of the two receptors and through competitive binding at the response element (Swevers et al., 2002). Both HR3 and E75 have critical roles in oogenesis and embryo development (Carney et al., 1997; Bialecki et al., 2002).

A portion of the HR3 cDNA was reportedly cloned from the American lobster (*Homarus americanus*) (El Haj et al., 1997) and E75 cDNA has been cloned from the tropical land crab (*Gecarcinus lateralis*) (Kim et al., 2005). Both nuclear receptor genes were annotated from the water flea (*D. pulex*) genome (Thomson et al., 2009). Their presence in crustaceans suggests that, as in insects, these nuclear receptors may have important roles in crustacean ecdysteroid signaling. The goals of the present study were: (1)

to clone HR3 and E75 from the same crustacean species *Daphnia magna*, (2) evaluate the expression of these receptors during development and growth, and (3) establish the responsiveness of these receptors to ecdysteroids.

Elucidation of the factors that transduce ecdysteroid signals in crustaceans could have significant impacts on identifying means of enhancing and optimizing ecdysteroid-regulated processes related to growth, development and reproduction in aquaculture applications. Ecdysteroid signal transduction is altered by chemicals that bind to either the EcR or the RXR as agonists or antagonists (Mikitani, 1996; Mu and LeBlanc, 2002, 2004a, b; Mu et al., 2005; Hopkins et al., 2008; Li et al., 2008; Wang and LeBlanc, 2009). While considered orphans, downstream nuclear receptor transcription factors, such as HR3 and E75, possess ligand-binding sites and could thus similarly serve as targets for disruption by environmental chemicals.

Materials and Methods

Water fleas

Water fleas (*D. magna*) used in this study were obtained from cultures maintained in our laboratory for over 17 years. Daphnids were reared in media reconstituted from deionized water as described previously (Wang et al., 2007). Cultured daphnids were maintained at a density of 40 adults per 800 ml of media and were fed twice daily with 2.0 ml ($1.4x10^8$ cells) of a suspension of unicellular green algae, *Pseudokirchneriella subcapitata*

and 1.0 ml (~4 mg dry weight) of Tetrafin™ fish food suspension (Tetra Holding Inc., Blacksburg, VA, USA) per 800 ml of media. All daphnids were housed in incubators set to 20°C with a 16/8 h light/dark cycle. Daphnids used in the experiments reproduced exclusively by parthenogenesis and were all female.

Full-length E75 cDNA derivation

Adult female daphnids were homogenized with a dounce homogenizer. The SV Total RNA Isolation System (Promega, Madison, WI) was used to isolate RNA from the homogenate. RNA yield was determined by absorbance at 260 nm and its purity was measured by the 260/280 nm absorbance ratio with a Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Montchanin, DE). RNA integrity was verified by formaldehyde agarose gel electrophoresis. RNA was reverse transcribed to cDNA with oligo dT primers using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI).

A 210 bp fragment was obtained by degenerate-based PCR. Primers were designed using the COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOP) program with a blocks format sequence alignment (Rose et al, 1998) of E75 from other species (GenBank accession numbers: AAY89587, AF092946, XP_971362, http://www.ncbi.nlm.nih.gov/). The codon usage table was set for *Artemia fransicana* as the closest related species. The primer sequences were: 5'-TGGTACTACTGTTCTTTGTCGAGTTTGYGGNGAYAA-3' and 5'-

ACAGCAATACACTTTTTAAGTCGACARTAYTGRCA-3'. The PCR product was amplified from 200 ng cDNA using 10 μL PCR Master Mix (Promega) and 0.4 μM of each primer. The first round of PCR cycling followed standard 3-step PCR (denaturation at 95°C for 1 min., annealing at 52°C for 45 sec, and extension at 72°C for 1 min) for 40 cycles. This PCR product was then used as template in a second round of PCR, performed using an annealing temperature of 56°C. The fragment was cloned into the vector pCR®4-TOPO using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Primer extension sequencing was performed by SeqWright Inc. (Houston, TX).

Rapid amplification of cDNA ends (RACE) was used to obtain full-length cDNA. 5'-RACE was performed using the SMART™ RACE kit (Clonetech, Mountain View, CA) and 3'-RACE was conducted using the GeneRacer™ kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Briefly, gene specific primer (GSP) sequences were: (5'-RACE): 5'-GGACGACCGACTGCATGAGCTGAAC -3', (5' nested RACE): 5'-CCAAATCGCACAGCATCACGACTCA -3', (3'-RACE): 5'-CTGCGAAGGTTGCAAGGGTTTCTTC -3', and (3' nested RACE): 5'-CAGTGCTCCATTCTTCGCATCAACC -3'. For 5'-RACE, the first strand cDNA synthesis was processed as per the recommendation of the supplier. The cDNA was subsequently used (30 ng) as template for PCR with 21 μL SuperMix High Fidelity reaction solution (Invitrogen), 0.2 μM Universal primer (SMART RACE kit), 0.2 μM GSP in a total

volume of 25 μL. The following touchdown PCR cycling protocol was used: denature at 94°C for 30 sec, anneal at 72°C for 3 min for the first 5 cycles, then drop the annealing temperature to 70°C and add an extension step of 72°C for 3 min for the next 5 cycles, and finally drop annealing temperature to 68°C for the remaining 25 cycles. The product from the first round of PCR was used to perform nested PCR, as above, using the 5' nested race primer at 0.2 μM.

For 3'-RACE, mRNA was reverse transcribed using the GeneRacer™ Oligo dT primer. PCR was performed with 22 μL SuperMix High Fidelity reaction solution (Invitrogen), 0.4 μM primers, and 100 ng template cDNA for a total volume of 25 μL, under the following conditions: denature at 94°C for 30 sec, anneal at 64°C and extend at 72°C for 3 min, for a total of 40 cycles. The template from the first round of PCR was re-amplified using the 3' nested RACE primer in a second round of PCR. Products of both 5' and 3' nested RACE were purified from a 1.2% agarose gel using Wizard® SV Gel and PCR Clean-Up System (Promega), and cloned and sequenced as above. The E75 amino acid sequence was determined and the molecular weight of the protein was calculated using ExPASy software (http://www.expasy.org/). Prediction of the DBD and LBD conserved domain locations within the sequence were made using NCBI protein-BLAST (Altschul et al., 1997).

Open-reading frame (ORF) HR3 cDNA derivation

A 148 bp fragment with identity to HR3 of other species (GenBank accession numbers: AAF36970, P31396, NP_001037012) was obtained by CODEHOP-PCR (as above). The hybrid consensus-degenerate primer sequences were as follows: 5'-TGATCGAGTTAATCGAAATCGATGYCARTAYTG -3' and 5'-GAGCAGCATCAGATTGAGCTCKCATYTGNGC -3'. One round of standard 3-step PCR was performed, using an annealing temperature of 52°C.

3'-RACE was performed as described for E75, using the GSP primer 5'-AGTCATCACCTGCGAGGGC -3'. Nested PCR was unnecessary. The RACE PCR product was cloned and sequenced. The remainder of the HR3 gene sequence was obtained through PCR using primers designed at the 5' and 3' ends of the predicted HR3 sequence derived from the *D. pulex* genome (Thompson et al 2009). The primers 5'-GGTACCGCCATGGAAGCTCCGGCCGTTCCG -3' and 5'-CTCGAGATCCACGGAAAAGAGTTCCTTGTG -3' were used with cDNA derived from adult female daphnid RNA and reagents/cycling parameters as described above for standard 3-step PCR. Conserved nuclear receptor domains were predicted as above.

The products were purified, cloned and sequenced. The amino acids of the HR3 protein were deduced based on the total nucleotide sequence of the ORF and the molecular

weight of the protein was calculated based on the amino acid constituency using ExPASy software.

Profiling gene expression over a molt cycle

Three hundred daphnids (<24 h old) were reared for 5 days (100 daphnids/1 L media containing food, micronutrients, and salts as described for culture media). On the fifth day, daphnids were divided among six 1-L beakers (50 daphnids/beaker) to accommodate the increased size of the organisms. On the tenth day, daphnids were individually distributed among 50 ml beakers containing 40 ml media. The next morning, any daphnid that had molted during the past day (i.e., an exoskeleton present in the beaker) was discarded and remaining animals were monitored every two hours for the presence of an exoskeleton in the beaker. Upon evidence of molting, the daphnid was designated as being at time 0 in its molt cycle and the animal was targeted for sampling at either time 0, 12, 24, 36, 48, and 60 hours. This process continued until ~30 animals were targeted for sampling at each time point. At sampling, animals were placed in RNALater™ (Qiagen, Valencia, CA, USA) solution in groups of 8-10 individuals (three replicates consisting of 8-10 animals per replicate for each time point) and stored at 4^{o}C until processed. At processing, RNALater^{TM} solution was removed and replaced with 175 µL of Promega^{TM} lysis buffer (Madison, WI, USA). Samples were homogenized with a dounce homogenizer and RNA was isolated, quantified, assessed for purity and integrity, and reverse transcribed to cDNA as described above. cDNA was quantified by absorbance as described above.

Profiling gene expression during embryo development

Embryos of developmental stages 1 through 6 (Kast-Hutcheson et al., 2001) were excised from the brood chamber of maternal organisms and combined to yield approximately 350 embryos of each developmental stage. These embryo pools represented the combined broods of 15-20 maternal organisms. RNA was isolated and cDNA prepared as described above.

Profiling gene expression in response to 20-hydroxyecdysone

Animals were staged for position in the molt cycle as described above. At 0 h, daphnids were individually transferred to either control media or media containing 1.0 μM 20-hydroxyecdysone. 20-Hydroxyecdysone was delivered to the media dissolved in absolute ethanol and both controls and 20-hydroxyecdsone-containing solution contained 0.001% ethanol. Animals were sampled at designed time intervals and processed for RNA isolation and cDNA preparation as described above.

Real-time RT-PCR

Relative targeted mRNA levels were assessed during the time course experiments using real-time RT-PCR. Primers were designed based upon the cDNA sequences derived in the present study for HR3 and E75. Primers used to measure EcR-A mRNA levels were based upon the previously published sequence for this cDNA (Wang and LeBlanc, 2009).

All primers were designed using ABI Primer Express software (Applied Biosystems, Foster City, CA). Primer sequences were as follows: E75 F: 5'-TCCGGAGAAGTATTCAACAAAAGA-3', E75 R: 5'-TGCGAAGAATGGAGCACTGT -3', HR3 F: F 5'- AGTCATCACCTGCGAGGGC-3', HR3 R: R 5'-GAACTTTGCGACCGCCG -3', EcR-A F: F 5'- CAGCGCTATGGAAGAATGGT -3', EcR-A R: R 5'-TCATCGACATGGACGAACTG -3'. Actin (accession number AJ292554) cDNA was also amplified and used in the normalization of transcripts as described previously (David et al., 2003; Zeis et al., 2003; Rider et al., 2005; Wang and LeBlanc, 2009). Amplicons generated ranged from 51 to 72 base pairs. Quantitative real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using default parameters. Amplification mixtures consisted of 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems), 300 nM primers, 250 ng template cDNA in a total volume of 25 µl. Primer concentrations were optimized following the manufacturer's recommendations. The reaction mixtures were first kept at 95^{o}C for 10 minutes, followed by 40 cycles with each cycle consisting of a temperature of 95°C for 15 sec followed by 60^{o}C for 1 min. After the PCR reactions, the melting temperature of PCR product was determined using the dissociation protocol provided by the instrument manufacturer. A single melting peak was detected for all samples indicating no amplification of non-target DNA. Furthermore, only a single amplification product was detected following electrophoresis in a 2% agarose gel and staining with ethidium bromide. The comparative C_T method ($2^{-\Delta\Delta C_T}$) was used to assess the relative levels of EcR-A, HR3 or E75 mRNA normalized to mRNA

levels of actin measured with the same cDNA sample. Results in Figs. 5 and 6 represent absolute abundance of gene products. Results in Fig. 7 are presented relative to mRNA levels measured at time 0. The time 0 levels were arbitrarily set at 1.0. Validation experiments, as described by the instrument manufacturer, confirmed that the efficiencies of the target and endogenous control (actin) amplifications were approximately equal (Applied Biosystems).

Statistical analysis

Significant differences in gene expression over a molting cycle and were determined for each gene compared with the respective "time 0" by ANOVA and Dunnett's test using JMP software (SAS Institue, Cary, NC). Significant differences in gene expression at each embryo stage compared with the respective stage 1 also were evaluated using ANOVA and Dunnett's test. Significant induction of genes from 20-hydroxyecdysone exposure was evaluated using Student's *t*-test.

Results

HR3 cDNA

Using RACE PCR, the full length open-reading frame cDNA for *D. magna* HR3 was obtained (GenBank accession number FJ755466) (Fig. 1). The cDNA was 1866 nucleotides in length and coded for a protein having a molecular weight of 67,935. The *D. magna* HR3 protein had the typical domain structure of the nuclear receptors (domains A through E), but lacked the F-domain associated with some nuclear receptors. The protein possessed a highly

conserved DNA-binding C domain with 95% and 80% identity to the *D. melanogaster* and *Homo sapiens* orthologs, respectively (Fig. 2). Adjacent to the C domain was a highly conserved 32 amino acid C-terminal extension with 97% and 62% identity to the *D. melanogaster* and *H. sapiens* orthologs, respectively. This extension of the DNA-binding domain has been implicated in monomeric DNA binding in other nuclear receptors (Wilson et al., 1993; Gearhart et al., 2003). The ligand-binding E-domain of the *D. magna* HR3 was 61% and 35% identical to the *D. melanogaster* and *H. sapiens* orthologs, respectively. These results indicate that daphnids, and perhaps crustaceans in general, express the nuclear receptor HR3. The receptor possessed a high degree of similarity to HR3 in *Drosophila* and likely binds DNA as a monomer to regulate gene transcription.

E75 cDNA

The nuclear receptor E75 also was successfully cloned from *D. magna* (GenBank accession number EF369510.1). This cDNA was considerably larger than that of HR3 and consisted of an open-reading frame of 2,826 nucleotides that coded for a protein having a molecular weight of 102,407 (Fig. 3). Daphnid E75 possessed a highly conserved DNA-binding C domain that was 100%, 100%, and 81% identical to those of orthologous receptor proteins in the tropical land crab *G. lateralis,* the fruitfly *D. melanogaster,* and the human *H. sapiens*, respectively (Fig. 4). The C domain possessed two zinc finger domains and a highly conserved C-terminal extension suggesting that unlike some E75 forms (Segraves and Hogness, 1990; Jindra et al., 2005), the cloned daphnid E75 possesses DNA-binding

characteristics. The hinge region (domain D) of the daphnid E75 was comparable in length to those of *G. lateralis* and *D. melanogaster*, but was considerably shorter than that of the human ortholog rev-erb-α The ligand-binding domain for daphnid E75 was 71%, 52%, and 42% identical to domain E of E75 from *G. lateralis*, *D. melanogaster,* and *H. sapiens*, respectively. The daphnid E75 ligand-binding domain contained all of the key histidine and cysteine residues that have been shown to function in the binding of a heme moiety (Reinking et al., 2005; de Rosny et al., 2006). In contrast to daphnid HR3 which possessed no F-domain, daphnid E75 possesses an extensive F-domain that was largely responsible for the increased molecular mass of the receptor as compared to HR3 (Fig. 4). *G. lateralis* and *D. melanogaster* also possess extended F-domains, though the degree of similarity among the F-domains among the three species was low. The *H. sapiens* ortholog rev-erb-α possesses no F-domain.

Receptor expression

Experiments were performed to determine whether the HR3 and E75 receptor genes are expressed in daphnids and whether levels of expression vary over the molt cycle of the organism. mRNAs from both receptor genes were detected throughout the molt cycle. HR3 and E75 mRNA levels were comparable early in the molt cycle. HR3 mRNA levels increased over time with an ~30-fold elevation in mRNA levels 48-hours into the molt cycle. This increase in HR3 levels corresponded to the temporal increase in ecdysteroid levels during the cycle (Martin-Creuzburg et al., 2007). Levels decreased significantly following

this peak (Fig. 5). In contrast, E75 mRNA levels were relatively constant through the molt cycle (Fig. 5).

Previous studies have shown that ecdysteroid levels are greatest early in embryo development (Mu and LeBlanc, 2004b). Daphnid embryos were evaluated at six stages of development (as described by (Kast-Hutcheson et al., 2001)) for relative levels of HR3 and E75 mRNA. HR3 mRNA levels were greatest early in embryo development and significantly declined to approximately 30% of maximum levels by stage 4 (Fig. 6). E75 mRNA levels were appreciably lower than those of HR3 throughout embryo development. E75 levels also declined significantly by stage 5 of embryo development (Fig. 6). Thus, both HR3 and E75 levels were co-elevated when ecdysteroid levels are reportedly highest in embryos, though expression of HR3 is appreciably greater.

The apparent coordinated expression of HR3 with ecdysteroids levels during both the molt cycle and during embryo development prompted the evaluation of changes in HR3 and E75 levels in response to exposure to exogenous 20-hydroxyecdysone. The ecdysteroid receptor EcR-A also was evaluated as a positive control for 20-hydroxyecdysone exposure. As described previously with insect EcR (Roesijadi et al., 2007), EcR-A mRNA levels increased rapidly with exposure to 20-hydroxyecdysone (Fig. 7A). After 24 h exposure, EcR-A mRNA levels decreased and, by 48 h, approached levels measured at time 0. HR3 mRNA levels also increased in response to 20-hydroxyecdysone exposure (Fig. 7B);

however, elevated mRNA levels were evident later than was observed for EcR-A (12 h versus 6 h). Untreated (control) daphnids also experienced an increase in HR3 mRNA levels (presumably due to increases in endogenous ecdysteroid levels associated with molting); however, induction occurred sooner and was greater among 20-hydroxyecdysone exposed organisms. E75 mRNA levels were slightly elevated among 20-hydroxyecdysone-treated daphnids by 48 h following exposure (Fig. 7C). These results demonstrate that the daphnid HR3 gene is ecdysteroid activated resulting in a significant elevation in HR3 levels just prior to molting. In contrast, E75 levels are relatively unresponsive to 20-hydroxyecdysone.

Discussion

20-Hydroxyecdysone mediates a cascade of gene regulatory events leading to the control of various aspects of development, growth, and reproduction. In insects, trans-regulatory elements along the 20-hydroxyecdysone signaling cascade are often characterized as early, early-late, and late genes. Early genes are activated in direct response to the 20-hydroxyecdysone/EcR complex; whereas, early-late and late genes are activated in response to protein products produced earlier in the cascade. HR3 and E75 are two regulatory proteins that contribute to ecdysteroid signaling cascades in insects. HR3 is recognized as an early-late gene and E75 as an early gene in the ecdysteroid signaling cascade (Segraves and Hogness, 1990; Horner et al., 1995; White et al., 1997). HR3 binds to the RORE motif of responsive genes to function as a constitutive activator to gene transcription (Swevers et al.,

2002; Reinking et al., 2005). In contrast, E75 is viewed as a negative regulator of HR3 mediated transcription (Swevers et al., 2002; Reinking et al., 2005). E75 has been shown to suppress the action of HR3 by either binding to HR3 or by competing with HR3 for DNA binding (White et al., 1997; Swevers et al., 2002; Reinking et al., 2005). Nitric oxide has been shown to be a ligand for E75 in *Drosophila* and ligand binding to E75 relieves it of its suppressive action towards HR3 (Reinking et al., 2005).

Despite the rather extensive characterization of the actions of HR3 and E75 in insect ecdysteroid signaling, little is known of the role of these proteins in crustaceans. A cDNA identified as HR3 was partially cloned from the lobster *H. americanus* (El Haj et al., 1997). This mRNA was expressed in muscle, epidermis, and eye stalk and was shown to be ecdysteroid-inducible in muscle. E75 was cloned from the shrimp *Metapenaeus ensis* (Chan, 1998) and the tropical land crab *G. lateralis* (Kim et al., 2005). E75 mRNA was measured in all tissues examined from both species. Whether E75 is ecdysteroid-inducible in decapods crustaceans, as in insects, was not determined in these studies. These efforts demonstrate that HR3 and E75 are expressed in decapods crustaceans but provide little information on their role in crustacean ecdysteroid signaling.

We recently identified genes for HR3 and E75 in the fully sequenced genome of *D. pulex* (Thomson et al., 2009). Presently, we show that HR3 is differentially expressed during the molt cycle of the related species *D. magna* and that this differential expression is due to

the elevated HR3 mRNA levels in response to ecdysteroids. Thus, the expression and hormonal responsiveness of daphnid HR3 is consistent with its role as a down-stream mediator of ecdysteroid signaling. The DNA-binding domain of the daphnid HR3 is 95% and 80% identical to the DNA-binding domains of *Drosophila* HR3 and human ROR. These latter receptors transactivate gene expression through recognition of the half-site: AGGTCA (Horner et al., 1995). The high degree of similarity between the daphnid HR3 and these orthologs suggests that the daphnid receptor also binds to this response element to activate gene expression. The DNA-binding domain is flanked by a highly conserved C-terminal extension that provides stability, particularly to monomeric receptors, to the binding interaction between receptor protein and its cognate DNA binding site (Wilson et al., 1993; Peters and Khan, 1999). Thus like its orthologs, the daphnid HR3 likely transactivates gene expression as a monomeric transcription factor.

The daphnid E75 cDNA also shares many common structural features with its orthologs. The daphnid E75 DNA-binding domain possesses two complete zinc finger motifs which implicates it as a DNA-binding protein and differentiates it from *Drosophila and Galleria* E75B which lack one zinc finger (Segraves and Hogness, 1990; Jindra et al., 2005). The C-terminal extension flanking the DNA-binding domain indicates that, like HR3, daphnid E75 can bind DNA response elements as a monomer. The 100% identity of the daphnid E75 DNA-binding domain with that of Drosophila E75A indicates that, like *Drosophila* E75A and HR3, daphnid E75 is capable of binding the AGGTCA half-site.

Studies with *Drosophila* receptors indicate that heme can bind to the ligand-binding domain of the E75 receptor and that nitric oxide and carbon monoxide are capable of modifying the function of E75 by binding to the heme moiety (Reinking et al., 2005). The daphnid E75 ligand-binding domain contains all of the cysteine and histidine residues that are critical to heme binding (Reinking et al., 2005; de Rosny et al., 2006). Thus, the daphnid E75 is also likely to be a heme binding protein and may be regulated by nitric oxide and carbon monoxide.

Two mechanisms have been identified in insects by which E75 may suppress the transcriptional activity of HR3. E75 has been shown to bind and competitively displace HR3 from its DNA binding site (Swevers et al., 2002). HR3 and E75 have also been shown to form a heterodimeric complex (Reinking et al., 2005). This dimer is capable of binding to the DNA binding site via HR3-DNA interaction (White et al., 1997). This complex suppresses gene activation in a manner that is dependent upon the E75 F-domain (Swevers et al., 2002). The function of the F-domain on some nuclear receptors is equivocal; however, evidence indicates that this domain serves to modulate the interaction of co-activators with the E-domain (Peters and Khan, 1999; Sladek et al., 1999). The binding of nitric oxide to the heme moiety lodged within the ligand-binding pocket of E75 apparently prevents association of E75 with HR3 resulting in the restoration of HR3 transcriptional activity (Reinking et al., 2005). The structure of the daphnid E75 indicates that it has DNA binding capability, heme

binding capability, and suppressive activity associated with an extended F-domain and may thus regulate the action of HR3 through both mechanisms.

HR3 mRNA levels were significantly induced during the molt cycle of the organisms with an ~30-fold elevation in mRNA levels measured 48 hours into the molt cycle. 20-Hydroxyecdysone levels reach their pre-molt apex at ~44 hours (Martin-Creuzburg et al., 2007) suggesting that the increase in HR3 was due to elevated 20-hydroxyecdysone levels. Embryonic levels of HR3 mRNA were greatest early in embryo development which also corresponds to the time of greatest ecdysteroid levels (Mu and LeBlanc, 2004b). Direct exposure of daphnids to exogenous 20-hydroxyecdysone confirmed that the pre-molt and early embryonic increases in HR3 levels were due to 20-hydroxyecdysone. We also observed that the ecdysteroid receptor (EcR-A) mRNA levels were significantly elevated by 20-hydroxyecdysone. However, while significant induction of EcR-A mRNA levels occurred within 6 hours of 20-hydroxyecdysone exposure, increased HR3 mRNA levels were evident beginning at 12 hours of exposure. These temporal differences in expression of these genes suggests that activation of the HR3 gene occurs subsequent to the immediate early gene responses to ecdysteroid/EcR/RXR signaling such as the induction of EcR-A. Interestingly, EcR-A was not induced over the molt cycle of the daphnids (Fig. 7A), which is similar to previous evaluations of EcR-A in *D. magna* (Kato et al., 2007). This lack of responsiveness to increased endogenous ecdysteroid levels suggests that the sensitivity of EcR-A to the pre-

molt increase in endogenous ecdysteroid levels may be attenuated by other regulatory factors.

In contrast to HR3, E75 mRNA levels were relatively constant with little responsiveness to 20-hydroxyecdysone. HR3 mRNA levels were in appreciable excess, relative to E75, early in embryonic development and immediately prior to molting. However, at other times HR3 and E75 mRNA levels were rather comparable. These assessments of relative abundance suggest that E75 may negatively regulate HR3 activity during some periods of development and growth, but may be sufficiently low to be permissive of HR3 activity during other periods.

In conclusion, the crustacean *D. magna* expresses the nuclear receptors HR3 and E75 in a fashion that implicates these transcription factors in crustacean ecdysteroid signaling. The receptor proteins show a high degree of structural similarity with those of *Drosophila* suggesting that, as in *Drosophila* and other insects, these receptors function coordinately to both positively and negatively regulate gene transcription.

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Figure Legends

Fig. 1. Nucleotide (lower case) and deduced amino acid (upper case) sequences of the *D. magna* HR3 open-reading frame cDNA. Shaded regions correspond to the location of primers used for real-time RT-PCR.

Fig. 2. Comparison of the *D. magna* HR3 amino acid sequence to those of *Drosophila melanogaster* (DmHR3, ABN49269.2) and *Homo sapiens* (ROR, NP_599022). GenBank accession number follows gene name in parentheses. The putative DNA-binding C domain and ligand-binding E-domain are underlined in the *D. magna* sequence. Shaded areas denote amino acid identity with *D. magna*.

Fig. 3. Nucleotide (lower case) and deduced amino acid (upper case) sequences of the *D. magna* E75 cDNA. Shaded regions correspond to the location of primers used for real-time RT-PCR.

Fig. 4. Comparison of the *D. magna* E75 amino acid sequence to those of the tropical land crab *Gecarcinus lateralis* (AAY89587.2), *Drosophila melanogaster* (E75A, AAF49282) and *Homo sapiens* (rev-erb α, CAB53540). GenBank accession number follows gene name in parentheses. The putative DNA-binding C domain and ligand-binding E-domain are underlined in the *D. magna* sequence. Boxed amino acids correspond to the zinc fingers.

Shaded areas denote amino acid identity with *D. magna*. Darker shaded areas correspond to conserved histidine (H) and cysteine (C) residues implicated with heme moiety binding. GenBank accession number follows gene name in parentheses.

Fig. 5. Absolute expression of HR3 (closed squares) and E75 (open circles) mRNA levels through the molt cycle of *D. magna.* Data are presented as mean and standard deviation (n=3). An asterisk denotes significant difference from the respective control for each gene at $P \leq 0.05$ (ANOVA, Dunnett's test).

Fig. 6. Absolute expression of HR3 (closed squares) and E75 (open circles) mRNA levels during six stages of embryo development. Data are presented as mean and standard deviation (n=3). An asterisk denotes significant difference from the respective stage 1 level for each gene at $P \leq 0.05$ (ANOVA, Dunnett's test).

Fig. 7. Relative expression of EcR-A (**A**), HR3 (**B**), and E75 (**C**) mRNA in untreated daphnids (circles) and in response to 1.0 μM 20-hydroxyecdysone (squares). Data are presented relative to mRNA levels at time 0 which was set at 1.0. Data are presented as mean and standard deviation (n=3). An asterisk denotes significant difference from the respective control value at each time point at $P \leq 0.05$ (ANOVA, Student's *t*-test).

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Figure 1 HR3

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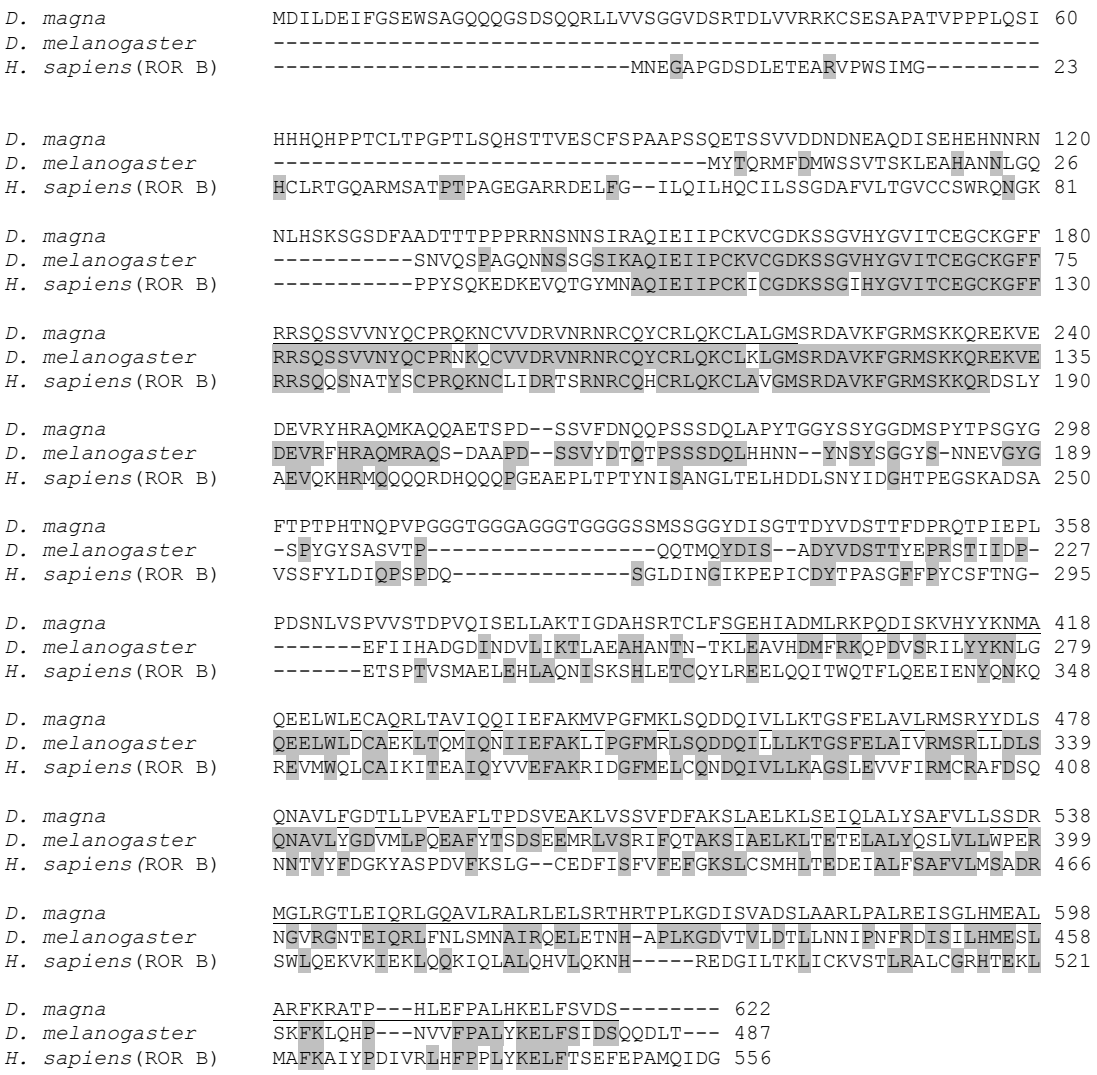
Figure 2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D. magna HHHQHPPTCLTPGPTLSQHSTTVESCFSPAAPSSQETSSVVDDNDNEAQDISEHEHNNRN 120
D. melanogaster ----------MYTQRMFDMWSSVTSKLEAHANNLGQ 26
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D. melanogaster -----------SNVQSPAGQNNSSGSIKAQIEIIPCKVCGDKSSGVHYGVITCEGCKGFF 75
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Figure 3 E75

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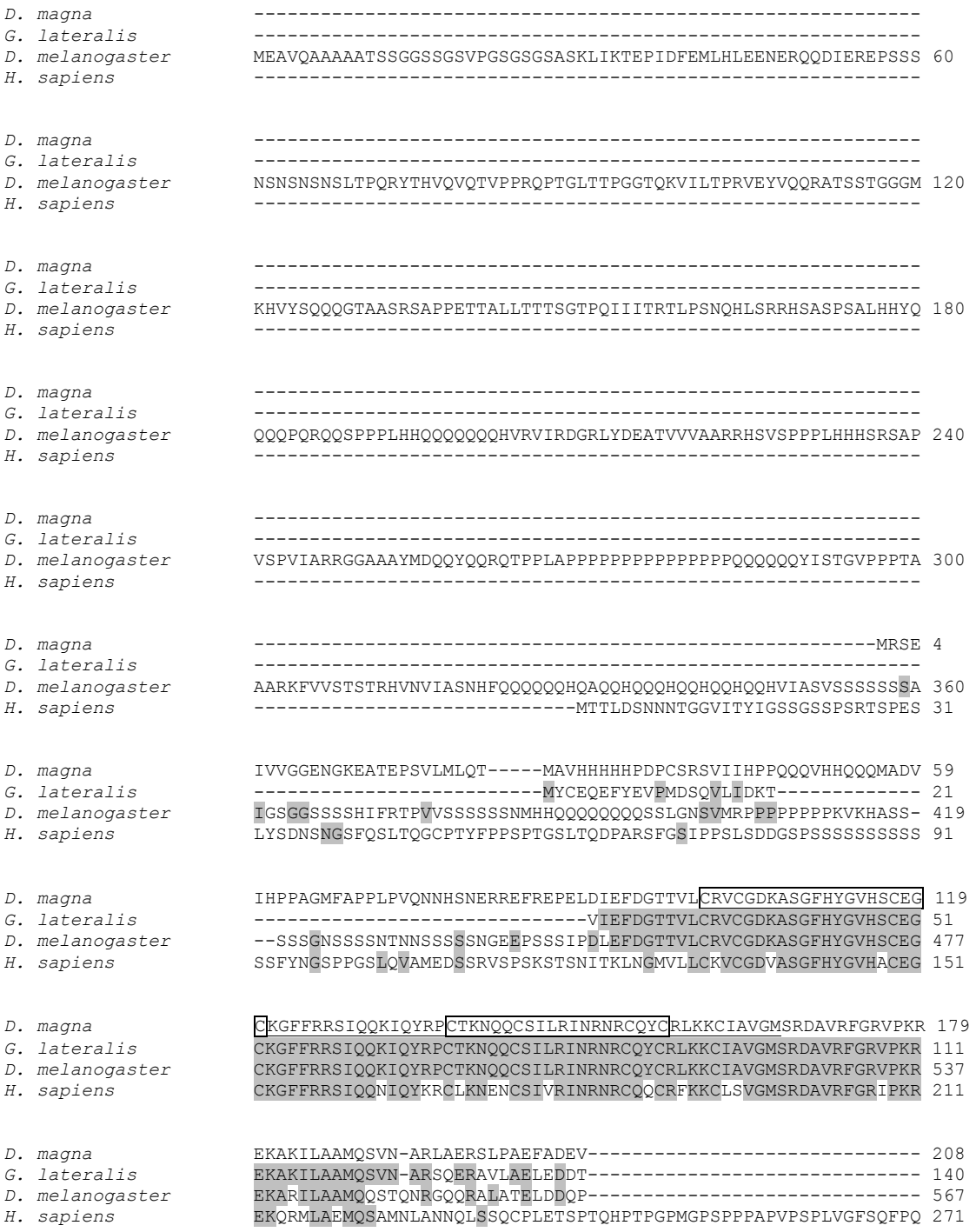
Figure 4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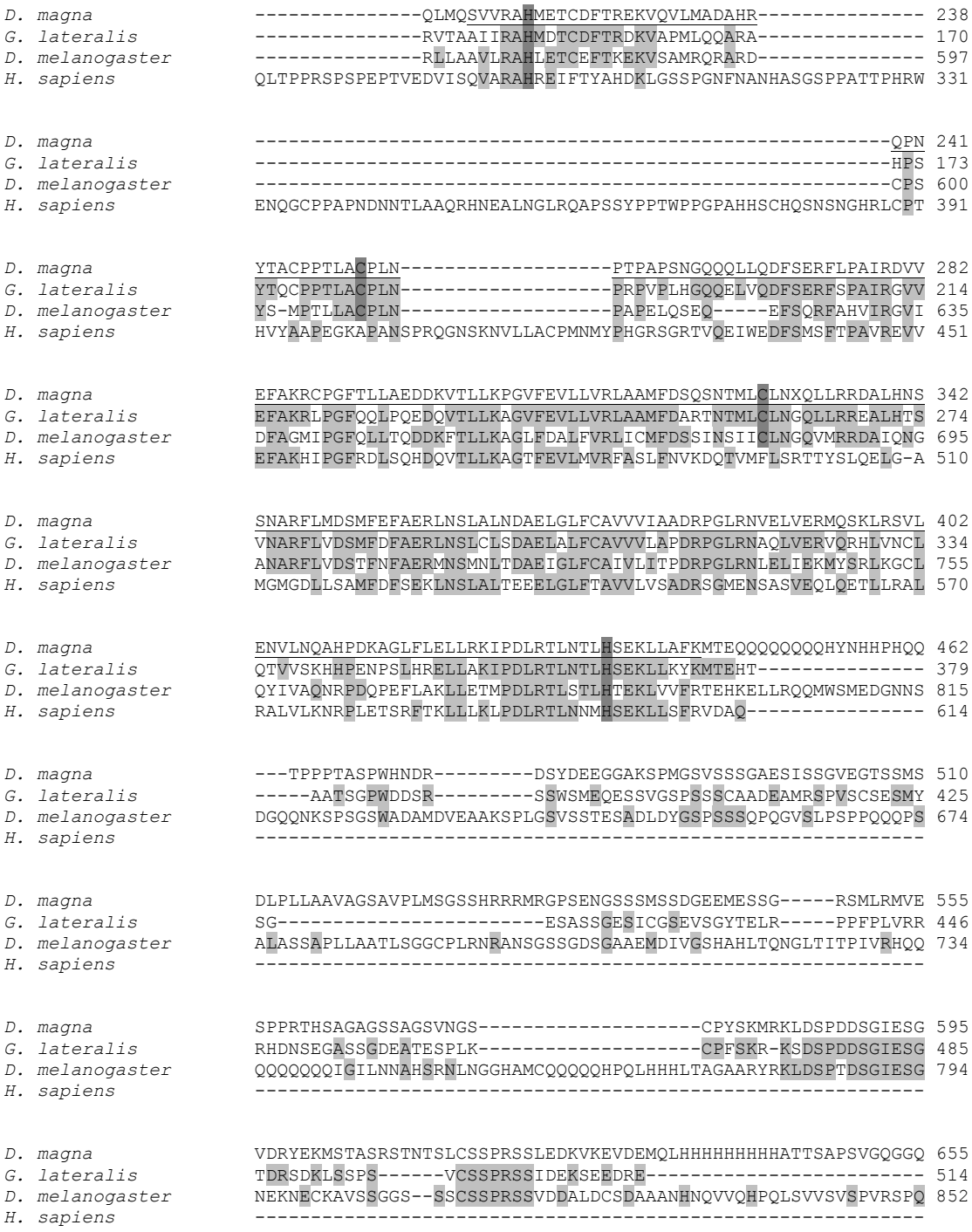
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G. lateralis -----AATSGPWDDSR---------SSWSMEQESSVGSPSSSCAADEAMRSPVSCSESMY 425
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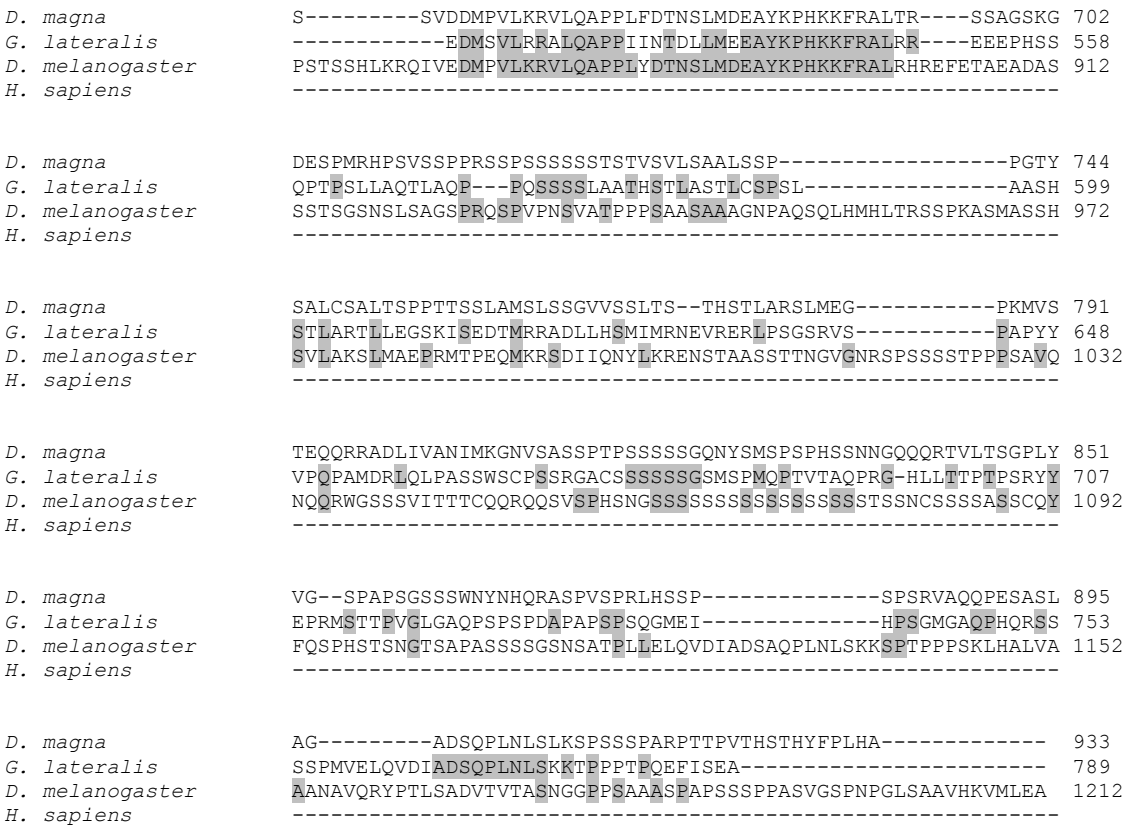
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H. sapiens ----------
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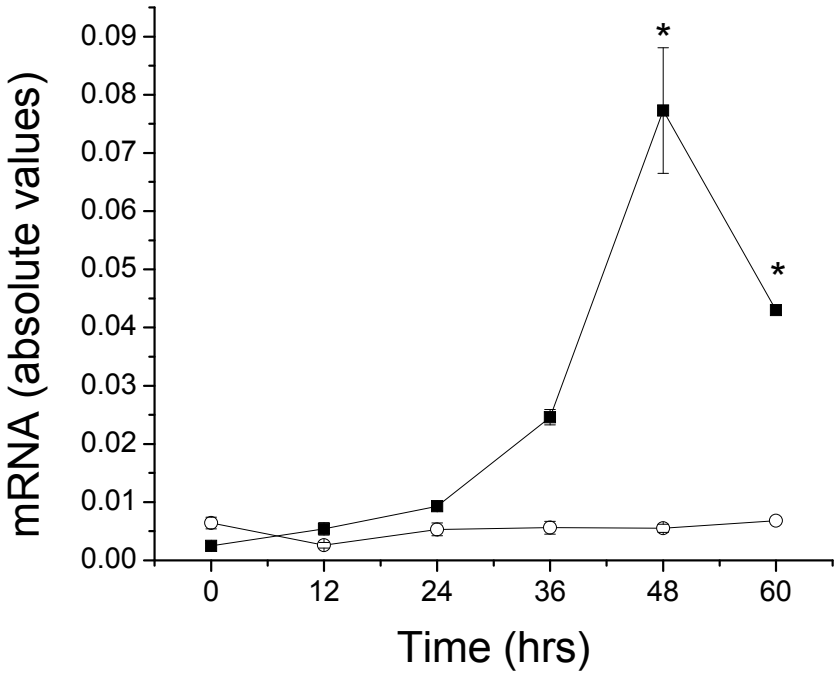
H. sapiens ----------

Figure 5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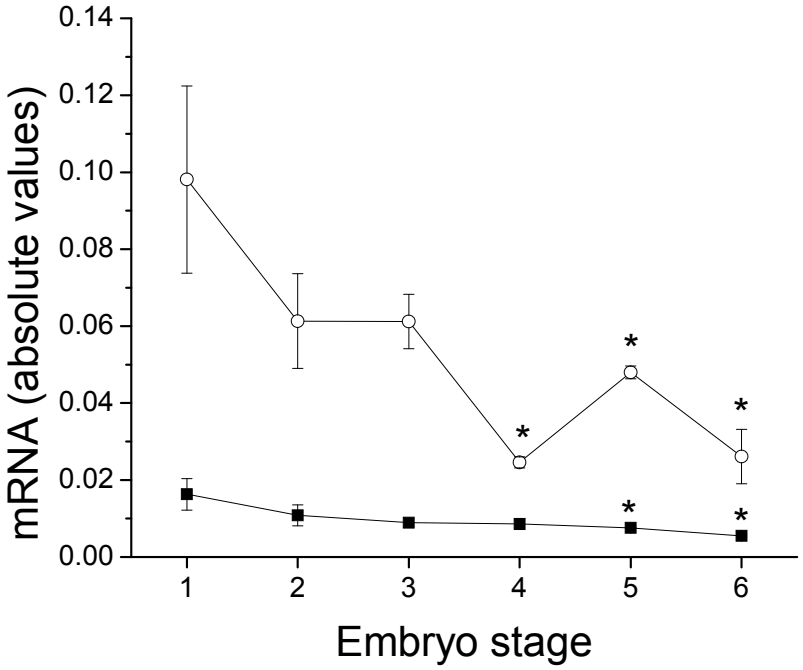
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
*
*
0
12
24
36
48
60
Time (hrs)

Figure 6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0.06
0.04
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mRNA (absolute values)
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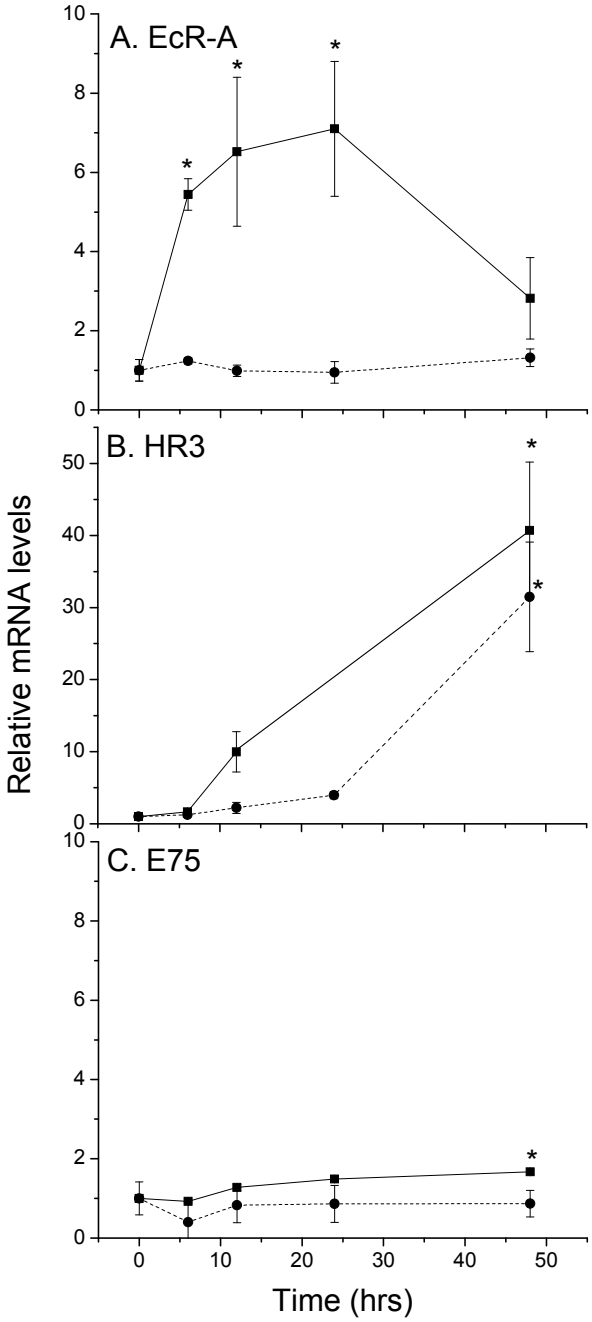
2
3
4
5
6
Embryo stage
*
*
*
*

Figure 7


A. EcR-A
B. HR3
C. E75
Relative mRNA levels
Time (hrs)
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2
4
6
8
10
0
10
20
30
40
50
0
10
20
30
40
50
*

CHAPTER THREE: INTERACTIONS OF THE CRUSTACEAN NUCLEAR RECEPTORS HR3 AND E75 IN THE REGULATION OF GENE TRANSCRIPTION

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Department of Environmental & Molecular Toxicology
North Carolina State University
Raleigh, NC 27695-7633

Abstract

Endocrine signal transduction occurs through cascades that involve the action of both ligand-dependent and ligand-independent nuclear receptors. In insects, two such nuclear receptors are HR3 and E75 that interact to transduce signals initiated by ecdysteroids. We have cloned these nuclear receptors from the crustacean *Daphnia pulex* to assess their function as regulators of gene transcription in this ecologically and economically important group of organisms. Both nuclear receptors from *D. pulex* (DpHR3 (group NR1F) and DpE75 (group NR1D)) exhibited a high degree of sequence similarity to other NR1F and NR1D group members that was indicative of monomeric binding to the RORE (retinoid orphan receptor element). DpE75 possesses key amino acid residues required for heme binding to the ligand binding domain. Next, we developed a gene transcription reporter assay containing a luciferase reporter gene driven by the RORE. DpHR3, but not DpE75, activated transcription of the luciferase gene in this system. Co-transfection experiments revealed that DpE75 suppressed DpHR3-dependent luciferase transcription in a DpE75 dose-dependent manner. Unexpectedly, low levels of DpE75 along with DpHR3 activated the luciferase gene to levels greater than that observed with DpHR3 alone. Finally, we evaluated the ability of nitric oxide to modulate the activity of DpE75 through interaction with the putative heme moiety located in the ligand binding domain. We found no evidence of a regulatory role for nitric oxide towards DpE75. These results demonstrate that DpHR3 functions as a transcriptional activator of genes regulated by the RORE and that DpE75 is a negative regulator of this activity. Taken together with the previous demonstration that daphnid HR3

is highly induced by 20-hydroxyecdysone, these results support the premise that HR3 is a major component of ecdysteroid signaling in crustaceans and is under the negative regulatory control of E75.

Introduction

The ecdysteroid signaling pathway is a major component of the endocrine axis controlling development and reproduction in arthropods. Numerous nuclear receptors, transcription factors, coactivators, and corepressors coordinate within this pathway to transmit the ecdysteroid-induced signal. Much is known about the structure, function, and interplay of these signaling components in insects [1]. Much less is understood about the genomic signaling pathway of ecdysteroid signaling in crustaceans, despite the significant ecological and economic importance of this subphylum and the demonstrated ability of many environmental contaminants to interfere with this pathway in these organisms [2-4]. Therefore, it is essential to gain more knowledge about ecdysteroid signaling in crustaceans in order to protect these organisms against disruptive environmental factors.

Ecdysteroids bind and activate the nuclear receptor heterodimer consisting of the ecdysteroid receptor (EcR) and the retinoid X receptor or Ultraspiricle (RXR/USP)[5-7]. This ligand-bound heterodimer activates transcription of a cascade of genes that regulate many physiological events including metamorphosis [8,9], embryogenesis [10], growth [10], differentiation [11], egg chamber development [12], ecdysis [13], diapause [14], reproduction [14], and behavior [14]. Two of these ecdysteroid-inducible transcriptional regulators in insects are the nuclear receptors HR3 (group NR1F) and E75 (group NR1D [15]. HR3 activates target downstream genes in the signaling pathway and E75 is best recognized as a negative regulator of HR3 transcriptional activation [16-18]. In *Drosophila melanogaster*,

the interaction between HR3 and E75 is further regulated by nitric oxide or carbon monoxide which binds to the heme moiety associated with E75 [19]. It is currently unknown whether a similar interplay of receptors operates in the ecdysteroid signaling pathway of crustaceans.

Studies involving receptor signaling in crustaceans of the genus *Daphnia* have been greatly facilitated by the recent sequencing of the *Daphnia pulex* genome (http://wFleaBase.org). We identified 25 nuclear receptor genes in the *D. pulex* genome [20]. Many of these receptors are orthologs to insect receptors involved in the ecdysteroid signaling pathway. In particular, we identified sequences for both E75 and HR3. We cloned, sequenced, and characterized expression patterns of these nuclear receptors from the closely related species, *Daphnia magna* [21]. The receptor sequences indicate that the proteins may contain structural characteristics similar to those of the *Drosophila* orthologs, suggesting that they play similar roles to the *Drosophila* receptors in ecdysteroid signaling.

An alignment of the *D. pulex* predicted gene sequence [20] with the *D. magna* cDNA sequence [21] displays discrepancies for both E75 and HR3. These differences may be due to species differences or errors in the splicing prediction of the sequences derived from the *D. pulex* genome information. Therefore, one objective of the present study was to elucidate the *D. pulex* cDNA sequences for HR3 (DpHR3) and E75 (DpE75) to establish the origin of these discrepancies. The second goal of the study was to functionally characterize these receptors by determining: (1) if either DpHR3 or DpE75 activates transcription of a reporter

gene under the control of the RORE; (2) if any regulatory interactions occur between DpHR3 and DpE75 ; and (3) if nitric oxide modifies the action of E75.

Materials and methods

Full-length E75 and HR3 derivation

Female daphnids (*Daphnia pulex*) (clone MP2 (Busey 16)) provided by Dr. Jeffery Dudycha, University of South Carolina, USA), were cultured as described previously [22]. Adults (>2 weeks after birth) were stored in RNAlater® (Ambion, Austin, TX, USA) at -20°C until sufficient tissue mass was collected for RNA isolation (approximately 30 mg wet weight). Daphnids were homogenized and RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI). RNA integrity was confirmed by formaldehyde agarose gel electrophoresis. The concentration of RNA was determined by absorbance at 260 nm and the purity determined by the 260/280 nm absorbance ratio, using a Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Montchanin, DE). The ImProm-II™ Reverse Transcription System (Promega) and oligo dT primers were used to reverse transcribe RNA into cDNA.

Primers were designed at the 5' and 3' ends of the predicted open reading frame (ORF) for both E75 and HR3 genes derived from the *Daphnia pulex* genome[20]. The primer sequences used were as follows:

DpE75 F: 5'-**GACGACGACAAG**ATGAGAAGTGAAATTGTTGTG-3',

DpE75 R: 5'-**GAGGAGAAGCCCGGT**TCAGCCCTTCATGATGTTGG-3',

DpHR3 F: 5'-**GACGACGACAAGATG**ATGGAAGCTCCGGCCGTT-3'

DpHR3 R: 5'-**GAGGAGAAGCCCGGT**TCAACTATCCACGGAAAAGAG-3'.

The bold portion of each primer corresponds to a ligase-independent cloning (LIC) extension sequence for cloning into an Ek/LIC vector (Novagen, EMD Biosciences, San Diego, CA, USA) which can be used for recombinant protein expression in *E. coli*. The genes were amplified by PCR using 75 ng cDNA, 22.5 µL high fidelity Supermix (Invitrogen, Carlsbad, CA), and 0.4 µM primers in a final volume of 25 µL. Cycling conditions were as follows: denature for 30 seconds at 94°C, anneal primers for 30 seconds at 57°C, and extend products for 3 minutes at 72°C for a total of 40 cycles, followed by a final extension for 7 minutes at 72°C. PCR products were purified from a 1.2% agarose gel using the Wizard® SV Gel and PCR Clean-up System (Promega). Purified products were cloned into the vector pCR®4-TOPO using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. The gene inserts were subsequently sequenced by primer extension (SeqWright Inc., Houston, TX). The amino acid sequence and molecular weight for both the DpHR3 and DpE75proteins were determined using ExPASy software (http://www.expasy.org/). The DpHR3 sequence was aligned with the ortholog from *Daphnia magna* (DmHR3 accession # FJ755466) and the predicted gene annotation DpHR3(P) derived from the *Daphnia pulex* genome [20] using ClustalW2 software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The DpE75 protein sequence was aligned with that of DmE75 (accession # 875485), the predicted gene annotation DpE75(P), and the orthologs of the tropical landcrab *Gecarcinus*

lateralis (accession # AAY89587) and the sand shrimp *Metapenapeus ensis* (accession # AF092946). Prediction of the DNA-binding domain and ligand-binding domain locations within the sequence were made based on the DpE75 and DpHR3 sequences, using NCBI protein BLAST[23].

Following release of an update of the *Daphnia pulex* genome, we discovered that the above cloning of DpE75 yielded a gene sequence that was missing 403 nucleotides in the C-terminus and this cDNA was used to produce proteins for the functional characterization of the receptors. Therefore, the primers E75 R2: 5'-**GAGGAGAAGCCCGGT**TCAGGCGTGAAGGGGAAAAT-3' and E75 F from above were used in PCR (as above) to obtain the full length cDNA which was used in comparative functional experiments with the truncated cDNA.

Transcriptional Reporter Assays

DpHR3 and DpE75cDNA was amplified using the primers

pMT-E75 F: 5'-GGTACCGCCATGGGAAGTGAAATTGTTGTG -3',

pMT-E75 R: 5'-TTCGAACTTCATGATGTTGGCGACGATG -3', or

pMT-E75 R2 (full length): 5'- CTCGAGGGCGTGAAGGGGAAAATAGTG-3', and

pMT-HR3 F: 5'-GGTACCGCCATGGAAGCTCCGGCCGTTCCG -3',

pMT-HR3 R: 5'-CTCGAGATCCACGGAAAAGAGTTCCTTGTG -3'.

Recognition sites for KpnI, BstBI and XhoI are underlined. DpHR3, and DpE75 (-403 nucleotides and full length) and HR3 cDNA were individually inserted into pMT/V5-His vectors (Invitrogen) at the KpnI and BstBI restriction sites and the KpnI and XhoI restriction sites, respectively. The reporter plasmid pEar1-Luc was a generous gift from K. Pardee and H. Krause [19] and contains the luciferase reporter gene, under the control of the RORE [24].

Transcription assays were performed in *Drosophila* Schneider (S2) cells. Cells were maintained in Schneider's medium (Gibco, Carlsbad, CA, USA) containing 10% heat inactivated fetal bovine serum (Gibco) and incubated at 23°C under ambient air atmosphere. Cells were seeded at a density of 3 $x10^6$ and transfected 16 hours after plating. Transfections were performed by calcium phosphate DNA precipitation with 5 µg pEar1-Luc, and various amounts (indicated in the figures) of pMT-HR3, pMT-E75 (-403 nt) or pMT-E75 (full length), and pPAC-β-gal, which served as a control for transfection efficiency and was a kind contribution from Dr. Robert Tjian (University of California, Berkeley). Cells were transfected for 24 hours at 23°C, washed, and then induced with the addition of $CuSO_4$ at a final concentration of 500 µM. Cells were provided 1.5 µM hemin (Sigma-Aldrich) dissolved in DMSO at the time of induction. In some experiments, cells were exposed for 24 hours to either 400 µM of the nitric oxide donor 2,2'-(Hydroxynitrosohydrazino) bis-ethanamine (DETA-NO) (Caymen Chemicals, Ann Arbor, MI, USA) or the vehicle 0.01 mM (final concentration) NaOH, following 24 hours of induction. Cells were harvested and luciferase and ß-galactosidase activities were measured according to manufacturer's protocols for the Luciferase Assay System and the β-galactosidase Enzyme Assay System

with Reporter Lysis Buffer (Promega). Luciferase values were normalized with ß-galactosidase activity levels and reported as relative to the untreated control cells (transfected with empty pMT-V5-His vector in lieu of pMT-E75 or pMT-HR3).

Immunoblotting

Expression of recombinant protein from the transfected genes was evaluated by Western blotting against the V5 tag. To prepare lysates, cells were first washed with PBS, then were lysed using NP-40 buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, pH 8.0), supplemented with protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Nucleic acids were sheared using a hand-held sonicator probe (Vibra Cell™, Danbury, CT, USA). Proteins were denatured by boiling the lysates at 95°C for 3 minutes. Protein concentrations were determined by the Bradford Assay [25] using commercially available reagents (Bio-Rad, Hercules, CA) against the standard, bovine serum albumin (Fraction V) (Sigma-Aldrich, St. Louis, MO). Samples were added to 2× SDS loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 25% glycerol) for electrophoresis. Monoclonal anti-V5-HRP antibody (Invitrogen) was used to detect both DpE75 and DpHR3 fusion proteins which each contained a V5 epitope. Proteins were separated by electrophoresis on a 10% NuPAGE Novex Bis-Tris gel and transferred to PVDF membrane using the iBlot™ Dry Blotting System (Invitrogen) according to manufacturer protocol. Protein bands were visualized by chemiluminescence using the ECL Substrate Western blot detection system (Pierce, ThermoScientific).

Statistical analysis

Differences among transfection groups in the reporter assay were assessed using ANOVA and Tukey-Kramer comparison analysis to compare all pairs of groups. Paired comparisons of untreated vs. NO treated cells were evaluated using Student's *t*-test, with significance established at $P < 0.05$. All analyses were performed using JMP statistical software (SAS Institute, Cary, NC, USA).

Results

DpHR3 cDNA

The ORF of the nuclear receptor DpHR3 was sequenced from *D. pulex* (GenBank accession # FJ755467; Fig. 1). This 1,953 nucleotide cDNA translates to a 649 amino acid protein with an estimated mass of 70,709. Alignment of the DpHR3 with the predicted HR3 (DpHR3(P)) and the *D. magna* HR3 (DmHR3) showed that total identity with the full length DpHR3 receptor was 94% for DpHR3(P) and 93% for DmHR3 (Fig. 2). The discrepancies between the predicted sequence DpHR3(P) and the sequenced cDNA (DpHR3) existed largely in errors in the identification of splice sites resulting in deletions in the predicted sequence in the 309 to 403 amino acid region (Fig. 2). Sporadic single amino acid differences between the predicted and actual DpHR3 sequences may be due to sequencing error or clone differences (clone Log50 was used in the genome sequencing effort) (http://daphnia.cgb.indiana.edu/projects/genome/). The deletions in the predicted sequence

also are largely responsible for the previously observed discrepancy between the predicted sequence and the cDNA derived from *D. magna* [21]. DmHR3 also contained several deletions in the first 100 amino acids of the protein (Fig. 2). Despite these differences between the three HR3 sequences, the DBD containing two zinc finger motifs was 100% identical among sequences. The LBD was close to 100% identical among sequences, with only two amino acid differences in the DmHR3 sequence. A high degree of identity in the DBD and LBD suggests functionality is likely conserved between DpHR3 and DmHR3.

DpE75 cDNA

The E75 full-length (2865 nucleotides) ORF cDNA was cloned from *D. pulex* (GenBank accession # FJ946916). This cDNA encoded a 955 amino acid protein with an estimated molecular mass of 104,136 (Fig.3). The full length amino acid sequence for DpE75 was aligned with that of the predicted *D. pulex* (DpE75(P)) sequence and that of *D. magna* (DmE75) (Fig. 4). Sequence identity between the predicted sequence and the actual DpE75 sequence was high (95%) with the single major difference being apparent errors in the selection of splice boundaries in the predicted sequence (DpE75 amino acid region 722-769). DmE75 shared 90% sequence identity to DpE75. Differences between DmE75 and DpE75(P) also were due to the error in predicted splice sites and sporadic amino acid differences. Species differences existed largely in the A/B and F domains. These results demonstrate a high degree of conservation between E75 of the two daphnid species.

The DpE75 protein DNA binding C domain (DBD) contains two zinc fingers and shares 100% identity with both DpE75(P) and DmE75. The ligand-binding E domain (LBD) of DpE75 is 99% and 97% identical to DpE75(P) and DmE75, respectively. The DpE75 ligand-binding domain contains histidine and cysteine residues situated in key positions to bind a heme moiety, as described previously [19,26]. Despite slight differences in sequence between the two *Daphnia* species, the close identity of the E75 functional domains between *D. pulex* and *D. magna* suggests that the orthologs maintain similar functions.

The DpE75 protein was also compared to protein sequences of E75 orthologs for the two crustacean species the tropical land crab (*G. lateralis*) and the sand shrimp (*M. ensis*). DpE75 shared 100% identity with both orthologs in the DBD and the C-terminal extension. The LBD of *G. lateralis* and *M. ensis* were 86% and 84% identical, respectively, to the LBD of DpE75. These orthologs contain the key heme-binding histidine and cysteine residues in the LBD. The highly conserved DBD and LBD of these proteins suggest that function of E75 is similar between the considered crustacean species.

Transcription Activation by DpHR3 and DpE75

Transient expression luciferase-based reporter assays were performed to determine if DpHR3 or DpE75 activates gene transcription driven by the RORE. The DpHR3 expression vector (pMT-HR3) was cotransfected into *Drosophila* S2 cells with the luciferase reporter plasmid (pEar1-Luc) which contains six RORE recognition sequences, located upstream of

the basal promoter for the luciferase gene[19]. Western blot analysis of cell lysates confirmed that both the DpHR3 and DpE75 proteins were expressed in induced cells. DpHR3 activated transcription approximately 15-fold over basal activation (Fig.5). DpE75 did not activate the luciferase reporter gene when transfected alone (Fig. 5). Cotransfection of DpHR3 and DpE75 with the reporter plasmid reduced transcriptional activation seen with DpHR3 alone to basal levels (Fig. 5).

DpHR3 luciferase activation was characterized in a concentration-response experiment in which increasing levels of the DpHR3 expression plasmid were transfected, resulting in an inverse-U shaped relationship with luciferase activation (Fig. 6). Next, a concentration-response analysis was performed to definitively characterize the observed suppression of DpHR3 activity by DpE75. Increasing concentrations of the DpE75 expression plasmid were cotransfected with a constant concentration of both DpHR3 and reporter plasmid. The resulting relationship was inverse, with reporter activation decreasing as levels of DpE75 levels increased (Fig.7). Interestingly, at the lowest concentration of transfected DpE75 plasmid (~500 times less than the amount DpHR3 plasmid), activational activity was significantly greater than that of DpHR3 transfected alone.

Finally, experiments were performed to determine if NO releases DpHR3 from the suppressive action of DpE75. NO had no effect on either the activation of luciferase by DpHR3 or the suppressive action of DpE75 on this activity (Fig. 8A). Collectively, these

results demonstrate that the crustacean receptor DpHR3 activates gene transcription driven by the RORE response element and DpE75 negatively regulates this activity. However, results provide no evidence that this interaction is modulated by NO.

DpE75 activity: Full length receptor vs. -403 nucleotides

We used the pMT-E75 (-403 nucleotides) expression plasmid as the source for DpE75 protein in the reporter assays described above. Therefore, upon discovery of the additional C-terminal nucleotides and cloning of this full sequence, we performed a comparative assays with pMT-E75 (full length) and pMT-E75 (-403 nucleotides) to determine whether the full F domain was necessary to detect any effect of NO. Both DpE75 proteins performed similarly with respect to the suppression of DpHR3 activity and their non-responsiveness to NO (Fig. 8A, B).

Discussion

The nuclear receptors HR3 and E75 are prominent contributors to ecdysteroid signaling in insects [16-18]. In the present study HR3 and E75 were cloned from the crustacean *Daphnia pulex* and used to determine whether either of these nuclear receptors activate the transcription of genes regulated by the RORE; if DpHR3 and DpE75 interact to regulate gene transcription; and, whether NO modifies the action of DpE75. We demonstrated that DpHR3 activates transcription of a reporter gene under the regulatory control of the RORE and DpE75 negatively regulates this activity of DpHR3. However,

despite the fact that DpE75 contains amino acids that coordinate heme binding, we found no evidence that NO modifies the action of DpE75.

The differences in DpHR3 and DmHR3 protein sequences generally fall outside of functional domains (i.e., DBD and LBD). The DBD of DpHR3, DmHR3, and the *Drosophila* DHR3 are highly similar ($\geq$95%) ([21], present study). The DBD of DHR3 binds an 11-bp DNA response element that consists of the half-site AGGTCA flanked on the 5' end by an A/T rich sequence, referred to as an RORE. DHR3 also contains a highly conserved C-terminal extension (CTE) to the DBD domain. This CTE contains a "GRIP-box" sequence, which has the consensus sequence (K/R)XGRZ(P/S), where X is any amino acid and Z represents a hydrophobic amino acid [27]. The GRIP-box typically provides stability to a monomer protein when binding DNA [28]. DpHR3 also contains a GRIP-box. The similarities between the structure of the DBD of *Daphnia* and *Drosophila* suggest that, like DHR3, DpHR3 binds the RORE as a monomer. DpE75, DmE75, and DE75 are 100% identical in their DBD. Furthermore, the daphnid E75 also contains the GRIP-box. Like HR3, DE75A binds the RORE [29] and daphnid E75 may similarly bind this response element.

HR3 is a member of the NR1F group of nuclear receptors which includes the RORs of vertebrates [30]. HR3 has been shown in many insects to be ecdysteroid inducible [31-33] and functions in ecdysteroid signaling to regulate aspects of embryogenesis and

metamorphosis [34,35] along with contributions to many other physiological processes (see Introduction). A portion of the HR3 cDNA covering the LBD was reportedly cloned from the American lobster (*Homarus americanus*) [36]. However, the putative LBD associated with this amino acid sequence exhibited low identity to the HR3 LBD from *Drosophila melanogaster* (19.6%) and *Manduca sexta* (22.2%). Either the sequence cloned from the lobster was not actually HR3 or the LBD of lobster HR3 differs significantly from those of insects and daphnids. Aside from *D. magna* [21] and *D. pulex* (present study), we are not aware of any other crustacean species from which the full HR3 cDNA has been sequenced.

E75 is a member of the NR1D group of nuclear receptors. Vertebrate rev-erbs also are members of this group [30]. E75 has been cloned from the tropical land crab (*G. lateralis*) [37] and the shrimp (*M. ensis*) [38]. Amino acid sequence alignment revealed that the DBD and LBD of daphnid DpE75 are highly similar to those of the malacostracan crustaceans. Not surprisingly, while the DBD of DmE75 and, therefore, DpE75 was 100% identical to the DBD of the Drosophila E75 [21], the LBD shared less identity with insect E75 LBD (52%) than the other crustacean species analyzed in the present study (land crab 86% and shrimp 84%). Therefore, based on these findings, it is apparent that the structure of E75 protein is highly conserved between crustacean species, but diverges somewhat from that of insects.

Functional analyses demonstrated that DpHR3 activated transcription of a reporter gene driven by the RORE. This result demonstrates that this crustacean representative of the NR1F group of nuclear receptors functions similarly to other group members. In contrast, DpE75 did not activate RORE-driven gene transcription. Rather, DpE75 suppressed transcriptional activation mediated by DpHR3. This suppressor activity of a crustacean E75 is consistent with the suppressive activity demonstrated with insect E75 [17,18], as well as, the vertebrate ortholog REV-ERB [39,40].

Two mechanisms have been described through which E75 can suppress the action of HR3. BmE75 from *Bombyx mori*, equipped with a complete DBD, has been shown to bind the RORE and is capable of competing with BmHR3 for response element occupancy [17]. In addition, BmE75 can bind directly to BmHR3 forming a ternary complex with the response element [17] which is presumably inhibitory towards BmHR3. *Drosophila* DE75B lacks one zinc finger in the DBD but remains capable of inhibiting DHR3 by complexing with DHR3 on the response element [18]. E75 of daphnids possesses two zinc fingers in the DBD and therefore is equipped to compete with daphnid HR3 for RORE occupancy but may also interact directly with the DpHR3 protein.

Co-transfection experiments with the daphnid receptors revealed that, in addition to the suppressive action of DpE75 on DpHR3 activity, low levels of DpE75 augment DpHR3 transcriptional activation. Increased luciferase activity may be explained by DpE75

protection of DpHR3 from degradation, therefore increasing the levels of DpHR3 available to bind the RORE. Alternatively, levels of DpHR3 transfected in this experiment may have caused toxicity to the cells and DpE75 may have protected the cells against this toxicity. The low level of DpE75 used in the experiment may have protected the cells while being present at a concentration insufficient to suppress DpHR3 activity resulting in enhanced transcription of the luciferase gene. Toxicity associated with high levels of DpHR3 could explain the inverted-U shaped curve of luciferase activity vs. increasing levels of DpHR3 expression vector observed in the present study. Further investigations are necessary to definitively characterize this interaction. However, these results suggest that an important and potentially unique regulatory interplay occurs between DpE75 and DpHR3.

DME75A [19] and human REV-ERBβ[41]bind heme which confers stability to the protein and provides for the binding of diatomic gases NO and CO [19,42]. The binding of NO to the heme group relieves the protein of its suppressive action towards HR3/ROR [19,42]. DpE75 protein possesses key histidine and cysteine residues required for heme binding in the LBD [19,26,42]. However, we found no evidence that NO interacts with DpE75 to relieve it of its suppressive activity towards DpHR3. Raghuram et al. [41] failed to detect NO binding to REV-ERB though such activity was subsequently reported by Pardee et al. [42]. DpE75 may not be responsive to diatomic gases. Alternatively, methodological issues, such as inadequate intracellular uptake of the NO donor may have been responsible for the lack of responsiveness of DpE75 to the gas.

In summary, results from the present study demonstrate that crustaceans express both HR3 and E75 and that these proteins function coordinately to regulate gene transcription via the RORE. DpHR3 activates transcription of genes driven by this response element and DpE75 suppresses this activity of DpHR3. Considering the high similarity in structure and function of these gene products in comparison to those of insects and the high level, rapid induction of daphnid HR3 in response to ecdysteroids [21], it is reasonable to conclude that these receptors are integral components of ecdysteroids signaling in crustaceans.

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Figure 1. Nucleotide (lower case) and deduced amino acid (upper case) sequences of the DpHR3 cDNA.

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atggaagctccggccgttccgggtgccaatccgcatcattcgatggatattctcgatgag
 M  E  A  P  A  V  P  G  A  N  P  H  H  S  M  D  I  L  D  E
attttcggcagtgaatggtcggctggccaacaacaggcagccgacagtcaacaacaacaa
 I  F  G  S  E  W  S  A  G  Q  Q  Q  A  A  D  S  Q  Q  Q  Q
caacgattattagttggtggtgtcgatacacggaccgaattggtggtgcgccgtaaaggt
 Q  R  L  L  V  G  G  V  D  T  R  T  E  L  V  V  R  R  K  G
ggtggtggtgaatcaggaggtcaacctcctcccggtgtgcctccgcctctccaatcgatc
 G  G  G  E  S  G  G  Q  P  P  P  G  V  P  P  P  L  Q  S  I
caccgacaccaccaacagcaacaacaacaacaacatccgccaacgtgcctgactcccggc
 H  R  H  H  Q  Q  Q  Q  Q  Q  Q  H  P  P  T  C  L  T  P  G
ccaacgttgagccaacattcgacgacggtcgagtcttgtttctcaccggccgctcccagc
 P  T  L  S  Q  H  S  T  T  V  E  S  C  F  S  P  A  A  P  S
agccaagagacgagcagcgtcgtcgacgacaacgacaacgaagcccaagacattagcgag
 S  Q  E  T  S  S  V  V  D  D  N  D  N  E  A  Q  D  I  S  E
cacgaacacaacaaccacaacaacctccaaagcaaagccggatccgattttgccgccgat
 H  E  H  N  N  H  N  N  L  Q  S  K  A  G  S  D  F  A  A  D
acaacaacaccgccgcccagaagaaatagtaacaattccatcagagcgcaaattgagatc
 T  T  T  P  P  P  R  R  N  S  N  N  S  I  R  A  Q  I  E  I
attccgtgcaaggtgtgcggcgacaagagttcgggcgtccattatggagtcatcacctgc
 I  P  C  K  V  C  G  D  K  S  S  G  V  H  Y  G  V  I  T  C
gagggctgcaagggtttcttccggcggtcgcaaagttccgtcgtcaactaccaatgcccg
 E  G  C  K  G  F  F  R  R  S  Q  S  S  V  V  N  Y  Q  C  P
agacaaaagaattgcgtcgtcgatcgcgtcaaccgcaaccgatgccaatactgccgccta
 R  Q  K  N  C  V  V  D  R  V  N  R  N  R  C  Q  Y  C  R  L
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```

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 D  I  S  V  A  D  S  L  A  A  R  L  P  A  L  R  E  I  S  G
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 L  H  M  E  A  L  A  R  F  K  R  A  T  P  H  L  E  F  P  A
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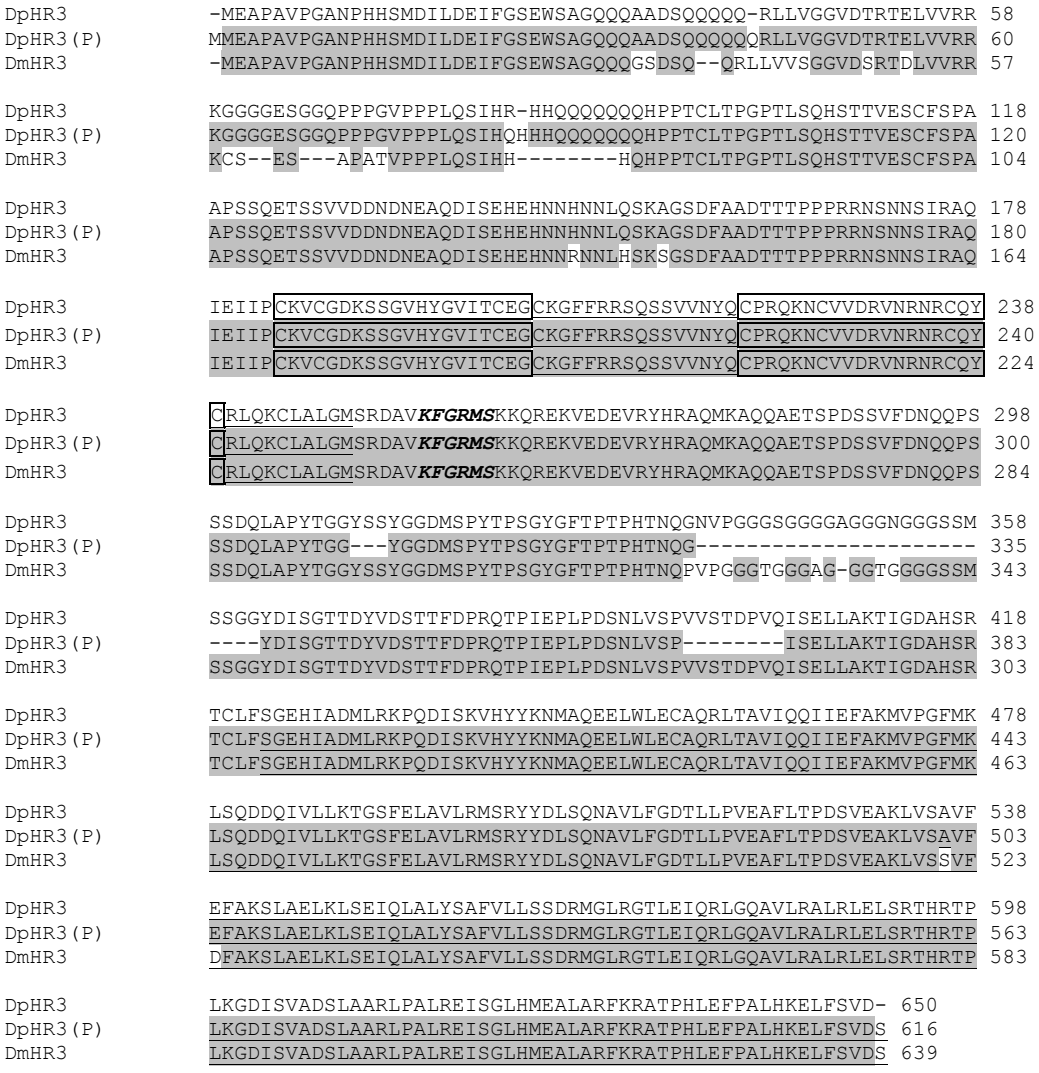
 L  H  K  E  L  F  S  V  D  S  -
```

Figure 2. Comparison of the DpHR3 amino acid sequence to the sequence predicted from the genomic sequence (DpHR3(P)), and DmHR3 from *Daphnia magna*. Shaded areas denote amino acid identity withDpHR3. The putative DNA-binding C domain and ligand-binding E domain are underlined. Boxed amino acids correspond to the zinc fingers. The GRIP-box is indicated by amino acid residues in bold, italics.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DmHR3 -MEAPAVPGANPHHSMDILDEIFGSEWSAGQQQGSDSQ--QRLLVVSGGVDSRTDLVVRR 57
DpHR3 KGGGGESGGQPPPGVPPPLQSIHR-HHQQQQQQQHPPTCLTPGPTLSQHSTTVESCFSPA 118
DpHR3(P) KGGGGESGGQPPPGVPPPLQSIHQHHHQQQQQQQHPPTCLTPGPTLSQHSTTVESCFSPA 120
DmHR3 KCS--ES---APATVPPPLQSIHH--------HQHPPTCLTPGPTLSQHSTTVESCFSPA 104
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DmHR3 APSSQETSSVVDDNDNEAQDISEHEHNNRNNLHSKSGSDFAADTTTPPPRRNSNNSIRAQ 164
DpHR3 IEIIPCKVCGDKSSGVHYGVITCEGCKGFFRRSQSSVVNYQCPRQKNCVVDRVNRNRCQY 238
DpHR3(P) IEIIPCKVCGDKSSGVHYGVITCEGCKGFFRRSQSSVVNYQCPRQKNCVVDRVNRNRCQY 240
DmHR3 IEIIPCKVCGDKSSGVHYGVITCEGCKGFFRRSQSSVVNYQCPRQKNCVVDRVNRNRCQY 224
DpHR3 CRLQKCLALGMSRDAVKFGRMSKKQREKVEDEVRYHRAQMKAQQAETSPDSSVFDNQQPS 298
DpHR3(P) CRLQKCLALGMSRDAVKFGRMSKKQREKVEDEVRYHRAQMKAQQAETSPDSSVFDNQQPS 300
DmHR3 CRLQKCLALGMSRDAVKFGRMSKKQREKVEDEVRYHRAQMKAQQAETSPDSSVFDNQQPS 284
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DpHR3(P) SSDQLAPYTGG---YGGDMSPYTPSGYGFTPTPHTNQG---------- 335
DmHR3 SSDQLAPYTGGYSSYGGDMSPYTPSGYGFTPTPHTNQPVPGGGTGGGAG-GGTGGGGSSM 343
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DpHR3(P) ----YDISGTTDYVDSTTFDPRQTPIEPLPDSNLVSP--------ISELLAKTIGDAHSR 383
DmHR3 SSGGYDISGTTDYVDSTTFDPRQTPIEPLPDSNLVSPVVSTDPVQISELLAKTIGDAHSR 303
DpHR3 TCLFSGEHIADMLRKPQDISKVHYYKNMAQEELWLECAQRLTAVIQQIIEFAKMVPGFMK 478
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DpHR3(P) LSQDDQIVLLKTGSFELAVLRMSRYYDLSQNAVLFGDTLLPVEAFLTPDSVEAKLVSAVF 503
DmHR3 LSQDDQIVLLKTGSFELAVLRMSRYYDLSQNAVLFGDTLLPVEAFLTPDSVEAKLVSSVF 523
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DmHR3 DFAKSLAELKLSEIQLALYSAFVLLSSDRMGLRGTLEIQRLGQAVLRALRLELSRTHRTP 583
DpHR3 LKGDISVADSLAARLPALREISGLHMEALARFKRATPHLEFPALHKELFSVD- 650
DpHR3(P) LKGDISVADSLAARLPALREISGLHMEALARFKRATPHLEFPALHKELFSVDS 616
DmHR3 LKGDISVADSLAARLPALREISGLHMEALARFKRATPHLEFPALHKELFSVDS 639

Figure 3. Nucleotide (lower case) and deduced amino acid (upper case) sequences of the DpE75 cDNA.

```
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 V  H  P  P  V  S  A  M  F  A  P  P  L  P  I  Q  N  E  R  R
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 E  F  R  E  P  E  L  D  I  E  F  D  G  T  T  V  L  C  R  V
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```

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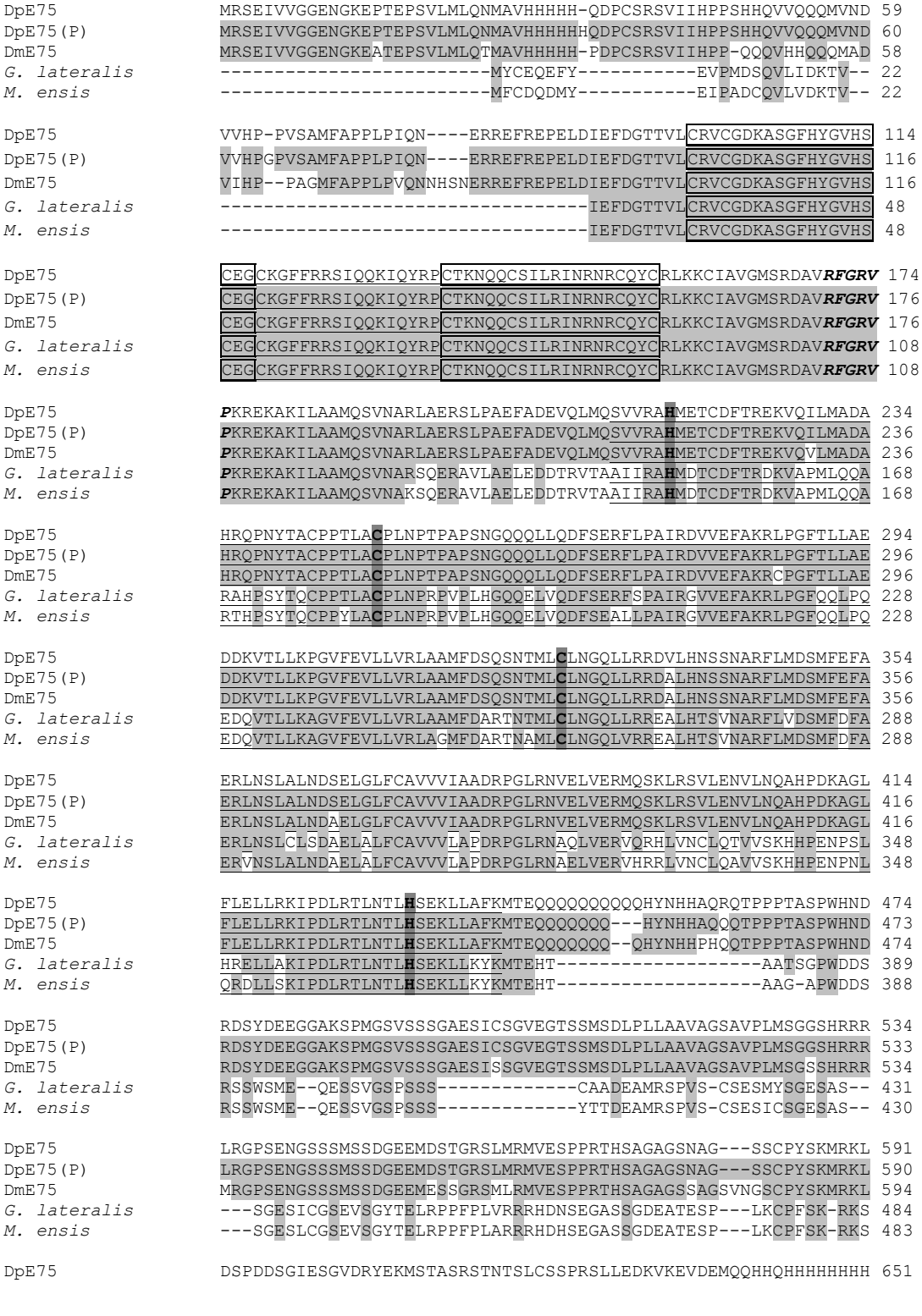
```

Figure 4. Comparison of the DpE75 amino acid sequence to the sequence predicted from the genomic sequence (DpE75(P)) and DmE75 from *Daphnia magna.* Shaded areas denote amino acid identity withDpE75. The putative DNA-binding C domain and ligand-binding E domain are underlined. Boxed amino acids correspond to the zinc fingers. The GRIP-box is indicated by amino acid residues in bold, italics. Histidines and cysteines required for heme binding are in bold and darkly shaded.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DmE75 MRSEIVVGGENGKEATEPSVLMLQTMAVHHHHH-PDPCSRSVIIHPP-QQQVHHQQQMAD 58
G. lateralis ----------MYCEQEFY----------EVPMDSQVLIDKTV-- 22
M. ensis ----------MFCDQDMY----------EIPADCQVLVDKTV-- 22
DpE75 VVHP-PVSAMFAPPLPIQN----ERREFREPELDIEFDGTTVLCRVCGDKASGFHYGVHS 114
DpE75(P) VVHPGPVSAMFAPPLPIQN----ERREFREPELDIEFDGTTVLCRVCGDKASGFHYGVHS 116
DmE75 VIHP--PAGMFAPPLPVQNNHSNERREFREPELDIEFDGTTVLCRVCGDKASGFHYGVHS 116
G. lateralis ----------IEFDGTTVLCRVCGDKASGFHYGVHS 48
M. ensis ----------IEFDGTTVLCRVCGDKASGFHYGVHS 48
DpE75 CEGCKGFFRRSIQQKIQYRPCTKNQQCSILRINRNRCQYCRLKKCIAVGMSRDAVRFGRV 174
DpE75(P) CEGCKGFFRRSIQQKIQYRPCTKNQQCSILRINRNRCQYCRLKKCIAVGMSRDAVRFGRV 176
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DpE75 PKREKAKILAAMQSVNARLAERSLPAEFADEVQLMQSVVRAHMETCDFTREKVQILMADA 234
DpE75(P) PKREKAKILAAMQSVNARLAERSLPAEFADEVQLMQSVVRAHMETCDFTREKVQILMADA 236
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M. ensis RTHPSYTQCPPYLACPLNPRPVPLHGQQELVQDFSEALLPAIRGVVEFAKRLPGFQQLPQ 228
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DmE75 DDKVTLLKPGVFEVLLVRLAAMFDSQSNTMLCLNGQLLRRDALHNSSNARFLMDSMFEFA 356
G. lateralis EDQVTLLKAGVFEVLLVRLAAMFDARTNTMLCLNGQLLRREALHTSVNARFLVDSMFDFA 288
M. ensis EDQVTLLKAGVFEVLLVRLAGMFDARTNAMLCLNGQLVRREALHTSVNARFLMDSMFDFA 288
DpE75 ERLNSLALNDSELGLFCAVVVIAADRPGLRNVELVERMQSKLRSVLENVLNQAHPDKAGL 414
DpE75(P) ERLNSLALNDSELGLFCAVVVIAADRPGLRNVELVERMQSKLRSVLENVLNQAHPDKAGL 416
DmE75 ERLNSLALNDAELGLFCAVVVIAADRPGLRNVELVERMQSKLRSVLENVLNQAHPDKAGL 416
G. lateralis ERLNSLCLSDAELALFCAVVVLAPDRPGLRNAQLVERVQRHLVNCLQTVVSKHHPENPSL 348
M. ensis ERVNSLALNDAELALFCAVVVLAPDRPGLRNAELVERVHRRLVNCLQAVVSKHHPENPNL 348
DpE75 FLELLRKIPDLRTLNTLHSEKLLAFKMTEQQQQQQQQQQHYNHHAQRQTPPPTASPWHND 474
DpE75(P) FLELLRKIPDLRTLNTLHSEKLLAFKMTEQQQQQQQ---HYNHHAQQQTPPPTASPWHND 473
DmE75 FLELLRKIPDLRTLNTLHSEKLLAFKMTEQQQQQQQ--QHYNHHPHQQTPPPTASPWHND 474
G. lateralis HRELLAKIPDLRTLNTLHSEKLLKYKMTEHT----------AATSGPWDDS 389
M. ensis QRDLLSKIPDLRTLNTLHSEKLLKYKMTEHT----------AAG-APWDDS 388
DpE75 RDSYDEEGGAKSPMGSVSSSGAESICSGVEGTSSMSDLPLLAAVAGSAVPLMSGGSHRRR 534
DpE75(P) RDSYDEEGGAKSPMGSVSSSGAESICSGVEGTSSMSDLPLLAAVAGSAVPLMSGGSHRRR 533
DmE75 RDSYDEEGGAKSPMGSVSSSGAESISSGVEGTSSMSDLPLLAAVAGSAVPLMSGSSHRRR 534
G. lateralis RSSWSME--QESSVGSPSSS----------CAADEAMRSPVS-CSESMYSGESAS-- 431
M. ensis RSSWSME--QESSVGSPSSS----------YTTDEAMRSPVS-CSESICSGESAS-- 430
DpE75 LRGPSENGSSSMSSDGEEMDSTGRSLMRMVESPPRTHSAGAGSNAG---SSCPYSKMRKL 591
DpE75(P) LRGPSENGSSSMSSDGEEMDSTGRSLMRMVESPPRTHSAGAGSNAG---SSCPYSKMRKL 590
DmE75 MRGPSENGSSSMSSDGEEMESSGRSMLRMVESPPRTHSAGAGSSAGSVNGSCPYSKMRKL 594
G. lateralis ---SGESICGSEVSGYTELRPPFPLVRRRHDNSEGASSGDEATESP---LKCPFSK-RKS 484
M. ensis ---SGESLCGSEVSGYTELRPPFPLARRRHDHSEGASSGDEATESP---LKCPFSK-RKS 483
DpE75 DSPDDSGIESGVDRYEKMSTASRSTNTSLCSSPRSLLEDKVKEVDEMQQHHQHHHHHHHH 651

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DpE75(P)            DSPDDSGIESGVDRYEKMSTASRSTNTSLCSSPRSSLEDKVKEVDEMQQH--HHHHHHHH 648
DmE75               DSPDDSGIESGVDRYEKMSTASRSTNTSLCSSPRSSLEDKVKEVDEMQLH--HHHHHHHH 652
G. lateralis        DSPDDSGIESGTDRSDKLSSPS------VCSSPRSSIDEKSEEDREEDMS---------- 528
M. ensis            DSPDDSGIESGTDRSDKLSSPS------VCSSPRSSIDEKE------------------- 518

DpE75               AAPSAG--GRVGQSSVDDMPVLKRVLQAPPLFDTNSLMDEAYKPHKKFRALTR-CAGSKG 708
DpE75(P)            AAPSAG--GRVGQSSVDDMPVLKRVLQAPPLFDTNSLMDEAYKPHKKFRALTR-CAGSKG 705
DmE75               ATTSAPSVGQGGQSSVDDMPVLKRVLQAPPLFDTNSLMDEAYKPHKKFRALTRSSAGSKG 712
G. lateralis        ---------------------VLRRALQAPPIINTDLLMEEAYKPHKKFRALRREEEPHSS 568
M. ensis            ------------------------------------------------------------

DpE75               EESPMRVPAVSSPPRSSPTSSSSSSSSSTSTVSVLSAALSSPPGTYSALCIALTSPPSTS 768
DpE75(P)            EESPMRVPAVSSP---------TSSSSSTSTVSVF----------------ALTSPPSTS 740
DmE75               DESPMRHPSVSSPPRSSP----SSSSSSTSTVSVLSAALSSPPGTYSALCSALTSPPTTS 768
G. lateralis        QPTPSLLAQTLAQPPQSSSSLAATHSTLASTLCSP----------------SLAASHSTL 612
M. ensis            ------------------------RGGPARTICR------------------------CC 530

DpE75               SSS-LLASSLSSGVVSSLTSTHSTLARSLMEGPKMVSTEQQRRADLIVANIMKGNVTGSS 827
DpE75(P)            SSSSLLASSLSSGVVSSLTSTHSTLARSLMEGPKMVSTEQQRRADLIVANIMKGNVTGSS 800
DmE75               S----LAMSLSSGVVSSLTSTHSTLARSLMEGPKMVSTEQQRRADLIVANIMKGNVSASS 824
G. lateralis        ARTLLEGSKISEDTMRRADLLHSMIMRNEVRERLPSGSRVSPAPYYVPQPAMDRLQLPAS 672
M. ensis            AR----------------------------LQRRPSSTRIC------------------- 543

DpE75               PTPSSSS--QNYCPSPLSSSSSSNGQQQQRTVLTSGPLYAGSSSPS---SWNYHHHQRSS 882
DpE75(P)            PTPSSSS--QNYCPSPLSSSSS-NGQ--QRTVLTSGPLYAGSSSPS---SWNYHHHQRSS 852
DmE75               PTPSSSSSGQNYSMSPSPHSSN-NGQQ-QRTVLTSGPLYVGSPAPSGSSSWNYN-HQRAS 881
G. lateralis        SWSCPSS-------RGACSSSSSSGSMSPMQPTVTAQPRGHLLTTP---TPSRYYEPRMS 722
M. ensis            SWRKPTT-------SPIKSSVRNVGKRS-LTPHSPPPPR----------SWSRRCLSLHS 585

DpE75               PVSPPHHHSSPSPSSSRIPQQQQQQQQ-PESVSSVGADSQPLNLSLKSPSSSPARPTTPV 941
DpE75(P)            PVSPP-HHSSPSPSSSRIPQQQQQQQQQPESVSSVGADSQPLNLSLKSPSSSPARPTTPV 911
DmE75               PVSPR-LHSSPSPS--RVAQQ-------PESASLAGADSQPLNLSLKSPSSSPARPTTPV 931
G. lateralis        TTPVGLGAQPSPSPDAPAPSP---------SQGMEIHPSGMGAQPHQRSSSSPMVELQVD 773
M. ensis            TRALWLRHTPPWPPVWRRPLA---------GLATACCESSIONAAYMENSISACCESSIO 636

DpE75               TQHNNTHYFPLHA- 954
DpE75(P)            TQHNNTHYFPLHA- 924
DmE75               THS--THYFPLHA- 942
G. lateralis        IADSQPLNLSKKTPPPTPQEFISEA 798
M. ensis            NAF---------------------- 639
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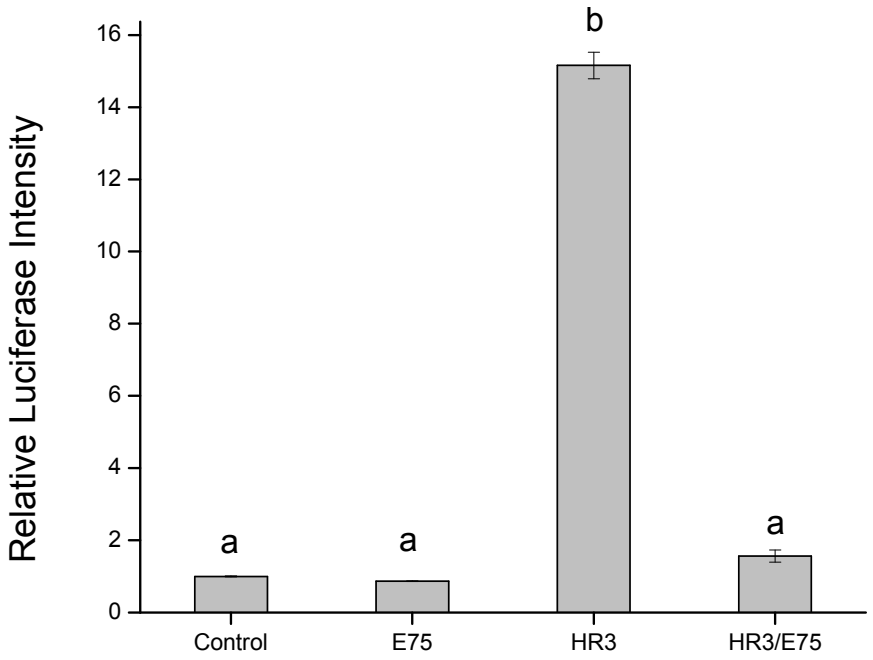


Figure 5. DpHR3 activation and DpE75 negative regulation of RORE-driven luciferase reporter assay. Amount of expression plasmid DNA transfected for each group was: 2.068 µg empty vector in control, 0.068 µg pMT-E75 in E75 group, 2 µg HR3 in HR3 alone group and 0.068 µg pMT-E75/2 µg HR3 in E75/HR3 group. Data bars represent mean± SEM (n=2) duplicate samples. Transfection groups not connected by the same letter are significantly ($p \leq 0.05$) different (ANOVA, Tukey-Kramer method).

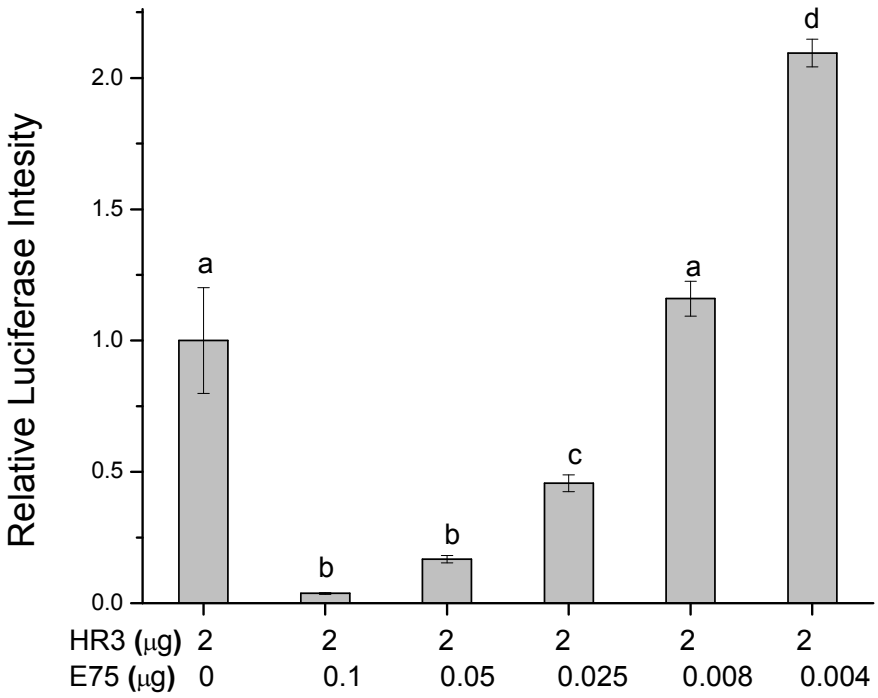


Figure 6. Concentration-response analyses of the transcriptional activity of DpHR3. Values under the x-axis represent the amount of each expression construct (pMT-HR3) transfected. Data bars represent mean ± SD (n=3). Transfection groups not connected by the same letter are significantly ($p \leq 0.05$) different (ANOVA, Tukey-Kramer method).

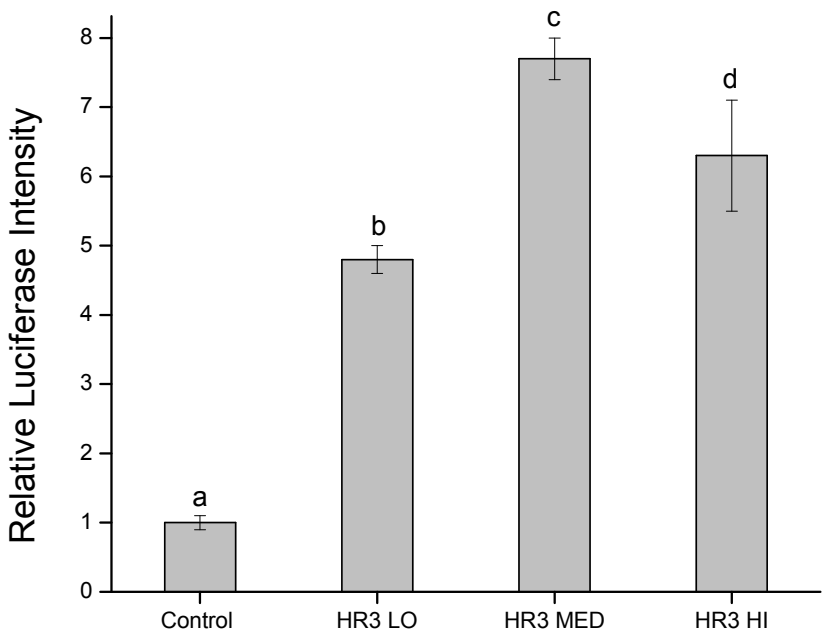
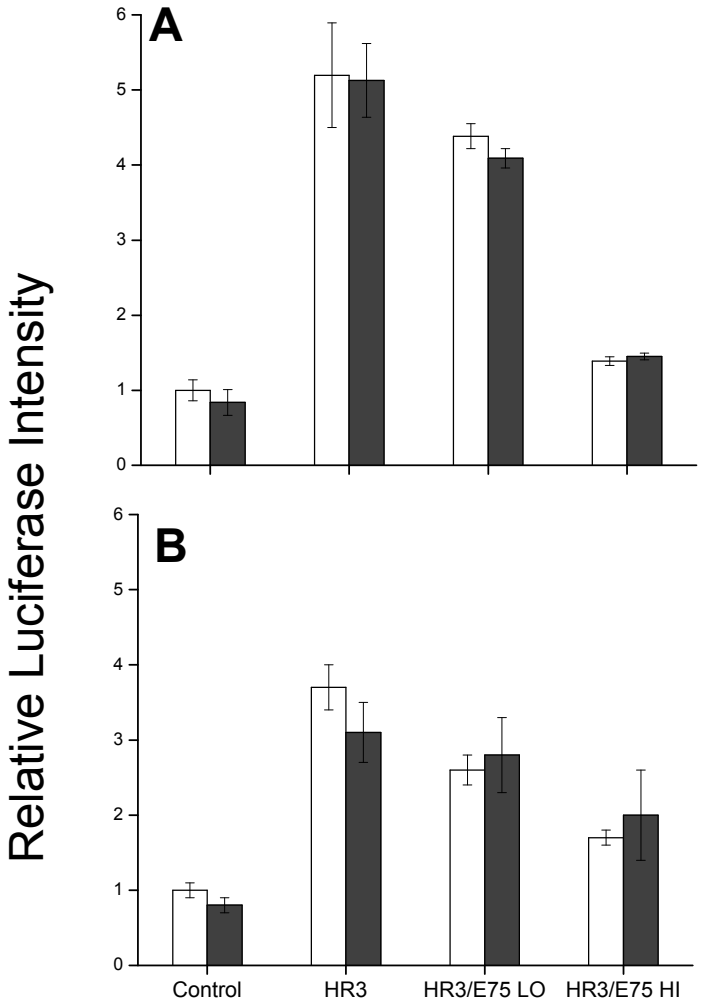


Figure 7. Concentration-response analyses of the effect of DpE75 on DpHR3 transcriptional activation. Values under the x-axis represent the amount of each expression construct (pMT-E75 or pMT-HR3) transfected. Data bars represent the mean± SD (n=3). Transfection groups not connected by the same letter are significantly ($p \leq 0.05$) different (ANOVA, Tukey-Kramer method).

Figure 8. DpE75 regulation of DpHR3 transcriptional activation in the absence (open columns) and presence (filled columns) of the NO donor DETA-NO (400μM). Amount of expression plasmid DNA transfected for each group was: 1.01 μg empty vector in control group, 1μg pMT-HR3 in HR3 alone group, 0.005 μg pMT-E75/1μg pMT-HR3 in E75/HR3 LO group, and 0.01 μg pMT-E75/1μg pMT-HR3 in E75/HR3 HI group. Panel **A** represents cells transfected with pMT-E75 (-403 nucleotides) and panel **B** represents cells transfected with pMT-E75 (full length). Data bars represent mean± SD (n=3). NO had no significant effect ($p<0.05$, Students *t*-test) on transcriptional activity under any of the treatment conditions.


A
B
Relative Luciferase Intensity
0
1
2
3
4
5
6
Control
HR3
HR3/E75 LO
HR3/E75 HI

SUMMARY AND CONCLUSIONS

Over the past two decades, contamination of the water supply by hormonally active compounds has been of significant concern to the scientific community. Studies related to this occurrence typically focus on biological consequences of steroid hormones and the resulting endocrine disruption experienced by exposed organisms. Sources of these hormones include waste from human pharmaceuticals which enter water bodies through municipal wastewater effluent. Additionally, contamination comes from veterinary pharmaceutical waste or concentrated animal feeding operations (CAFOs) where animals injected with steroid hormones release these compounds which are carried into surrounding waters through rain-water runoff. In the present study, we similarly evaluated potential endocrine disrupting properties of field runoff contaminants. However, in contrast to classical hormones, the chemicals of interest in this study, nitrate and nitrite, are not generally recognized as hormones. Instead, as demonstrated in the present study, nitrates and nitrites can be transformed to a potent signaling molecule, nitric oxide, which could have profound implications for the reproductive health of exposed wildlife.

Nitrate and nitrite (jointly referred to as NO_x) were first suggested to be potential endocrine disrupting chemicals with consequences to exposed wildlife in a commentary by Guillette and Edwards, (2005). These authors propose that wildlife reproductive endocrine disruption is linked to nitrate contaminant exposure in aquatic environments. They provide many examples of studies that lend support to this hypothesis. They propose that nitrates and

nitrites must be considered when determining the cause and mechanisms behind population or species declines. Furthermore, determining the mechanism of action for nitrate and nitrite impacts on reproductive health is critical in order to: 1) determine if these contaminants are, in fact, acting upon the endocrine system and 2) predict the susceptibility of different species to these compounds by determining if the mechanism is applicable to the physiology or genomic makeup of the species of concern. The research presented in this work attempts to determine the mechanism for nitrate and nitrite-associated developmental and reproductive toxicity in an ecologically relevant aquatic species, *Daphnia magna*.

The research in Chapter One focuses on characterizing toxicity associated with exposure of daphnids to NO_x. We determined the concentration-response profiles resulting from chronic exposure to nitrate, nitrite and a nitric oxide (NO) donor for both abnormal embryo development and fecundity. The toxicity associated with nitrate and nitrite exposure was consistent with anti-ecdysteroid-induced toxicity, suggesting that the action of this steroid hormone was compromised. Furthermore, this toxicity was also consistent with NO-induced toxicity, suggesting that toxicity was a result of nitrate and nitrite conversion to NO. Therefore, we hypothesized that NO, derived from nitrate and nitrite, causes reproductive and developmental toxicity by interfering with ecdysteroid signaling. This raised the question: are arthropod cells capable of converting NOx to NO? We demonstrated that arthropod cells (*Drosophila* S2) are capable of converting NO_3^- and NO_2^- to NO in a substrate concentration-dependent and cell density-dependent manner. Finally, we confirmed that developmental toxicity associated with NO_2^- could be attributed to NO because

administration of the NO-scavenger ß-carotene (Arroyo *et al.* 1992) offered significant protection to developing daphnids as was also observed using the NO-donor compound.

Nitrate and nitrite conversion to NO in rats decreased corticosterone and testosterone levels, by binding and inhibiting the action of cytochrome P450 enzymes involved in steroidogenesis (Panesar and Chan 2000). Additionally, Maniere et al. (2003) demonstrate increased cGMP concentrations and subsequent decreased ovarian ecdysteroid biosynthesis in response to NO donor exposure in the blowfly *Phormia regina.* Based on the apparent anti-ecdysteroid phenotype of NO_x-exposed daphnids, we hypothesized that steroidogenesis of ecdysteroid was similarly compromised by NO_x-derived NO. We measured ecdysteroid levels in daphnids following exposure to a NO donor. We were unable to detect significant, consistent decreases in 20-hydroxyecdysone levels in parental organisms or neonates under various exposure scenarios.

Next, we considered alterative targets for the effects of NO on ecdysteroids signaling. We hypothesized that NO_x-derived NO interferes with ecdysteroid signaling by binding and altering the function of a component of the pathway. We identified the nuclear receptor E75 (of the NRD1 group) as a likely candidate for interactions with NO. The E75 ortholog in insects binds a heme moiety in the ligand binding domain (LBD) (de Rosny *et al.* 2006; Reinking *et al.* 2005). Nitric oxide modulates the regulatory function of the receptor through binding to this heme group (Reinking *et al.* 2005). Therefore, we characterized daphnid E75 to determine if the receptor might serve as a target for NO binding.

Little is known about the action of E75 and its nuclear receptor heterodimer partner, HR3, in crustacean ecdysteroid signaling. The intent of the research presented in Chapter Two was to clone E75 and HR3 from *Daphnia magna*, evaluate expression of both receptors throughout growth and development, and determine the pattern of responsiveness for each receptor to ecdysteroids. We successfully cloned these receptors and determined that the predicted protein sequences for both receptor proteins share common structural features with insect and human orthologs. E75 additionally shared high sequence identity with a crustacean ortholog from the land crab *Gecarcinus lateralis*, indicating that the findings in this study are potentially applicable to other crustaceans. The high degree of identity in the DNA-binding domain region of both HR3 and E75 with the respective insect orthologs suggests that, as in insects, these receptors can bind to the retinoid orphan receptor element (RORE) as monomers. Additionally, the ligand binding domain of daphnid E75 contains conserved histidine and cysteine residues, shown to bind the heme group in both the fruitfly (*D. melanogaster*) (de Rosny *et al.* 2006; Reinking *et al.* 2005) and human (*H. sapiens*) (Raghuram *et al.* 2007; Yin *et al.* 2007) orthologs, indicating that *D. magna* E75 may similarly bind heme.

Expression profiles E75 and HR3 mRNA from *D. magna* were determined in molt-synchronized organisms and through embryo development. While E75 levels remained relatively constant through the molt cycle, HR3 mRNA levels were significantly elevated with a peak occurring coincident with a previously documented pre-molt surge of 20-hydroxyecdysone (Martin-Creuzberg *et al.* 2007). HR3 levels also were highly elevated

early in embryo development and progressively declined commensurate with the progressive decline in 20-hydroxyecdysone levels through embryo development (Mu and LeBlanc 2004). In contrast, E75 mRNA levels were present at low levels, as compared to HR3, early in embryo development and minimally declined through development. Taken together, these results suggest that HR3 is significantly induced by 20-hydroxyecdysone, while E75 is minimally responsive to this hormone. Indeed, exposure of molt-synchronized daphnids to exogenous 20-hydroxyecdysone significantly induced HR3 and minimally induced E75 mRNA levels above basal expression, confirming that these receptors are responsive to ecdysteroid.

The study presented in Chapter Three was performed to functionally characterize daphnid E75 and HR3 in regards to regulating gene transcription. Moreover, the intent of this study was to determine if NO alters this regulatory activity, thereby implicating this point in the ecdysteroid signaling pathway as a target for toxicity associated with exogenous NO exposure. These experiments were performed with *Daphnia pulex* whose genome had been recently sequenced. In this study, the *Daphnia pulex* E75 and HR3 genes were cloned and sequenced. We determined that the protein sequences for these genes in *D. pulex* maintain high identity with the protein sequences from *D. magna*, indicating that structure and perhaps function is preserved between the two *Daphnia* species.

A reporter assay consisting of a luciferase reporter gene driven by the RORE was constructed to determine whether *D. pulex* HR3 or E75 transactivated RORE-driven gene expression. D. *pulex* HR3 activated the luciferase reporter in a ligand-independent fashion;

whereas, E75 was not able to activate transcription of the reporter. When cotranfected, *D. pulex* E75 suppressed the transcriptional activation of the reporter by HR3 in an E75 concentration-dependent manner. At the lowest level of E75 co-transfected with HR3, reporter activation was actually increased above that of HR3 transfected alone. This result was unexpected because E75 transfected alone showed no activation of the reporter, indicating that an additive effect was unlikely. Possible explanations for this increased activity of HR3 include: (1) high levels of HR3 (i.e., control) were toxic to the cells causing reduced transactivation of the reporter. E75 ameliorated this toxicity and increase transcriptional activation by HR3; or (2) E75 reduced the degradation of HR3 resulting in elevated HR3 activity at non-suppressive concentrations of E75. Both possibilities are under continued evaluation. Finally, we assessed the ability of NO to reverse the E75 suppression of HR3 transcriptional activation. Experiments revealed no evidence that NO regulates the activity of E75 in daphnids. This result is interesting, in that it indicates that regulation of these receptors differs between crustaceans and insects. However, based on this conclusion, the mechanism by which NO_x-derived NO causes anti-ecdysteroid remains undetermined.

Alternative candidate mechanisms of NO_x-induced anti-ecdysteroid toxicity exist that should be addressed in future research. Nitric oxide readily reacts with the iron of heme proteins and therefore, often serves as a high affinity ligand for heme-containing proteins (Jain and Chan 2003). Therefore, identification of alternative heme binding nuclear receptors, enzymes or cofactors involved in ecdysteroid signaling may facilitate identification of other likely targets for the action of exogenous NO. Alternatively, Champlin and Truman (2000)

demonstrate that local NO production and ecdysteroid signaling operate reciprocally via a non-genomic pathway to tightly regulate coordination of optic lobe development in the moth, *Manduca seta*. Exogenous NO may target and deregulate a similar mechanism for development in daphnids.

Conversely, nitrite as opposed to nitric oxide could be responsible for altering ecdysteroid signaling, potentially through direct binding and subsequent inhibition of the ecdysteroid receptor. In daphnids, the potency of the nitric oxide donor and nitrite were comparable, whereas nitrate was a much less potent inducer of anti-ecdysteroid-like abnormalities. Additionally, nitrite is an oxidation or breakdown product of NO. Therefore, the observed toxicity in daphnids may potentially have been a result of nitrite action in all three treatment groups, where in addition to direct nitrite treatment; both nitrate cellular conversion and nitric oxide breakdown yield nitrite. Veselik et al. (2008) demonstrate that both nitrite and nitrate-derived nitrite interacts with the LBD of the estrogen receptor-alpha (ER-α) in human breast cancer cells. Therefore, toxicity in daphnids could be a result of interactions of nitrite with the LBD of susceptible nuclear receptors in the ecdysteroid signaling pathway. More information about ecdysteroid signaling in daphnids is necessary to test these alternative mechanisms of NO_x action.

Overall, this research underscores the deficiency that exists in our understanding of endocrine signaling for species potentially targeted by environmental endocrine disrupting contaminants. While great strides have been made in the area of endocrine disruptor research, a misconception persists that many contaminants (such as NO_x) pose no threat to

the health of exposed organisms under currently accepted safe levels. However, assessment of risk associated with exposure to these chemicals is often incomplete because the lack of knowledge on the endocrinology of many economically and ecologically important aquatic species (including crustaceans) leads to an inadequate representation of susceptible species. If toxicity testing endpoints exclude these susceptible species, the potential result is underestimation of risk and many undetected instances of endocrine disruption due to under-regulated environmental contaminant exposures.

Nitrate and nitrite are widespread aquatic contaminants for which levels in the environment continue to rise. Over the past decade, warning signs related to the potential underlying endocrine toxicity of these contaminants have developed through studies that identify negative consequences on reproduction and development in exposed wildlife. The present study not only demonstrates that direct exposure of the model crustacean species to nitrate and nitrite results in developmental and reproductive toxicity, but also demonstrates the necessity for further investigation of invertebrate endocrinology to define the mechanism of toxicity. Continued efforts of this type will strengthen toxicity testing for risk assessment of NO_x through improved mechanistic understanding and may therefore potentially support rationale for tighter regulation of these compounds.

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