ABSTRACT

RIDER, CYNTHIA. Development and Application of an Integrated Addition and Interaction Model of Mixture Toxicity (under the direction of Gerald A. LeBlanc)

The focus of this work was to elucidate general principles of chemical interactions among mixture constituents using mathematical modeling approaches. Mixtures of diverse acting chemicals displaying both toxicokinetic and toxicodynamic interactions were assessed. Endpoints measured in these assessments ranged from acute toxicity to molecular adaptive responses in *Daphnia magna*.

An Integrated Addition and Interaction (IAI) model of mixture toxicity was constructed and validated using a ternary mixture of organophosphates (malathion and parathion) and the P450 inhibitor piperonyl butoxide. Individual chemical concentration-response parameters and binary interaction data were used in the model. Modeled data was compared to experimentally derived data from *Daphnia magna* acute toxicity assays. The IAI model provided a good fit to the data. Results indicated that toxicokinetic interactions could be quantified and incorporated into mixture toxicity models.

In chapter two, we used the Integrated Addition (IA) model to assess a quaternary mixture of organophosphates and polar narcotic chlorophenols. Deviation of experimental results from IA model predictions indicated a slightly synergistic interaction between the two chemical groups. Mechanistic experiments implied that the interaction was toxicodynamic. The

interaction was quantified and incorporated into an IAI model, which provided a better fit to the data than models of simple additivity. We developed original approaches for assessing toxicodynamic interactions and incorporating them into a modeling framework.

In the next phase of the program, we identified and characterized a molecular endpoint for use in mixture modeling. In chapter three, we explored endocrine-mediated stress signaling in daphnids towards this end. We determined that juvenoid hormones induce hemoglobin production at both the mRNA and protein levels. Comparison of hemoglobin induction to a classic model of sex determination in daphnids indicated that both processes are controlled by the methyl farnesoate-mediated stress-signaling pathway. Hemoglobin expression in daphnids provided a promising molecular endpoint for use in mixture toxicity assessments.

Based on findings in chapter three, we focused more specifically on hemoglobin 2 (hb2) as a tool to further elucidate the methyl farnesoate signaling pathway in daphnids. We identified the cis-acting juvenoid response element (JRE) that is likely responsible for activating the hb2 gene in response to juvenoids. This sequence resembled known monomeric nuclear receptor response elements. The JRE was used to capture a specific protein that may prove to be the juvenoid receptor. We identified hb 2 as a highly inducible molecular endpoint which responds to multiple signaling pathways. These characteristics make hb2 an ideal endpoint for mixture toxicity analyses.

In chapter five, induction of the hb2 gene was used to model molecular interactions of chemical mixtures. We demonstrated that the ubiquitous herbicide atrazine induced hb2

gene expression, while having no obvious relatedness to juvenoid hormones. Additive effects of binary mixtures of atrazine and the juvenoid pyriproxyfen were modeled and experimentally assessed to determine whether atrazine induced the hb2 gene via the juvenoid signaling pathway. Results indicated that atrazine was not inducing hb2 through this endocrine pathway. We demonstrate that an adaptive molecular response can be accurately modeled using standard additive approaches.

In summary, mathematical models of mixture toxicity can be used to predict the joint effects of diverse chemicals displaying interactions, identify unexpected interactions, and elucidate potential mechanisms of interaction.

DEVELOPMENT AND APPLICATION OF AN INTEGRATED ADDITION AND INTERACTION MODEL OF MIXTURE TOXICITY

by

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Chair of Advisory Committee

This work is dedicated to my husband, my sister, my parents, and my little brown dog for their support, encouragement, and love

BIOGRAPHY

Cynthia Rider was born on July 4th, 1976 in New Port News, Virginia. Her nomadic parents moved the family from Virginia to Slidell, LA by way of Providence, RI, Houston, TX and Kwajalein in the Marshall Islands. Cynthia was approximately eight years old when she decided that she wanted to be an environmental scientist, although she was not exactly sure what that entailed until much later. She received her B.S. degree at Tulane University, New Orleans, LA in Environmental Studies with a coordinate major in Ecology, Evolution, and Organismal Biology in 1994. Through the wonderful work study program at Tulane University, Cynthia had the opportunity to hold a number of interesting jobs. She was a library assistant in the Latin American library, a second grade teacher's assistant in a New Orleans public school, and a lab assistant in Dr. John McLachlan's Environmental Endocrinology lab at the Center for Bioenvironmental Research at Tulane and Xavier Universities. She worked under the direction of a then post-doc, Dr. Ann Cheek, who was instrumental in her development as a scientist. Ann later put Dr. Gerald LeBlanc at North Carolina State University in touch with Cynthia when he was in need of a lab technician. At that time, Cynthia was living on the couch in her sister's one bedroom apartment in Austin, TX contemplating what to do with her life. Cynthia worked in Dr. LeBlanc's Environmental Endocrine Toxicology lab as a technician for two years. During this time, she met her future husband in a kickboxing class. In 2001, she began her work in the Environmental and Molecular Toxicology program at North Carolina State University. The product of the subsequent four and a half years is contained in the following work.

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INTRODUCTION

Mixtures of chemicals from anthropogenic sources are ubiquitous. In a national survey of water quality, USGS scientists detected mixtures of two or more chemicals in 75% of streams sampled (Kolpin et al, 2002) and in 47% of groundwater samples (Squillace et al, 2002). Accordingly, regulatory agencies (e.g. ATSDR, NIOSH, and US EPA) have been working towards development of methods for assessing mixture toxicity (Monosson, 2005; US EPA, 1986; US EPA, 2000; ATSDR, 2004). The infinite number of potential mixture formulations precludes direct assessment of mixture toxicity as the sole method for hazard characterization. Modeling approaches offer a practical complement. Mathematical models incorporate data from individual chemical exposures to predict mixture toxicity. These models are flexible and can be used to rapidly generate large amounts of data to test hypotheses regarding mixture toxicity. However, the incorporation of chemical interactions into mathematical models to account for deviation from standard additivity has been problematic (Hertzberg and MacDonnell, 2002; Borgert, 2004). Developing reliable methods for assessing toxicity of mixtures containing chemicals that display interactions would significantly enhance our ability to predict hazard associated with mixtures.

Mathematical Model Background

Mathematical models of mixture toxicity have the potential to be extremely useful tools in ecological risk assessment. Rigorous validation contributes to the reliability and accuracy of these models. Careful consideration should be given to the test organisms and experimental design used in validation procedures. Additionally, environmental relevance should be at the

forefront of test system selection. Researchers have used a number of species to assess mathematical models of mixture toxicity including fish (Könemann, 1981; Hermens and Leeuwangh, 1982), microcrustaceans (Hermens et al, 1984; Olmstead and LeBlanc, 2005), algae (Walter, 2002; Faust, 2003), and bacteria (Backhaus, 2000; Altenburger, 2000). These species display a wide range of organizational complexity, as well as compatibility to high-throughput testing. The microcrustacean, *Daphnia magna*, lies in the middle of the spectrum of model organisms. It is a complex organism containing organs and discrete systems, yet it is small, exhibits high fecundity, and is easily cultured in the lab. Additionally, daphnids are a key component of aquatic ecosystems. Most importantly, there is a wealth of data available on the toxicity of individual chemicals to daphnids, which could be applied to mixture toxicity modeling. Taken together, these factors make daphnids an ideal test organism with which to build and validate mathematical models of mixture toxicity.

Concentration addition and response addition are the dominant concepts underlying mathematical mixture toxicity models (Greco, 1992). Concentration addition applies to mixtures of chemicals with the same or similar mechanisms of action, while response addition pertains to chemicals with different mechanisms of action. Concentration addition, originally described by Loewe and Muischnek (1926), contends that concentrations of chemicals with the same mechanism of action can be added once their different potencies are accounted for. Concentration addition is similar to the toxic equivalency factor approach often associated with dioxin congeners (Safe 1990). The concept of concentration addition is represented by

$$\sum_{i=1}^{n} \frac{C_i}{ECx_i} = 1$$

where C_i is the concentration of the individual chemical i and ECx_i is the concentration of the chemical that produces an effect equal to that of the entire mixture. The concentration addition model has been used to accurately predict the toxicity of mixtures of like-acting chemicals (Könemann, 1981; Altenburger, 2000; Faust, 2001).

Bliss (1939) developed the model of response addition, also known as independent joint action. According to this concept, individual chemicals act at different target sites that lead to the same measured response. In effect, individual chemicals elicit a certain response level independent of other chemicals in the mixture. Although biochemically rational, there has been debate surrounding the likelihood that parallel processes could be completely independent given the complex and interconnected nature of whole organisms (Hermens and Leeuwangh, 1982). The concept of response addition is based on probability theory and is expressed as:

$$R_{mix} = 1 - \prod_{i=1}^{n} (1 - R_i)$$

Where R_i represents the response elicited by individual chemical i and R_{mix} corresponds to the mixture response. Many researchers have found that the toxicity of dissimilarly acting chemical mixtures is better characterized using response addition than using concentration addition (Backhaus 2000; Walter, 2002; Faust, 2003).

Selection of a universal model for mixture toxicity has been an important scientific goal. Berenbaum (1985) advocated the application of concentration addition to all mixtures. Drescher and Bodecker (1995) found that when individual chemicals display typical concentration-response relationships, the concentration addition model generally predicted greater mixture toxicity than response addition. They concluded that concentration addition provides more

conservative estimates of mixture toxicity than response addition. Consequently, the US EPA (2000) recommends using concentration addition as the basis for determining the toxicity of non-interacting chemical mixtures. This simple and conservative approach provides a reasonable starting point for the regulatory purpose of hazard characterization. The greater scientific goal, however, is to develop more accurate modeling approaches that are based on solid mechanistic ground. Towards this end, several researchers have combined concepts of concentration and response addition into a comprehensive model that explicitly considers the mechanisms of toxicity for individual mixture constituents (Teuschler *et al*., 2004; Altenburger *et al.*, 2005; Olmstead and LeBlanc, 2005).

The Integration of Additivity Models

Combining concentration and response addition represents a paradigm shift in mathematical mixture modeling. Previously, concentration addition and response addition were regarded as the two available alternatives for calculating non-interacting mixture toxicity. In the 1992 Saariselkä Agreement, a group of six prominent mixture scientists reached a consensus that both concentration and response addition were logical and reasonable reference models for determining the toxicity of non-interacting mixtures (Greco, 1992). The Integrated Addition model represents advancement because it does not require the researcher to choose the "more correct" model, but instead provides a mechanistically-directed application of both addition models.

The model of Integrated Addition proposed by Olmstead and LeBlanc (2005) and a similar approach by Altenburger et al. (2005) are conceptually indistinguishable and differ only

in their data fitting methods. Altenburger et al. (2005) used several different models to fit individual chemical toxicity data, whereas Olmstead and LeBlanc (2005) used a logistic fit exclusively. Therefore, the following general description applies to both models. The first step in this mixture toxicity modeling approach is to clearly characterize individual chemical concentration-response relationships. Mixture constituents which have the same mechanism of action are grouped into cassettes. A concentration addition model is then used to calculate the toxicity of the chemicals within each mechanism-based cassette. Finally, the overall mixture toxicity is calculated by combining the toxicities of the cassettes using response addition. The Integrated Addition model has been used to accurately predict the toxicity of a wide range of non-interacting mixtures containing constituents with diverse mechanisms of action (Altenburger, 2004; Altenburger, 2005; Olmstead and LeBlanc, 2005). This approach provides a solid foundation for characterizing chemical interactions in mixtures. In effect, the presence, intensity, and direction (synergistic or antagonistic) of an interaction can be determined in relation to this baseline.

Chemical Interactions

Toxicology has traditionally focused on single chemical exposures; therefore, interaction data is relatively sparse. In an exhaustive assessment of available interaction data, Borgert et al (2001) provided criteria for determining the suitability of interaction studies for use in mixture toxicity risk assessments. The criteria for acceptable data included: selection of environmentally relevant concentrations, clear definition of "no interaction" (i.e. additivity), and the use of statistical methods for determining whether mixtures deviate from additivity. In a review of current problems with interaction assessments, Hertzberg and MacDonnell (2002) recommended

that researchers should abandon attempts to identify individual cases of "synergy" or "antagonism" and instead work towards elucidation of basic tenants of mixture toxicity based on mechanistic principles. Durkin and colleagues (1995), for example, attempted to develop methods for identifying classes of chemicals that display consistent interactions. Haddad et al. (2000) explored whether data from binary exposures could be incorporated into models of more complex mixtures. They found that interactions quantified in binary exposures were maintained in higher order mixtures.

Chemical interactions can be classified mechanistically as toxicokinetic or toxicodynamic (Andersen and Dennison, 2004). Toxicokinetic interactions occur when one chemical changes the concentration of a second chemical at its site of toxicity. For example, one chemical can induce an enzyme that metabolizes a second chemical, thereby decreasing the active concentration of the second chemical. Toxicodynamic interactions occur when a chemical interferes with the response elicited by another chemical. Toxicokinetic interactions are thought to be more prevalent than toxicodynamic interactions. Examples of interactions occurring in mixtures include enhanced hepatotoxicity of carbon tetrachloride with kepone pre-exposure (Klingensmith and Mehendale, 1982) and endocrine-related interactions between hormone receptor antagonists and hormone synthesis inhibitors (Mu and LeBlanc, 2004). We know that chemical interactions occur in mixtures, the issue is how to account for these interactions in mathematical models of mixture toxicity.

Bliss (1939) and Finney (1945) initially discussed the incorporation of modifying factors into mixture toxicity calculations to describe chemical interactions. However, they did not

clearly convey the nature of these modifying factors or methods for obtaining them. The current EPA recommended method for incorporating interactions into mixture analysis is based on a weight of evidence approach described by Mumtaz and Durkin (1995) and expanded upon by Hertzberg (1999). Available interaction data is used to estimate the magnitude of the interaction. This term (measured or a default value of five) is dependent upon the concentration of the interacting chemical and is used to modify the effective concentration of the target chemical under the presumption of a toxicokinetic interaction. This approach is useful for predicting chemical combinations that could be hazardous, but it does not provide a precise quantification of the mixture toxicity. A quantitative approach that incorporates interactions into mixture toxicity models was described by Mu and LeBlanc (2004). This approach used experimentally derived coefficients of interaction, called K-functions, to modify the concentrations of the target chemicals, again, under the presumption of a toxicokinetic interaction. Mu and LeBlanc (2004) were able to accurately model the toxicity of an interacting binary mixture of endocrine active chemicals using this approach.

Endocrine Toxicity

Mixtures of endocrine disrupting chemicals in the environment warrant special attention (Squillace et al, 2002). Endocrine systems perceive and respond to very subtle changes in endogenous hormone activity (Colborn and Thayer, 2000). Therefore, it is conceivable that relatively low concentrations of endocrine-active compounds could disrupt normal endocrine function. The use of sub-toxic endpoints that are responsive to endocrine-active chemicals in mixtures analysis is one way to ensure that potentially harmful chemical combinations are detected before overt population level effects occur. Early in this research program, we found

that hemoglobin accumulation in daphnids is hormonally regulated. Accordingly, daphnid hemoglobin expression could serve as a molecular indicator of endocrine disruption in invertebrates.

Hemoglobin

The accumulation and increased oxygen affinity of hemoglobin in daphnids exposed to low oxygen conditions are well-studied phenomena (Fox and Phear, 1953; Kobayashi *et al.*, 1988; Zeis *et al.*, 2003). Accumulation of hemoglobin in daphnids leads to a visible color change from pale to red. Daphnids that are able to accumulate hemoglobin in low oxygen conditions demonstrate increased performance as compared to their uninducible counterparts (Pirow et al., 2001). Increased levels of hemoglobin in daphnids correspond to higher respiration and filtration rates, which in turn allow daphnids to continue functioning normally in low oxygen conditions (Fox *et al.*, 1951; Koh *et al.*, 1997). Hemoglobin accumulation also has implications for population survival. In a model of the population-level effects elicited by physical (increased temperature and low dissolved oxygen) and chemical stressors in *Daphnia magna*, Koh et al. (1997) incorporated hemoglobin as an adaptive response.

In addition to observations of the physiological effects of hemoglobin accumulation in daphnids, much is also known about its molecular properties. Hemoglobin exists in daphnids as an extracellular respiratory protein synthesized in the fat cells and epipodites, or leg appendages (Goldmann *et al.*, 1999). Daphnid hemoglobin is a two-domain protein composed of multiple subunits that combine to form different species of hemoglobin depending on oxygen conditions (Kimura *et al.*, 1999; Zeis *et al.* 2003). Additionally, three of the four identified hemoglobin

genes in *Daphnia magna* are fully sequenced (Tokishita *et al.*, 1997; Kimura *et al.*, 1999). The extensive knowledge of hemoglobin structure and function combined with its dramatic inducibility and link to population survival under low oxygen conditions, make hemoglobin an ideal molecular endpoint for use in mixture analyses.

Research Outline

The hypothesis was tested in this work that mixtures of diverse acting chemicals displaying various interactions could be accurately modeled using an Integrated Addition and Interaction approach. The model was evaluated using a range of chemicals and measured endpoints. Experiments were designed to elucidate principles of chemical interactions in mixtures.

In chapter one, we used a ternary mixture of two organophosphates (malathion and parathion) and the P450 inhibitor piperonyl butoxide to construct and validate an Integrated Addition and Interaction model of mixture toxicity. First, we characterized individual chemical concentration response relationships. We then assessed the antagonistic effect of piperonyl butoxide on the toxicity of each of the organophosphates in binary combinations. We confirmed that the organophosphates inhibited acetylcholinesterase activity in daphnids and showed that piperonyl butoxide abrogated this effect. The interactions were quantified and applied to the model as K-functions that modified the concentrations of each of the organophosphates in the mixture. Finally, we validated the model by comparing actual data generated from a series of thirty formulations of the ternary mixture to modeled data. We found that the Integrated Addition and Interaction model provided a good fit to the data. In this study, we furthered the

fledgling practice of quantitatively incorporating interactions into mathematical models of mixture toxicity.

In chapter two, we used the Integrated Addition and Interaction model to elucidate the mechanism of an unforeseen synergistic interaction between organophosphates and chlorophenols. We demonstrated with experimental data that application of inappropriate additivity models (i.e. concentration addition or response addition) evoked false conclusions regarding the type and magnitude of the observed interaction. Mechanistic studies suggested that the interaction was toxicodynamic in nature. K-functions were generated describing the effect of organophosphates on chlorophenol toxicity. These K-functions were applied to the Integrated Addition and Interaction model to describe a toxicodynamic interaction. Model predictions were compared to data generated from thirty formulations of the quaternary mixture. The Integrated Addition and Interaction model incorporating a toxicodynamic interaction between cassettes provided a better fit to the data than models of simple additivity.

The next goal of the program was to determine the ability of the Integrated Addition and Interaction model to evaluate additive effects at the molecular level. In chapter three, we explored endocrine-mediated stress signaling in daphnids in an effort to identify appropriate molecular endpoints for use in mixtures modeling. We determined that juvenoid hormones induce hemoglobin production at both the mRNA and protein levels. Further, we found that different juvenoids exhibited corresponding potencies with regards to hemoglobin induction and stimulation of male offspring production. A series of daphnid clones that either had, or did not have, the ability to produce male offspring were tested for their ability to accumulate hemoglobin

following exposure to the juvenoid hormone methyl farnesoate. We concluded that hemoglobin is induced by the same methyl farnesoate-mediated stress-signaling pathway that controls sex determination in daphnids.

In chapter four, we used hemoglobin 2 (hb2) as a tool to further elucidate the methyl farnesoate signaling pathway in daphnids. We identified the cis-acting juvenoid response element (JRE) that is likely responsible for activating the hb2 gene in response to juvenoids. Electrophoretic mobility shift assays (EMSA) and transcription experiments were used to identify a six base nucleotide sequence (commonly known as a nuclear receptor half-site) separated by a one nucleotide spacer to an eleven nucleotide A/T sequence that binds the putative juvenoid nuclear receptor following activation by juvenoid hormones. This JRE resembles response elements known to bind monomeric nuclear receptors. A nucleotide probe was used to capture and precipitate a candidate protein that may prove to be the juvenoid receptor. This research identified a site on the hb2 gene that could be used to model and experimentally evaluate the combined action of chemicals that may target a common molecular endpoint.

In chapter five, induction of the hb2 gene was used to model molecular interactions of chemical mixtures. Prior experiments demonstrated that juvenoid hormones induced the hb2 gene via the JRE. In the present study, we demonstrated that the ubiquitous herbicide atrazine induced hb2 gene expression, while having no obvious relatedness to juvenoid hormones. Additive models of joint action, as used in previous chapters, were exploited to evaluate whether atrazine induced the hb2 gene via the juvenoid signaling pathway. We hypothesized that if

atrazine induced gene expression through the JRE, the magnitude of the induction response of atrazine, in combination with the juvenoid hormone pyriproxyfen, would conform to a model of concentration addition. Conversely, if atrazine induced hb2 gene expression by some alternative signaling pathway, the combined actions of the two chemicals would better conform to a model of response addition. Results indicated that atrazine was not inducing hb2 via the JRE. These experiments also demonstrated that molecular interactions among mixture constituents can be modeled using conventional approaches.

In conclusion, mathematical models of mixture toxicity were used to successfully predict the toxicity of combinations of chemicals exhibiting a broad range of properties and mechanisms of action. Additionally, models were used to assess diverse endpoints that spanned the spectrum from general integrated effects (immobilization in daphnids) to specific molecular effects (hb2 induction). Experimental data were generated to develop and validate models and subsequently, models were used to generate mechanistic hypotheses. The combination of modeling and mechanistic studies contributed to our understanding of the combined action of chemicals in mixtures.

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An Integrated Addition and Interaction Model for Assessing Toxicity of Chemical Mixtures

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Abstract

The high propensity for simultaneous exposure to multiple environmental chemicals necessitates the development and use of models that provide insight into the toxicity of chemical mixtures. In this study, we developed a mathematical model that combines concepts of concentration addition, response addition, and toxicokinetic chemical interaction to assess toxicity of chemical mixtures. A ternary mixture of acetylcholinesterase inhibiting organophosphates (malathion and parathion) and the P450 inhibitor piperonyl butoxide was used to model toxicity. Concentration-response curves were generated for individual chemicals as well as for mixtures of the chemicals using acute toxicity tests with *Daphnia magna*. The toxicity of binary combinations of malathion and parathion adhered to the principles of concentration addition. The contribution of piperonyl butoxide to mixture toxicity was integrated using a model for response addition. Piperonyl butoxide also modified the toxicity of the organophosphates by inhibiting their metabolic activation. The antagonistic effects of piperonyl butoxide towards the organophosphates were quantified as coefficients of interactions (K-functions) and incorporated into the mixture model. Finally, toxicity of the ternary mixture was modeled at 30 different mixture formulations using three additive models that assumed no interaction (concentration addition, response addition, and integrated addition) and using the integrated addition and interaction (IAI) model. Toxicity of the 30 mixtures was then experimentally determined and compared to model results. Only the IAI model accurately predicted the toxicity of the mixtures. The IAI model holds promise as a means for assessing hazard of complex chemical mixtures.

Key words: synergy, cumulative toxicity, predictive model, toxicodynamic, hazard assessment, risk assessment

Introduction

Surveys of agricultural and urban streams and groundwater have brought public attention to widespread chemical mixture contamination (Kolpin, 2002; Battaglin, 2003). The infinite number of potential chemical combinations (in terms of both constituents and concentrations of constituents) limits the utility of standard toxicity testing methods for establishing hazard associated with chemical mixtures. Modeling approaches could augment the standard toxicity testing paradigm when evaluating hazards associated with exposure to chemical mixtures. Chemical constituents of a mixture can elicit similar action, dissimilar action, or interaction (Bliss, 1939; Cassee, 1998). Models of mixture toxicity have focused primarily on quantifying the "no-interaction" scenarios, while cases of interaction often appear as qualitative observations (Hertzberg and MacDonell, 2002). Concentration addition (Loewe additivity) and response addition (Bliss independence) (Greco, 1992) are commonly used to model the toxicity of non-interacting chemicals within a mixture.

Concentration addition models rely upon the assumption that mixture components contribute to toxicity through a common mechanism of action. Calculating mixture toxicity based upon concentration addition requires assessing the relative contribution of each constituent to the total toxicant pool. The toxicity of this pool is then modeled as a single toxicant. Concentration addition is the basis of the "toxic equivalency" approach commonly used to assess toxicity of chemicals of the same class such as dioxins (Safe, 1990). Ample evidence supports the use of the concentration addition model for assessing mixtures toxicity of like-acting chemicals (Könemann, 1981; Deneer, *et al*., 1988; Altenburger *et al*., 2000). The response addition model, also referred to as the independent joint action model, has been used to compute

toxicity of mixtures when chemical constituents have different mechanisms of action (Backhaus, 2000; Walter, 2002). In the response addition model, combined effects of the chemicals are based upon the probability that individual constituents of the mixture will affect the exposed organisms.

The concentration addition and response addition models are limited in their application to complex mixtures in that they do not address chemical interactions. Toxicokinetic interactions can occur between chemicals in which one chemical alters the effective concentration of another (Andersen and Dennison, 2004). Alternatively, toxicodynamic interactions can occur between chemicals in which one chemical influences the response of the organism to another chemical (Andersen and Dennison, 2004). Both toxicokinetic and toxicodynamic interactions can significantly impact the toxicity of chemical mixtures. The importance of addressing chemical interactions was highlighted by the US EPA in their recommendations for evaluating risk associated with chemical mixtures (US EPA, 2000).

Recently, Altenburger, Schmitt, and Schüürmann (2005) and Olmstead and LeBlanc (2005) demonstrated that concentration addition and response addition models could be integrated into a comprehensive model for use in evaluating toxicity of non-interacting chemical mixtures. The intent of the present study was to expand this approach to incorporate interactions among chemical constituents when they are predicted to occur. Important issues addressed in this work include: (1) evaluating whether single interaction modifiers can be applied to classes of chemicals and (2) establishing whether clearly-defined binary interactions persist in higher order combinations. The strength of the integrated addition and interaction (IAI) model was assessed

by comparing model results to experimentally-determined toxicity of 30 different derivations of a ternary mixture.

Materials and Methods

Daphnid culture

All toxicological experiments were performed with the daphnid *Daphnia magna.* Daphnids were acquired from long-standing cultures in our laboratory that were originally obtained from the US Environmental Protection Agency, Mid-Continent Ecology Division - Duluth, MN. Daphnids were maintained in reconstituted deionized water (192 mg/L $CaSO_4 \bullet H_2O$, 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 in 1 L beakers at a density of ~50 daphnids/L medium and culture medium was changed three times per week. Adult daphnids were discarded after three weeks and replaced with neonates. Culture beakers and all experiments were maintained in incubators with a 16/8 hour light/dark cycle at a constant temperature of 20°C. Culture daphnids were fed 2.0 mL (1.4×10^8 cells) of the unicellular green algae *Selenastrum capricornutum* and 1.0 mL (4 mg dry weight) of Tetrafin® fish food suspension (Pet International, Chesterfill, New South Wales, Australia). The *Selenastrum* was cultured in the laboratory using Bold's basal medium.

Acute toxicity assays

Chemicals used in mixture analyses (malathion, parathion, and piperonyl butoxide) were acquired from ChemServices (West Chester, PA). Absolute ethanol was used as the carrier for all of the chemicals. All toxicity assessments were initiated with neonatal (≤24 hours old)

daphnids. Each treatment consisted of two 50 mL beakers containing 40 mL of exposure medium and 10 neonates. *Selanastrum* (7 x 10^6cells) and fish food homogenate (0.2 mg dry weight) were provided to each beaker as food at the start of each exposure. All beakers, including controls, contained 0.01% carrier (ethanol). Beakers were labeled on the bottom and randomly rearranged, so that the exposure concentration in each beaker was not known to the investigator when assessing response of organisms. At 48 hours, neonates were evaluated for response. The response endpoint, immobilization, was judged by the inability of the neonate to occupy the water column during 10 seconds of observation.

Acetylcholinesterase analyses

Acetylcholinesterase activity was measured according to Ellman et al (1961) as modified for use with microtiter plates (Fisher *et al*., 2000) with minor additional modifications. Exposure groups consisted of three 250 mL beakers containing 200 mL solution and 40 neonates (≤24 hours old). Algae ($1.4x10^7$ cells) and fish food (0.4 mg dry weight) were added to each beaker once per day. Solutions were renewed at 24 hours. Following the 48-hour exposure period, neonates were transferred to 1.5 mL microfuge tubes. Media was removed from tubes; neonates were rinsed, and homogenized in 35 µL ice cold 0.02 M phosphate buffer, pH 8.0 with 1% Triton-X-100 using a Teflon pestle. An additional 315 µL phosphate buffer, pH 8.0 without Triton-X-100 was then added and samples were mixed. Samples were centrifuged at 14,000 g for 4 minutes at 4°C and supernatant was transferred to a clean pre-cooled microfuge tube. Approximately 100 µL of the supernatant was stored at -20°C for protein analysis. The following solutions were added to each well in a 96-well plate: 100 µL of 8 mM 5,5'-dithio-bis(2-nitrobenzoate) (D-1830 Sigma), 50 µL supernatant (phosphate buffer with 0.1% Triton-X-

100 was used for supernatant blanks), 50 µL of 16 mM acetylthiocholine iodide (A-5751 Sigma). Absorbance was measured kinetically for 15 minutes at 420 nm using a Fusion™ Universal Microplate Analyzer (PerkinElmer, Boston, MA). Protein was measured according to Bradford (1976) using Bio-Rad Protein Assay dye concentrate (Hercules, CA) and a standard curve generated with bovine serum albumin. The molar extinction coefficient (13,300 $M^{-1} \cdot cm^{-1}$) (Masson *et al*., 2004) was used to calculate the amount of yellow anion, 5-thio-2-nitrobenzoate, formed over 15 minutes and this rate was normalized to the amount of protein added to the assay (nmol/min/mg). Analyses of variance and Tukey-Kramer HSD were used to determine if significant ($P \leq 0.05$) differences existed between treatments.

Individual chemical toxicity

Exposure concentrations for each chemical were selected, based upon preliminary experiments that would span response levels from 0 to 100%. The percentage response was plotted against exposure concentration on a log scale and fit with a sigmoidal line using Origin™ software (Microcal™ Software Inc., Northampton, MA). The logistic equation representing the sigmoidal fit to the data is:

$$R = \frac{1}{1 + \left(\frac{EC50}{C} \right)^{\rho}} \qquad \text{Equation 1}$$

where R is the response (% immobilization), C is the chemical concentration, ρ is the power or slope of the curve, and $EC50$ is the exposure concentration eliciting immobilization in 50 % of exposed animals. These individual concentration-response curves were subsequently used in mixture modeling as described below.

Mixture modeling

Concentration addition According to Olmstead and LeBlanc's (2005) integrated addition model, like acting chemicals are assigned to a common cassette (i.e., grouping). Toxicity associated with the cassette is then calculated using a concentration addition approach. Accordingly, malathion and parathion were assigned to a common cassette, the organophosphate (OP) cassette. To establish whether the toxicity of the chemicals within the OP cassette conformed to a concentration addition model, five ratios (Table 2) of the chemicals (malathion:parathion) were each tested at six different concentrations. Parathion concentrations were expressed in terms of malathion equivalents. All five ratios were equitoxic based upon characterization of the toxicity of the individual OPs. The six concentrations of each binary mixture used in the experiments were selected to define the concentration-response curve for the mixture. The joint toxicity of these binary mixtures of like-acting chemicals was computed using the following equation (Olmstead and LeBlanc, 2005):

$$R = \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{C_i}{EC50_i}\right)^{\rho'}}} \qquad \text{Equation 2}$$

where R is the response to the mixture, C_i is the concentration of chemical i in the mixture, $EC50_i$ is the concentration of chemical i that causes a 50% response, and ρ' is the average power associated with the chemicals in the cassette. The average power was used because chemicals within a cassette should have similar slopes, as was the case with malation and parathion. Concentration-response results from each binary mixture were then used to calculate $EC50$ values as described for individual chemicals. Analyses of variance were performed to detect

significant ($P \leq 0.05$) differences among the five ratios using SAS 8.2 software (SAS institute, Cary, NC).

Response Addition The concept of response addition was used by Olmstead and LeBlanc (2005) to compute the joint toxicity associated with the different chemical cassettes within a mixture. The response addition model was used because each cassette is assumed to elicit a response through different mechanisms. The response addition model can be depicted as:

$$R = 1 - \prod_{i=1}^{n} (1 - R_i) \qquad \text{Equation 3}$$

where R represents the response to the mixture and R_i is the response to chemicals in cassette i.

Equations 2 and 3 were integrated to establish the response associated with individual cassettes within a mixture and to sum the responses associated with the cassettes (Olmstead and LeBlanc, 2005). The resulting equation is a combination of concentration and response addition equations:

$$R = 1 - \prod_{I=1}^{N} \left\{ 1 - \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{C_i}{EC50_i} \right)^{\rho'}}} \right\} \qquad \text{Equation 4}$$

Chemical interactions

The ability of one chemical in the mixture to modify the effective concentration of another was defined by coefficients of interactions or K-functions (Finney, 1942; Mu and

LeBlanc, 2004). Specifically, K-functions, defined the degree to which the concentration of PBO in the mixture altered the effective concentration (i.e., oxon metabolite) of either organophosphate in the mixture. K-functions were described by experimentally deriving the effect of concentrations of PBO on the EC50 values derived for each organophosphate. K-functions were calculated for each of the PBO concentrations with the following equation:

$$K = \frac{EC50_{OP}}{EC50_{OP+PBO_x}} \qquad \text{Equation 5}$$

where $EC50_{OP}$ is the concentration of organophosphate that immobilized 50% of the exposed animals and $EC50_{OP+PBOx}$ is the EC50 of the organophosphate when exposure occurred in the presence of x concentration of PBO. These K-functions were then plotted against the concentration of PBO from which they were derived. The logistic equation that defined this relationship was used to calculate K-functions when modeling mixture toxicity. K-functions were integrated into this model to describe toxicokinetic interactions between PBO and the organophosphates:

$$R = 1 - \prod_{I=1}^{N} \left\{ 1 - \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{k_{a,i}(C_a) \times C_i}{EC50_i} \right)^{\rho'}}} \right\} \qquad \text{Equation 6}$$

where $k_{a,i}$ represents a function describing the extent to which chemical a (PBO) present in the mixture at concentration C_a alters the effective concentration of chemical i (malathion or parathion).

The response to thirty combinations of the three chemicals was computed using the concentration addition model (equation 2), the response addition model (equation 3), the integrated addition model (equation 4) and the IAI model (equation 6). In addition, the actual toxicity of the 30 mixtures was measured and results compared to the four model results. The 30 mixture formulations were designed so that each of the three chemicals was present in a range of magnitudes in relation to the other two chemicals within the mixture (i.e., The ratio of the three chemicals varied among the mixture formulations). Model predictions were compared to experimental data using coefficients of determination (r^2; Zar, 1996). An r^2 value of 0.7 or greater was considered a good fit of the observed data to the model (http://www.qualityamerica.com/knowledgecente/knowctrCoefficient_of_Determination_R_S.htm).

Results

Individual chemical toxicity analyses

The IAI model requires toxicity description for the individual chemicals within a mixture. Concentration-response curves were generated for malathion, parathion and piperonyl butoxide (Fig. 1) from which EC50 values and corresponding 95% confidence intervals, and power of the curves (ρ) were derived (Table 1). The logistic equation provided a good fit to the malathion ($r^2 = 0.987$), parathion ($r^2 = 0.987$), and piperonyl butoxide ($r^2 = 0.998$) concentration-response data. The two organophosphates exhibited similar toxicity characteristics. Piperonyl butoxide was considerably less toxic as compared to the organophosphates and had a power approximately one-half that of the organophosphates.

Cassette assignment

According to the IAI model, the organophosphates would be assigned to the same cassette and toxicity associated with the cassette would be assessed using a concentration addition approach. The validity of using concentration addition to model the toxicity associated with the organophosphate cassette was determined using several combinations of the two organophosphates deemed to be equitoxic based upon concentration additivity. Indeed, the concentration-response assessments of these binary mixtures were statistically indistinguishable (Table 2). Therefore, the contributions of malathion and parathion to the toxicity of the final mixtures were modeled as a single organophosphate cassette.

The common mode of action of the organophosphates – the inhibition of acetylcholinesterase activity – was confirmed experimentally (Fig. 2). In contrast, piperonyl butoxide did not inhibit acetylcholinesterase activity. Piperonyl butoxide was, therefore, assigned to its own cassette where the toxicity of this mixture component was integrated into the toxicity of the mixture using the response addition model.

Chemical interaction

We hypothesized that piperonyl butoxide would interact with the constituents of the organophosphate cassette in a manner that would modify the toxicity associated with this cassette. The ability of piperonyl butoxide to abrogate the acetylcholinesterase-inhibiting potential of each organophosphate was demonstrated directly (Fig. 2). The antagonistic effect of piperonyl butoxide on the toxicity of the organophosphates was further demonstrated by the progressive shifting of the concentration-response curves for malathion (Fig. 3A) and parathion

(Fig. 3B). This modifying effect of piperonyl butoxide was quantified as concentration-dependent K-functions (Fig. 4). These K-functions were used in the final IAI model to modify the effective concentrations of malathion and parathion as dictated by the concentration of piperonyl butoxide in the mixture.

Mixtures toxicity assessment

The toxicity of 30 combinations of the ternary mixture (Table 3) was experimentally determined and compared to predicted toxicity using the concentration addition model (equation 2), the response addition model (equation 3), the integrated addition model (equation 4) and the IAI model (equation 6). Neither the concentration addition, response addition nor integrated addition models accurately described the toxicity of the mixtures (r^2 <0.10). Rather, all models grossly overestimated mixture toxicity (Figs. 5A-C). However, the IAI model provided a good (r^2 = 0.712) assessment of the toxicity of the various mixture formulations (Table 3, Fig. 5D). Toxicity was accurately estimated within a factor of 2 for 83% of the mixture formulations.

Discussion

The results of this study demonstrate that toxicokinetic interactions can be incorporated into an integrated addition model to assess mixture toxicity. Recent studies have shown that concentration and response addition models can be used in combination to create a comprehensive additive model to calculate the toxicity of non-interacting chemical mixtures (Teuschler *et al*., 2004; Altenburger *et al*., 2005; Olmstead and LeBlanc, 2005). Here, we build upon that modeling framework by incorporating toxicokinetic interactions between mixture constituents.

By definition, chemical interactions represent a deviation from simple additivity when modeling mixture toxicity. To quantify these interactions, the expected additive toxicity of the mixture must first be determined. Choosing the appropriate model to assess additivity is essential for accurate interpretation of interaction results. US EPA guidelines for assessing mixture toxicity suggest a default model of concentration addition (2000). This recommendation is based on a tendency towards more conservative estimates of mixture toxicity with concentration addition than with response addition modeling (Drescher and Boedecker, 1995). However, indiscriminate application of concentration addition lacks a sound mechanistic basis and therefore increases the uncertainty associated with predicting mixture toxicity. The integrated addition model described in recent works (Altenburger *et al*., 2005; and Olmstead and LeBlanc 2005) provides a mechanism-based alternative to assessing mixture toxicity. Initially, chemicals with similar mechanisms of action are placed into groups, or cassettes. The toxicity within each cassette is modeled with concentration addition and overall toxicity of the different cassettes is then modeled with response addition (Fig. 6). The integrated addition models presented by Altenburger et al (2005) and Olmstead and LeBlanc (2005) are conceptually equivalent and differ only slightly in their methods of calculation. The integrated addition model represents a significant advance in assessing toxicity of non-interacting chemical mixtures. This model, however, is not equipped to manage interactions among chemicals that impact toxicity of the mixture.

The possibility of significant synergistic interactions occurring between two or more chemicals in the environment is perhaps the most compelling reason to study mixture toxicity.

Well-defined examples of synergy include enhanced hepatotoxicity of carbon tetrachloride with pre-exposure to kepone (Klingensmith and Mehendale, 1982) and interactions involving hormone receptor antagonists and hormone synthesis inhibitors (Mu and LeBlanc, 2004). Interactions often can be predicted based on mechanisms of action of constituent chemicals. For example, the P450 inhibitor piperonyl butoxide used in the present study, was hypothesized to antagonize the toxicity of malathion and parathion by decreasing their metabolic activation. However, some interactions will not be apparent from constituent mechanisms of action. The integrated addition model has the potential to identify these unexpected interactions. In effect, significant deviation of experimental results from model predictions implies interaction. Once the source of the interaction is identified, either through inference or experimentation, quantification and incorporation of the interaction into the model follow.

Toxicokinetic interactions can be incorporated into mixture assessments via a qualitative "weight of evidence" approach or a quantitative approach. The two approaches are conceptually quite similar in that both modify the effective concentrations of chemicals in an effector concentration-dependent manner. However, the approaches differ significantly in their application. The "weight of evidence" approach (Mumtaz and Durkin, 1992; modified by Hertzberg *et al*., 1999) is currently recommended in the EPA mixture toxicity guidelines (2000). Briefly, interaction terms that define the effect of one chemical upon another are generated based upon the predicted magnitude of interaction (experimentally determined or default value) as a function of the concentrations of the interacting chemicals. Hazard quotients (exposure level divided by reference dose or reference concentration) of individual chemicals in the mixture are multiplied by the interaction term. The modified hazard quotients are then summed to arrive at

the hazard index of the mixture (Hertzberg and MacDonell, 2002). The hazard index is dimensionless and simply provides a general estimate of the hazard associated with the mixture. It is useful for identifying potentially hazardous mixtures, but it does not provide an accurate calculation of mixture toxicity. Alternatively, a strictly quantitative approach was described by Mu and LeBlanc (2004), which is based on the concept of k-values, or K-functions, first introduced by Finney (1942). This approach involves quantification of the progressive shift in the concentration-response curve of a chemical elicited by increasing concentrations of the effector chemical.

The primary goal of this work was to establish whether modifying functions (i.e., K-functions) could be used to augment the integrated addition model to account for chemical interactions that impact toxicity of mixture constituents. A secondary aim of this work was to increase our understanding of how mechanism-based classes of chemicals, or cassettes, function in mixtures. For example, evidence suggests that certain classes of chemicals display consistent patterns of interaction (Durkin *et al*., 1995). Such consistency raises the possibility that K-functions could be generated that describe the effect of one cassette of chemicals upon another cassette. However, displaying the same type of interaction does not imply that the chemicals exhibit the same magnitude of interaction. In the present work, piperonyl butoxide demonstrated substantial antagonism with both malathion and parathion; however, the degree of antagonism was significantly different between the two organophosphates necessitating the generation of K-functions specific to each organophosphate. Application of K-functions based on malathion/piperonyl butoxide interactions to the entire organophosphate cassette significantly underestimated mixture toxicity (data not shown). Further, some organophosphates (e.g.,

dichlorvos) do not require metabolic activation, but are detoxified by P450s. These compounds might appropriately be assigned to the organophosphate cassette to calculate joint organophosphate toxicity, but they would require K-functions that describe a synergistic, and not antagonistic, interaction with piperonyl butoxide.

The three concepts describing mixture behavior originally identified by Bliss (1939) over 60 years ago are mathematically integrated in the IAI model. The IAI model provided reasonable predictions of the toxicity of a ternary mixture tested at thirty unique formulations. The model represented a significant improvement over basic addition models. The variability that did exist between observed and modeled results may be due to several factors. Inherent biological variability resulting in different responses of organisms between assays may have contributed to some of the observed variability. The assumption that K-functions derived in binary exposures are unaffected when used in higher order chemical mixtures has not been exhaustively tested. Further testing of the IAI model with increasingly complex mixtures will help to elucidate basic principles and limitations associated with K-function application.

This model is relatively simple in its application and requires input parameters that are typically available from standard concentration-response analyses. However, quantification of interactions among chemicals requires rigorous experimentation. Future studies may reveal whether limited but targeted experimentation can provide the information required to quantify interactions. Additional studies also are required to develop means of describing interactions where the response to a chemical modifies the organism's response to another chemical in the mixture. Such toxicodynamic interactions are less common (Hertzberg and McDonell, 2002),

but may still be important contributors to mixture toxicity. The IAI model holds promise to increase the accuracy of hazard and risk assessments of chemical mixtures by reducing uncertainty in estimating mixture toxicity.

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Table 1. Concentration-response parameters of individual chemicals used in mixture toxicity modeling. Toxicity of chemicals was assessed in 48-hour acute toxicity tests measuring immobilization in *Daphnia magna*. The EC50, 95 % confidence interval, and power were calculated from a logistic fit to the concentration-response data.

Chemical	EC50 (μM)	95% Confidence Interval (μM)	Power (ρ)
Malathion	0.0107	0.0105-0.0108	18
Parathion	0.0113	0.0112-0.0115	23
Piperonyl butoxide	6.37	6.26-6.49	11

Table 2. Concentration-response toxicity evaluation of five ratios of malathion:parathion predicted to be equitoxic based upon concentration addition modeling. EC50s and 95% confidence intervals were calculated from a logistic fit to the concentration-response data generated for each ratio. Concentrations are expressed in malathion equivalents.

Ratio	EC50 (μM)	95% Confidence Interval (μM)
1:0	0.00889	0.00843-0.00939
2:1	0.00876	0.00844-0.00910
1:1	0.00874	0.00737-0.0102
1:2	0.00905	0.00865-0.00949
0:1	0.00867	0.00737-0.0102

Table 3. Formulations of the 30 ternary mixtures of malathion, parathion, and piperonyl butoxide (PBO) used to model (IAI model; equation 6) and measure (with *D. magna*) mixture toxicity.

Mixture (#)	Malathion (μM)	Parathion (μM)	PBO (μM)	Response (% immobilization)	
				Modeled	Measured
1	0.0271	0.0193	1.81	0	0
2	0.0190	0.0305	1.90	0	0
3	0.0350	0.0161	2.00	0	0
4	0.0158	0.0395	2.11	0	0
5	0.0444	0.0119	2.22	0	0
6	0.0117	0.0500	2.34	14	5
7	0.0553	0.0066	2.46	0	0
8	0.0065	0.0623	2.59	85	85
9	0.0681	0.0000	2.72	0	0
10	0.0000	0.0767	2.87	99	60
11	0.0453	0.0323	3.02	0	0
12	0.0318	0.0510	3.18	27	30
13	0.0585	0.0268	3.34	0	15
14	0.0264	0.0659	3.52	95	60
15	0.0741	0.0198	3.71	0	15
16	0.0195	0.0835	3.90	100	70

17	0.0924	0.0110	4.11	1	20
18	0.0108	0.1041	4.32	100	80
19	0.1138	0.0000	4.55	3	55
20	0.0000	0.1281	4.79	100	95
21	0.0756	0.0540	5.04	87	45
22	0.0531	0.0852	5.31	100	60
23	0.0978	0.0448	5.59	71	50
24	0.0441	0.1101	5.88	100	95
25	0.1238	0.0331	6.19	57	65
26	0.0326	0.1395	6.52	100	100
27	0.1543	0.0184	6.86	70	75
28	0.0181	0.1738	7.22	100	100
29	0.1900	0.0000	7.60	87	75
30	0.0000	0.2140	8.00	100	100

Figure Legends

Figure 1. Concentration-response profiles of the individual chemicals used in the mixtures toxicity assessment: malathion (A), parathion (B) and piperonyl butoxide (C). Data points represent the percentage of immobilized daphnids. Data were fit using a logistic equation (equation 1).

Figure 2. Acetylcholinesterase activity in daphnids following exposure to mixture constituents. Treatment abbreviations: C, control; M, malathion; P, parathion; PBO, piperonyl butoxide. **A**: Malathion was evaluated at 0.01 μM and PBO at 0.1 μM. **B**: Parathion was evaluated at 0.01 μM and PBO at 0.1 μM. Bars represent the mean and standard deviation for 3 replicate treatments. Treatments with the same letter were not significantly different (Analyses of variance and Tukey-Kramer HSD, $P \leq 0.05$).

Figure 3. Impact of piperonyl butoxide on organophosphate concentration-response curves. Concentration-response curves were generated for malathion (A) and parathion (B) alone (□) and in combination with increasing concentrations of piperonyl butoxide (■). Data points represent the percentage of immobilized daphnids. Data were fit using equation 1. All piperonyl butoxide concentrations tested were below the *no observed effect level* for piperonyl butoxide alone.

Figure 4. The relationship between piperonyl butoxide concentrations and modifying effects on malathion (A) and parathion (B) as defined by K-functions. K-functions were derived for each concentration of PBO evaluated (Fig. 3) using equation 5. Parameters used in equation 5 were

derived from the concentration-response curves depicted in Fig. 3. A curve was fit to the plot using equation 1.

Figure 5. Comparison of observed data to results generated from concentration addition (A), response addition (B), integrated addition (C), and integrated addition and interaction models (D). The solid line represents a 1:1 relationship between modeled and predicted data. Observed data were generated in toxicity tests with *Daphnia magna* using thirty concentrations of the ternary mixture of malathion, parathion and piperonyl butoxide described in Table 3. Concentration addition, response addition, integrated addition, and integrated addition and interaction results were calculated with equations 2, 3, 4 and 6 respectively.

Figure 6. Schematic representation of the integrated addition and interaction model. $R_{mixture}$ represents the response of the mixture. Similar-acting chemicals are placed in a cassette. Toxicity associated with the cassette is calculated using the concentration addition model. Toxicokinetic interactions between chemicals are incorporated as modifiers of concentrations of chemicals within the cassettes. Total mixture toxicity associated with all cassettes is calculated using the response addition model.

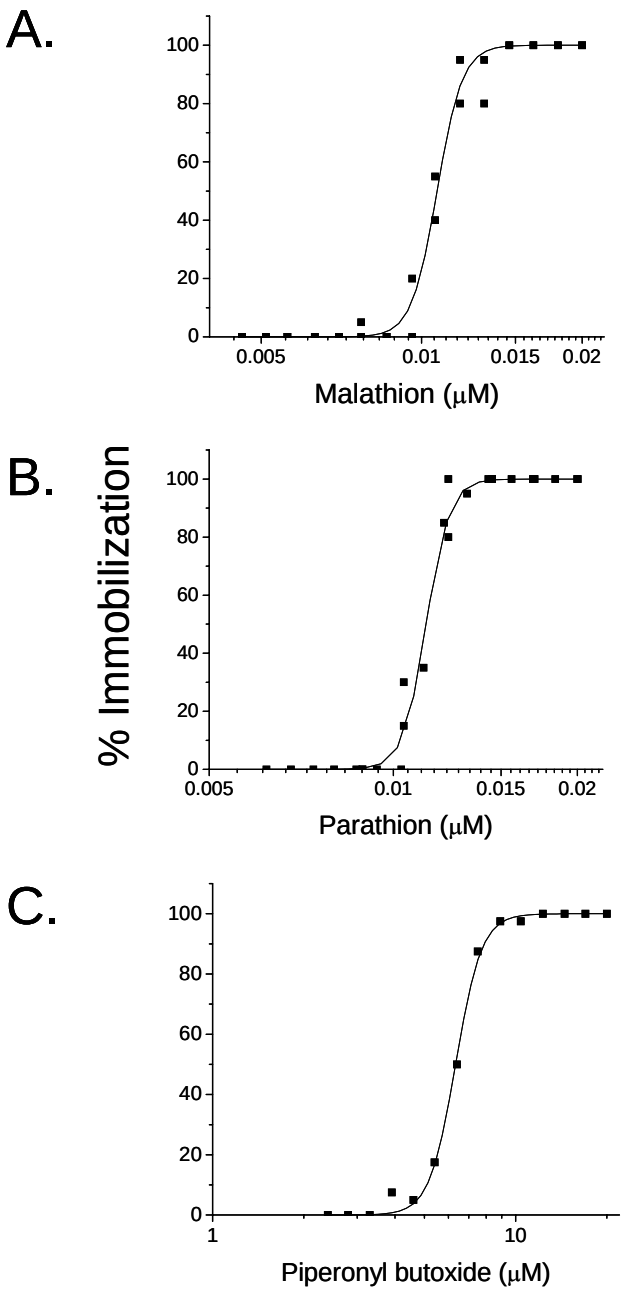
A.
100
80
60
40
20
0
0.005
0.01
0.015
0.02
Malathion (μM)
B.
% Immobilization
100
80
60
40
20
0
0.005
0.01
0.015
0.02
Parathion (μM)
C.
100
80
60
40
20
0
1
10
Piperonyl butoxide (μM)

Figure 1

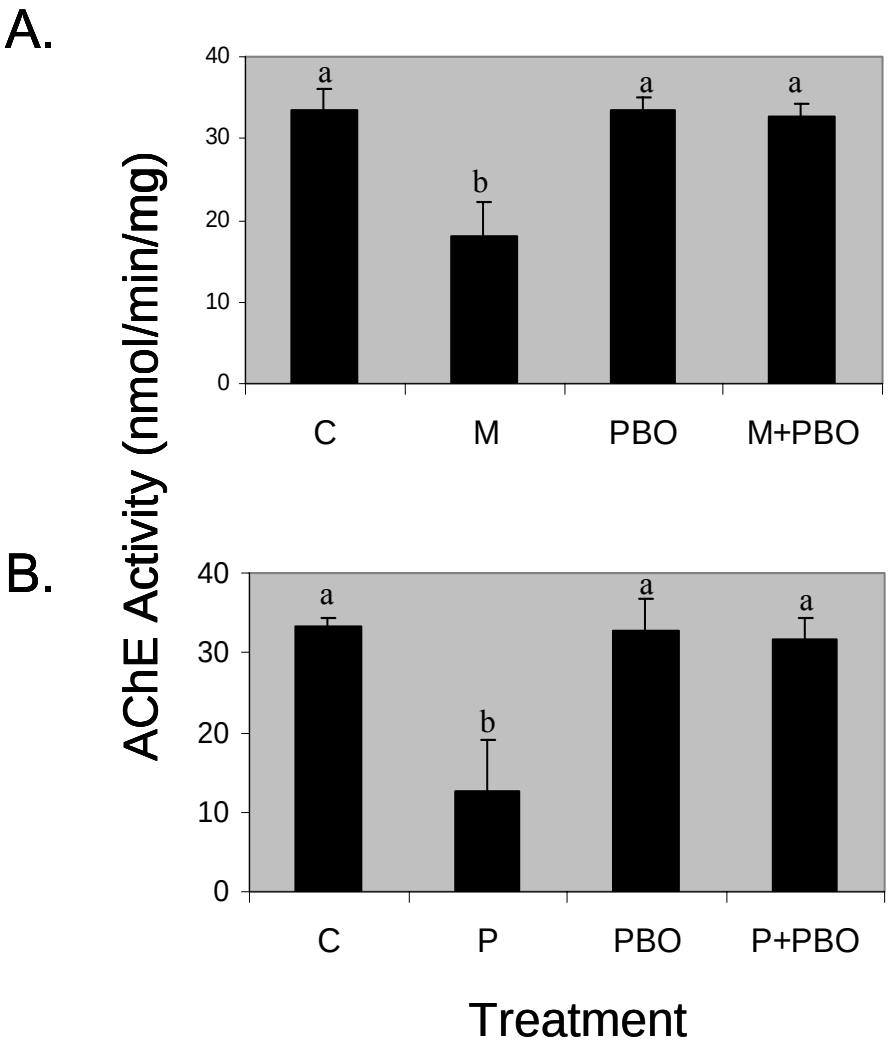
A.
B.
AChE Activity (nmol/min/mg)
40
30
20
10
0
a
b
a
a
C
M
PBO
M+PBO
a
b
a
a
C
P
PBO
P+PBO
Treatment

Figure 2

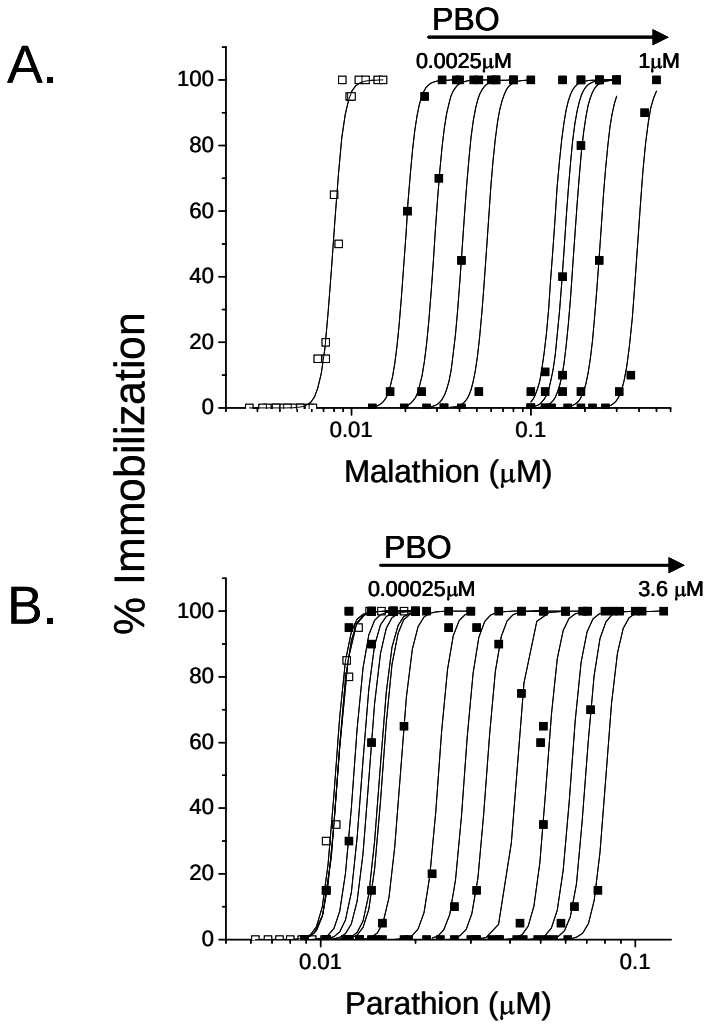
A.
PBO
0.0025μM
1μM
100
80
60
40
20
0
0.01
0.1
Malathion (μM)
% Immobilization
B.
PBO
0.00025μM
3.6 μM
100
80
60
40
20
0
0.01
0.1
Parathion (μM)

Figure 3

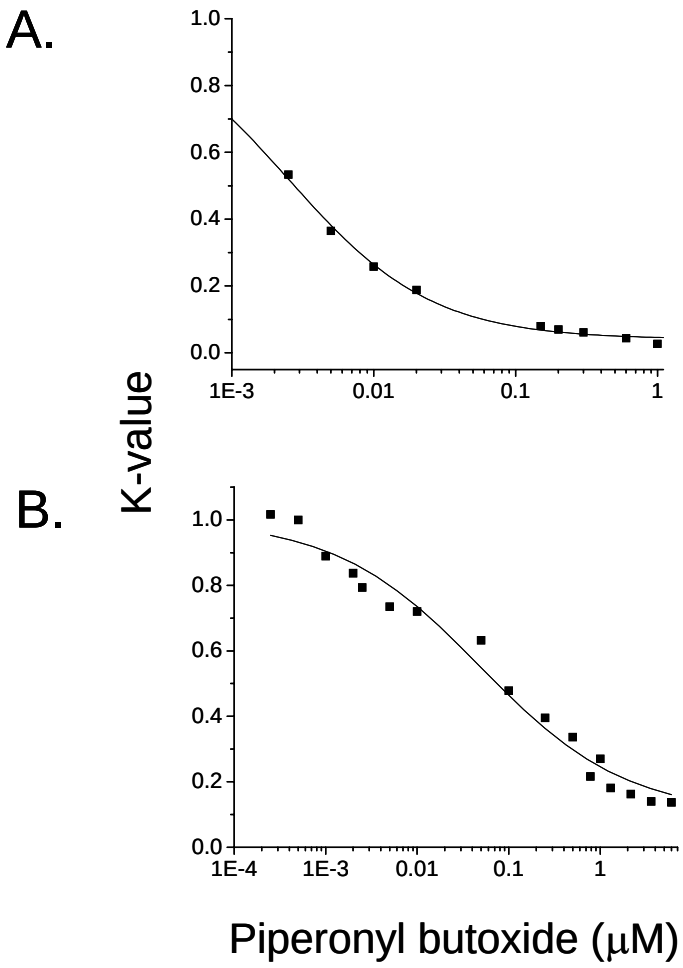
A.
B.
K-value
1.0
0.8
0.6
0.4
0.2
0.0
1E-3
0.01
0.1
1
1E-4
Piperonyl butoxide (μM)

Figure 4

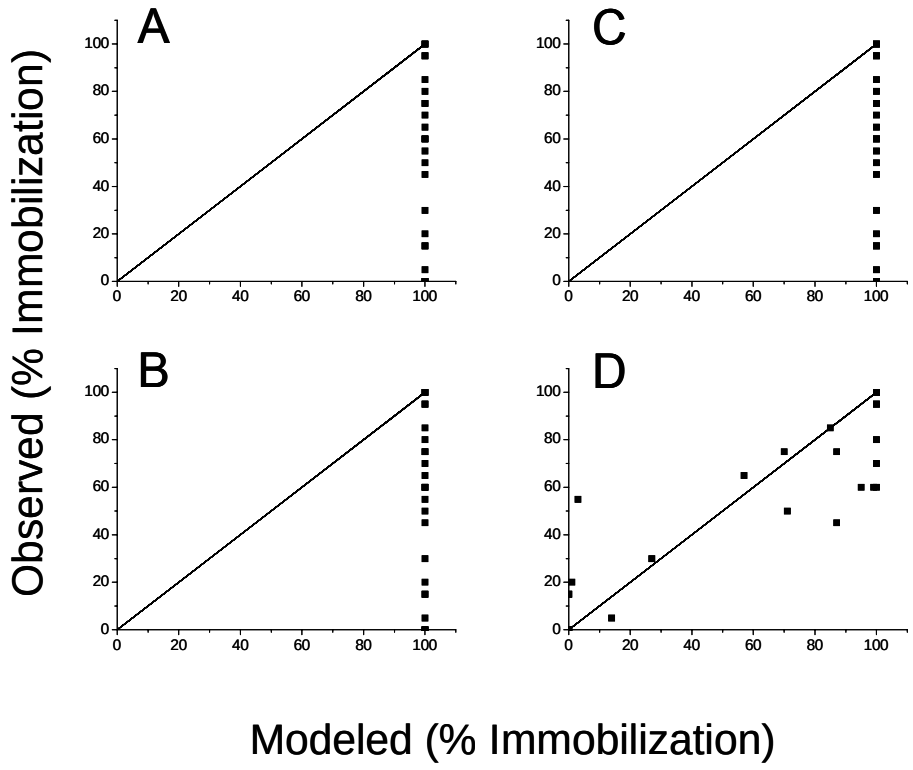
A
B
C
D
Observed (% Immobilization)
Modeled (% Immobilization)
0
20
40
60
80
100

Figure 5

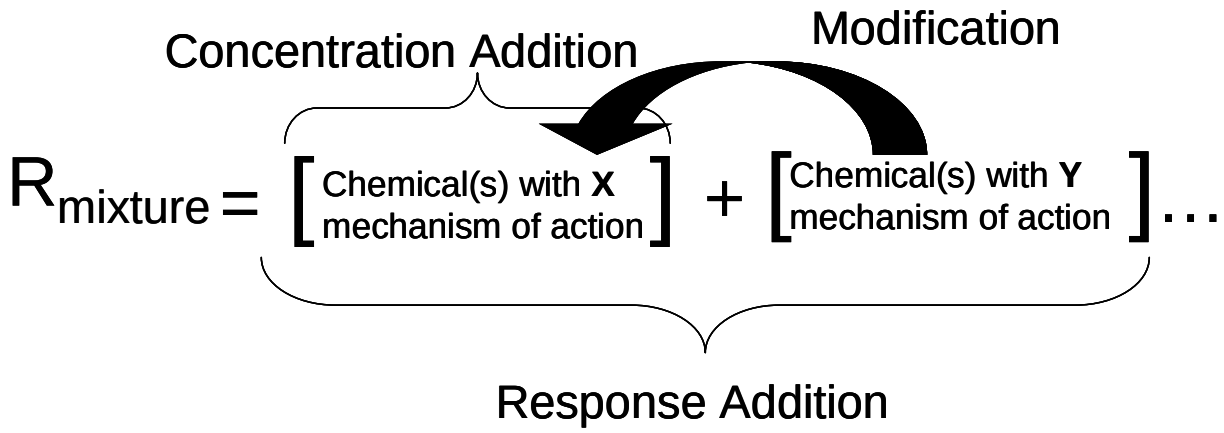
Concentration Addition
Modification
R mixture =
Chemical(s) with X mechanism of action
+
Chemical(s) with Y mechanism of action
...
Response Addition

Figure 6

Assessment of Chemical Interactions in Mixtures using Modeling and Mechanistic Approaches

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Abstract

Models of mixture toxicity can be powerful tools for assessing the hazard associated with complex chemical mixtures. Chemical interactions pose a major challenge to model development because they can be difficult to predict and incorporate into a modeling framework. In this study, we used our Integrated Addition model to predict the toxicity of quaternary mixtures of organophosphate insecticides and polar narcotics. The joint measured toxicity of malathion, parathion, 4-chlorophenol, and 2-chlorophenol to *Daphnia magna* was best modeled an Integrated Addition (IA) model as compared Concentration Addition (CA) or Response Addition (RA) models. However, deviation of experimental data from IA model predictions indicated a synergistic interaction between the two classes of chemicals. Mechanistic studies revealed no toxicokinetic interaction as chlorophenols did not affect the bioaccumulation or acetylcholinesterase-inhibiting activity of the organophosphates and organophosphates did not affect the bioaccumulation of chlorophenols. Analysis of the daphnid energy budget revealed a synergistic action of the chemicals on carbohydrate stores suggesting a possible toxicodynamic interaction. Next, we quantified this interaction through the use of coefficients of interaction (K-functions) and incorporated the K-functions into the IAI modeling framework to describe a synergistic toxicodynamic interaction. The resulting model provided a better fit to the data than non-interacting additivity models, but did not fully account for the magnitude of the interaction.

Key words: synergy, cumulative toxicity, mixture modeling, integrated addition, hazard assessment

Introduction

Societal reliance on chemicals for individual, agricultural, and industrial use has led to widespread environmental contamination by chemical mixtures. Mathematical models are often used to assess or predict the toxicity of defined chemical mixtures based upon an assumption of additivity. A major impediment to using models to predict mixture toxicity is the potential for chemicals to interact. Although the direction of an interaction (synergistic or antagonistic) may be evident from constituent mechanisms of action, the threshold and magnitude of the interaction must be determined experimentally. Developing approaches for characterizing and incorporating interactions into mixture toxicity models is required for widespread application of models to predict toxicity of complex chemical mixtures.

Chemical mixture toxicity models are predominately based on concepts of concentration addition and response addition. Concentration addition applies to mixtures of chemicals with the same mechanism of action. Response addition is used to model mixtures of chemicals with different mechanisms of action. Models incorporating concepts of both concentration and response addition have been used to accurately assess toxicity of chemical mixtures (Teuschler *et al*, 2004; Altenburger *et al*., 2005; Olmstead and LeBlanc, 2005; Rider and LeBlanc, 2005). These models provide means for assessing the toxicity of non-interacting chemical mixtures.

Chemical interactions can be inferred by a deviation of experimentally-determined mixture toxicity from additive model predictions of mixture toxicity. Chemical interactions within a mixture can be mechanistically classified as toxicokinetic or toxicodynamic (Andersen and Dennison, 2004). Toxicokinetic interactions occur when one chemical alters the effective

concentration of another chemical. Toxicodynamic interactions occur when one chemical modifies the response of an organism to another chemical (Andersen and Dennison, 2004). The majority of known interactions are toxicokinetic in nature (Hertzberg and McDonnel, 2002).

Current regulatory guidelines for incorporating interactions into mixture toxicity assessments are based upon a weight of evidence approach (Mumtaz and Durkin, 1992; US EPA, 2000). Available interaction data are used to estimate the type and magnitude of chemical interactions in the mixture. Interaction terms are then applied to modify affected chemical concentrations. Finally, the modified chemical concentrations are used to calculate the interaction-modified Hazard Index using a concentration addition model. This approach aids in the identification of mixtures that may be hazardous due to synergistic interactions. However, the interaction-modified Hazard Index relies on the assumptions that concentration addition is an appropriate framework for calculating mixture toxicity and that all interactions are toxicokinetic in nature. Additionally, the quantitative impact of the interactions is a gross estimate.

Recent studies have shown that concentration addition and response addition models can be combined in an Integrated Addition (IA) model to assess toxicity of mixtures (Olmstead and LeBlanc, 2005). We have further shown that the IA model can be modified to incorporate interactions among mixture constituents (Rider and LeBlanc, 2005). The primary objective of the present study was to further validate the IA model by using a quaternary mixture involving chemicals with two mechanisms of action. In the course of the experiments, we identified an unexpected interaction between the two groups. We attempted to elucidate the mechanism of

this interaction and incorporate the interaction into an IAI model. Novel methods for assessing and incorporating toxicodynamic interactions into mixture toxicity models are addressed.

Materials and Methods

Daphnid culture

The microcrustacean daphnid *Daphnia magna* was used in all experiments. Daphnids were originally acquired from US Environmental Protection Agency, Mid-Continent Ecology Division - Duluth, MN and have been maintained in our laboratory for over ten years. Daphnid media consisted of reconstituted deionized water (192 mg/L $CaSO_4 \bullet H_2O$, 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}). All daphnid cultures and experiments were housed in incubators set to 20° C with a 16 h-8 h light-dark cycle. Culture daphnids were maintained at a density of ~50 animals per 1 L beaker. Cultures were changed three times per week and adults were discarded and replaced with neonates after three weeks. Culture daphnids were fed twice a day with 2.0 mL (1.4×10^8 cells) of the unicellular green algae *Selenastrum capricornutum* and 1.0 mL (4 mg dry weight) of Tetrafin® fish food suspension (Pet International, Chesterfill, New South Wales, Australia) prepared as described previously (Baldwin and LeBlanc, 1994). The *Selenastrum* was cultured in the laboratory using Bold's basal medium.

Acute toxicity assays

Malathion and parathion were acquired from Chem Service, Inc. (West Chester, PA). 4-Chlorophenol and 2-chlorophenol were purchased from Sigma-Aldrich (St. Louis, MO). Chemicals were dissolved in absolute ethanol, which was added to all beakers, including

controls, at 0.01%. Neonatal (≤24 hours old) daphnids were selected from cultures for use in acute toxicity assessments. Each treatment consisted of two 50 mL beakers with 40 mL of exposure medium and 10 neonates. Daphnids were fed *Selenastrum capricornutum* (7 x 10^6cells) and fish food homogenate (0.2 mg dry weight) at the start of each exposure. Daphnid response was assessed at 48 hours. The response measurements were performed blind; beakers were rearranged randomly and labels were not visible to the evaluator. The measured response was immobilization, defined as the inability of the neonate to occupy the water column during 10 seconds of observation.

Individual chemical toxicity measurements

Exposure concentrations for each chemical were selected, based upon preliminary experiments that would span response levels from 0 to 100%. The percentage response was plotted against exposure concentration on a log scale and fit with a sigmoidal line using Origin™ software (Microcal™ Software, Inc., Northampton, MA). The logistic equation representing the sigmoidal fit to the data is:

$$R = \frac{1}{1 + \left(\frac{EC50}{C}\right)^{\rho}} \qquad \text{Equation 1}$$

where R is the response (% immobilization), C is the chemical exposure concentration, ρ is the power or slope of the curve, and *EC50* is the exposure concentration eliciting immobilization in 50 % of exposed animals. These individual concentration-response curves were subsequently used in mixture modeling as described below.

Integrated Addition (IA) model

The IA model requires that chemicals in a mixture be assigned to cassettes based upon mechanism of action. All chemicals that elicit toxicity via the same mechanism of toxicity are assigned to one cassette. In the present study, the acetylcholinesterase inhibitors malathion and parathion were assigned to the organophosphate (OP) cassette and the polar narcotics 4-chlorophenol and 2-chlorophenol were assigned to the chlorophenol (CP) cassette. The toxicity associated with all chemicals within a cassette was computed using a Concentration Addition (CA) model. To confirm that chemicals were appropriately assigned to cassettes, five ratios (Table 2) of the chemicals in each cassette were evaluated for toxicity at six different concentrations and compared to CA model predictions. All five ratios were equitoxic based upon characterization of the toxicity of the individual chemicals. The six concentrations of each binary mixture used in the experiments were selected to define the concentration-response curve for the mixture. The joint toxicity of these binary mixtures of like-acting chemicals was computed using the following CA equation (Olmstead and LeBlanc, 2005):

$$R = \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{C_i}{EC50_i}\right)^{\rho'}}} \qquad \text{Equation 2}$$

where R is the response to the mixture, C_i is the concentration of chemical *i* in the mixture, $EC50_i$ is the concentration of chemical *i* that causes a 50% response, and ρ' is the average power associated with the chemicals in the cassette. Concentration-response results from each binary mixture were then used to calculate *EC*50 values as described for individual chemicals. Analyses of variance were performed to detect significant ($P \leq 0.05$) differences among the five ratios using SAS 8.2 software (SAS institute, Cary, NC). No significant differences among the binary mixtures would confirm proper cassette assignment.

The joint toxicity of the different cassettes within the mixture was calculated using a Response Addition (RA) model (Olmstead and LeBlanc 2005, Rider and LeBlanc, 2005). The following equation describes RA:

$$R = 1 - \prod_{i=1}^{n} (1 - R_i) \qquad \text{Equation 3}$$

where R represents the response to the mixture and R_i is the response to cassette i.

Equations 2 and 3 were integrated to establish the response associated with individual cassettes within a mixture and to sum the responses associated with the cassettes (Integrated Addition (IA) model, Olmstead and LeBlanc 2005):

$$R = 1 - \prod_{I=1}^{N} \left\{ 1 - \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{C_i}{EC50_i} \right)^{\rho'}}} \right\} \qquad \text{Equation 4}$$

Mechanism of interaction

Chemical accumulation The effect of 4-clorophenol on malathion accumulation over time was assessed by measuring the quantity of [^{14}C] malathion (Sigma-Aldrich; specific activity = 5.6 mCi/mmol) in daphnids exposed to [^{14}C] malathion alone and in the presence of 4-chlorophenol. Likewise, the effect of malathion on 4-chlorophenol accumulation was assessed by measuring the quantity of [^{14}C] 4-chlorophenol (Sigma-Aldrich; specific activity = 7.09 mCi/mmol) in daphnids exposed to [^{14}C] 4-chlorophenol alone and in the presence of malathion. In the

malathion accumulation experiment, 7-Day-old daphnids were exposed to either media alone, 0.010 μM [^{14}C] malathion or 0.010 μM [^{14}C] malathion and 30 μM 4-chlorophenol. In the 4-chlorophenol accumulation experiment, daphnids were exposed to media alone, 30 μM [^{14}C] 4-chlorophenol, or 30 μM [^{14}C] 4-chlorophenol and 0.0060 μM malathion. Chemical concentrations were selected that were well tolerated by the daphnids and were likely to display an interaction based on preliminary experiments. Exposures were performed in triplicate with 250 mL beakers containing 100 mL media and 5 daphnids. Chemical uptake by daphnids was assessed after 0, 1, 2, 4, 8, and 24 hours of exposure. At the end of each exposure period, daphnids were weighed and transferred to 7 mL scintillation vials containing 0.50 mL Soluene 350 (Perkin Elmer, Boston, MA). Vials were incubated in a 55°C waterbath overnight. Daphnid tissue was completely dissolved during incubation. The solution then was transferred to a clean scintillation vial and 4.0 mL Ultima Gold scintillation fluid (Perkin Elmer) was added to each vial. Radioactivity was measured on a Tri-Carb 2200CA liquid scintillation analyzer (Perkin Elmer).

Acetylcholinesterase activity Acetylcholinesterase activity was measured according to Ellman (1961) as modified for use with microtiter plates (Fisher et al. 2000). Exposure groups consisted of two 250 mL beakers containing 200 mL solution and 40 neonates (≤24 hours old). Algae ($1.4x10^7$ cells) and fish food (0.4 mg dry weight) were added to each beaker once per day. Solutions were renewed at 24 hours. Following the 48-hour exposure period, neonates were transferred to 1.5 mL microfuge tubes. Media was removed from tubes; neonates were rinsed, and homogenized in 35 μL ice cold 0.02 M phosphate buffer, pH 8.0 with 1% Triton-X-100 using a Teflon pestle. An additional 315 μL phosphate buffer, pH 8.0 without Triton-X-100 was

then added and samples were mixed. Samples were centrifuged at 12,000 g for 4 minutes at 4°C and supernatant was transferred to a clean pre-cooled microfuge tube. Approximately 100 µL of the supernatant was stored at -20°C for protein analysis. The following solutions were added to each well in a 96-well plate: 100 µL of 8 mM 5,5'-dithio-bis(2-nitrobenzoate) (D-1830 Sigma), 50 µL supernatant (phosphate buffer with 0.1% Triton-X-100 was used for supernatant blanks), 50 µL of 16 mM acetylthiocholine iodide (A-5751 Sigma). Absorbance was measured kinetically for 15 minutes at 420 nm using a Fusion™ Universal Microplate Analyzer (PerkinElmer, Boston, MA). Protein was measured according to Bradford (1976) using Bio-Rad Protein Assay dye concentrate (Hercules, CA) and a standard curve generated with bovine serum albumin. The molar extinction coefficient (13,300 $M^{-1} \cdot cm^{-1}$) (Masson et al 2004) was used to calculate the amount of 5-thio-2-nitrobenzoate, formed over 15 minutes and this rate was normalized to the amount of protein added to the assay (nmol/min/mg). Significant ($P \leq 0.05$) differences between treatments were determined by Analysis of Variance (ANOVA) and Tukey HSD using JMP software (SAS, Cary, NC).

Cellular energy budget Cellular energy budget was measured according to De Coen and Janssen (1997) with minor modifications. Exposure groups consisted of 100 neonatal daphnids (≤ 24 hours) in 1 L beakers. Beakers were provided with algae (7.0×10^7 cells) and fish food (2 mg dry weight) twice daily. Treatment groups included solvent control, 0.0060 µM malathion, 22 µM 4-chlorophenol and 0.0060 µM malathion with 22 µM 4-chlorophenol. Following 48-hours of exposure, daphnids were collected in 1.5 mL microfuge tubes and snap frozen in liquid nitrogen.

Energy stored Energy stores were measured as carbohydrate, protein, and lipid content of the organisms. Three samples (each consisting of 100 snap-frozen daphnids) per treatment were processed for each measurement. To measure carbohydrate content, frozen daphnids were homogenized in 200 μL deionized water using a Teflon pestle. Next, 100 μL of 15% trichloroacetic acid (TCA) was added to the homogenate, samples were vortexed, and incubated on ice for 10 minutes. A 10 minute 3000 rpm centrifugation at 4°C followed. The supernatant was transferred to a pre-cooled 1.5 mL microfuge tube, while the pellet was resuspended in 5% TCA. Following a second centrifugation, the supernatant was added to the previously collected supernatant. The supernatant was vortexed and 250 μL was transferred to a 2 mL microfuge tube to which was added 250 μL 5% phenol and 1 mL hydrosulfuric acid. Samples were vortexed. From each sample, 300 μL was transferred in triplicate to wells of a 96 well microtiter plate. The plate was incubated in the dark for 15 minutes and absorbance was measured on a Fusion™ Universal Microplate Analyzer (PerkinElmer, Boston, MA) at 490 nm. Assays were performed with known amounts of glucose in place of tissue to generate a standard curve.

For protein analysis, frozen daphnid samples were homogenized in 200 μL 0.5 M, pH 6.8 Tris HCl with a Teflon pestle. Homogenate was centrifuged at 6000 rpm for 10 minutes at 4°C. Protein concentration of the supernatant was analyzed according to Bradford (1976) with a bovine serum albumin standard.

Lipids were extracted from frozen daphnids according to Bligh and Dyer (1959). Frozen daphnids were homogenized in 200 μL deionized water using a teflon pestle. The following were added to the homogenate: 500 μL chloroform, 500 μL methanol, and 250 μL deionized

water. Samples were then centrifuged at 10,000 rpm at 4°C for 10 minutes. The top phase was discarded and 100 μL of the lipid phase was transferred in triplicate to glass tubes. Tubes were placed in a 60°C waterbath for 30 minutes. Next, 500 μL hydrosulfuric acid was added to each tube. The tubes were vortexed to mix and incubated at 200°C for 15 minutes. Following cooling, samples were diluted with 250 μL deionized water. A 250 μL aliquot of each sample was transferred to each well of a 96 well microtiter plate. Absorbance was measured with a Fusion™ Universal Microplate Analyzer at 340 nm. Known amounts of tripalmitin were used in place of sample to make a standard curve.

Energy consumed Energy consumption was measured as cellular respiration rates. Frozen daphnid samples were homogenized in 400 μL homogenizing buffer (0.1 M phosphate buffer at pH 8.5 with 0.05 M Tris HCl, 75 μM $MgSO_4$, 1.5% polyvinylpyrrolidone, and 0.1% Triton-X-100) with a Teflon pestle. Homogenate was centrifuged at 3000 rpm for 10 minutes at 4° C. The supernatant was transferred to clean 1.5 mL microfuge tubes and vortexed briefly. The following components were added to each well of a 96-well microtiter plate: 180 μL buffered substrate solution (0.1 M phosphate buffer at pH 8.5 with 0.05 M Tris HCl, 0.1% Triton-X-100, 1.7 mM NADH, 0.25 mM NADPH), 60 μL supernatant, and 60 μL of 2 mg/ml p-iodonitrotetrazolium violet (SIGMA I-8377). The plate was read kinetically for 15 minutes at an absorbance of 490 nm on a Fusion™ Universal Microplate Analyzer. Absorbance over time was graphed and only the linear portion was used in analysis.

Calculation of energetics The energy of combustion for each of the stored energy compartments (17500 mJ/mg carbohydrate, 24000 mJ/mg protein, 39500 mJ/mg lipid (Gnaiger, 1983)) was

used to convert quantities into energetic equivalents. Cellular respiration rates were determined as the rate of oxygen consumption in the samples as measured by the rate of formazan generation and the 2:1 relationship between μmol formazan formed and μmol O_2 consumed. Oxygen consumed per daphnid was converted into energy units using the conversion factor 484 kJ/mol O_2 (Gnaiger, 1983).

Treatment groups were analyzed for significant differences with ANOVA and Tukey HSD using JMP software (SAS).

The Integrated Addition and Interaction Model

Coefficients of interaction or K-functions were used to describe the effect of one cassette on the toxicity of the other cassette (Finney 1942, Mu and LeBlanc 2004, Rider and LeBlanc, 2005). Results from the mechanistic studies and previous data suggested that organophosphates were more likely than chlorophenols to be the actuating chemical group. Therefore, K-functions were experimentally determined to quantify the effect of organophosphates on the toxicity of chlorophenols. K-functions were calculated using the following equation:

$$K = \frac{EC50_{CP}}{EC50_{CP+OP}} \qquad \text{Equation 5}$$

where $EC50_{CP}$ is the concentration of the target chemical (CP) that immobilized 50% of the exposed animals and $EC50_{CP+OP}$ is the EC50 of the target chemical when exposure occurred in the presence of a concentration (OP) of the actuating chemical (i.e., the chemical responsible for modifying toxicity). A series of K-functions were derived over the effective concentration range of OP. These K-functions were then plotted against the concentration of actuating chemical (OP) from which they were derived. The logistic equation that defined this relationship was used to calculate K-functions for any concentration of OP present in a mixture. K-functions

were integrated into the model to describe toxicodynamic interactions by applying K-functions to modify the response of the target cassette as folows:

$$R = 1 - \prod_{I=1}^{N} \left\{ 1 - \left(k_{a,i}\left(C_a\right)\right) \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{C_i}{EC50_i} \right)^{\rho'}}} \right\} \qquad \text{Equation 6}$$

where $k_{a,i}(C_a)$ represents the K-function describing the extent to which cassette *a* (actuating cassette) present in the mixture at concentration *C* alters the response elicited by the target cassette *i*.

Mixtures Assessment

Models were assessed with data generated from thirty combinations of OP and CP chemicals. Measured responses were compared to modeled responses calculated with the three aditive mixture models: CA, RA, and IA in an effort to identify the best-suited model. Interactions among mixture constituents were inferred by the deviation of measured responses to modeled responses. K-functions were then applied to the IA model to describe a toxicodynamic interaction. The fit of observed data to each of the models was assessed using coefficients of determination (r^2; Zar, 1996). A high r^2 value (>0.70) indicated that the experimental data were well represented by the model.

Results

Individual chemical concentration-response assessment

Concentration-response relationships were defined for the individual chemicals used in the quaternary mixture assessment from which toxicity parameters were derived (Fig. 1). The logistic equation provided a good fit to acute toxicity data for malathion ($r^2 = 0.940$), parathion ($r^2 = 0.950$), 4-chlorophenol ($r^2 = 0.917$), and 2-chlorophenol ($r^2 = 0.955$). The concentration-response curves for the specific-acting organophosphates were steeper than for the polar narcotic chlorophenols (Table 1). Additionally, the chlorophenols were significantly less toxic than the organophosphates (Table 1).

Cassette assignment

The IA model requires that chemicals with the same mechanism of action be grouped together in cassettes. The toxicity associated with each cassette is then calculated using the CA concept. Previously, we demonstrated that the organophosphates (OP) malathion and parathion abide by concentration addition and should therefore be assigned to a single OP cassette (Rider and LeBlanc, 2005). To validate the assignment of the chlorophenols (CP) to a CP cassette, we assessed the toxicity of five ratios of 2-chlorophenol to 4-chlorophenol (Table 2) at six concentrations. The resulting concentration-response relationships were statistically equivalent. These results corroborate our assumption that the chlorophenols elicit toxicity through the same mechanism of action and justify their assignment to the same cassette.

Mixture Toxicity Modeling

The toxicity of thirty mixtures of the four compounds were experimentally determined (Table 3) and compared to additive model predictions (Fig. 2). The IA (Fig. 2A) and RA (Fig. 2C) models tended to under predict the toxicity of the mixtures ($r^2 = 0.726$ and 0.378,

respectively), while the CA model (Fig. 2B) over estimated toxicity ($r^2 < 0.1$). In summary, all additive models inadequately predicted toxicity, though the IA model yielded the best results. The under-prediction of toxicity using the IA model indicated a synergistic interaction between cassettes.

Mechanism of interaction

Mechanistic experiments were performed to determine whether the observed interaction was toxicokinetic or toxicodynamic in nature and to determine which group was actuating the interaction. In the mechanistic studies, malathion and 4-chlorophenol were used as representative chemicals from the OP and CP cassette, respectively. To test for a toxicokinetic interaction, we assessed the effect of 4-chlorophenol on malathion uptake (Fig. 3A) and the effect of malathion on 4-chlorophenol uptake (Fig. 3B). In both cases, there was a time-dependent increase in radiolabeled chemical uptake (Fig. 3). The accumulation of neither compound was significantly affected by the other compound, which argues against toxicoinetic interaction resulting in altered chemical uptake. Furthermore, acetylcholinesterase activity measured in daphnids differentially treated with the two compounds indicated that 4-chlorophenol did not modify the effective concentration of malathion at the organophosphate target site (Fig. 4). Results suggested that the observed interaction was not toxicokinetic in nature.

Further assessment of a potentially toxicodynamic interaction required an endpoint broad enough to detect a response-level interaction, yet specific enough to be informative. Towards this end, we assessed the energy budget in daphnids exposed to malathion, 4-chlorophenol and a

combination of the two compounds. We measured energy stored in lipid, protein, and carbohydrate compartments, as well as energy consumed via respiration. Additive effects between the two chemicals were observed in measurements of energy stored in lipid and protein compartments, and energy consumed (Fig. 5B-D). A synergistic effect was observed in the carbohydrate compartment (Fig. 5A). 4-Chlorophenol significantly decreased energy stored in all compartments and energy consumed (Fig. 5). The interaction took place in the only compartment in which malathion alone significantly affected the endpoint (i.e. carbohydrate energy store). This is also the compartment in which 4-chlorophenol alone had the least pronounced effect. These results seem to indicate that OP is driving the interaction. Additionally, results identify a specific area for further targeted mechanistic studies.

Incorporating the Interaction into the IAI Model

For the derivation of K-functions, malathion and 4-chlorophenol were used as proxies for OP and CP, respectively. The degree to which sub-toxic levels of malathion shifted the 4-chlorophenol concentration-response curve (represented by K-functions) is depicted in Figure 6. The toxicodynamic interaction models incorporating OP modification of CP toxicity improved model predictions and provided a relatively good fit to the experimentally determined data (r^2 = 0.833), but tended to under-predict mixture toxicity (Fig. 7).

Discussion

Chemical interactions are identified by deviation of observed data from that predicted by models of simple additivity (Hertzberg and MacDonnell, 2002). Kodell and Pounds (1991) describe circumstances in which application of a single additivity model, either CA or RA, could

result in erroneous conclusions regarding the type of interaction (synergy or antagonism) present in a mixture. Results from our study support the conclusion that model selection can drastically impact assumptions about chemical interactions within a mixture.

Application of a CA model to a quaternary mixture of chlorophenols and organophosphates resulted in considerable over-estimation of mixture toxicity and therefore evidenced an antagonistic interaction (Fig. 2B). On the other hand, both the RA and IA models provided reasonable predictions of mixture toxicity though some synergism among chemicals was implied (Fig. 2C and 2A). Mixture researchers have typically utilized a single model as the default model to define simple additivity in an attempt to establish a universal method for calculating mixture toxicity (Berenbaum, 1985). In 1992, a group of prominent mixture scientists concluded that either CA or RA were appropriate default models (Greco *et al*.), whereas, the USEPA mixture guidelines recommend using CA exclusively (USEPA, 2000). In the present study, a default model of CA would have led to the conclusion that an antagonistic interaction existed between the chlorophenols and organophosphates. Additionally, use of either CA or RA as default models would have indicated a more significant degree of interaction than that seen with the IA model (Fig. 2). Here, we provide a concrete case in which inappropriate selection of a default additivity model influences conclusions regarding both the type and magnitude of an interaction.

In the IA model, like-acting chemicals are grouped and assessed using concentration addition and these mechanism-based groups are combined using response addition. This approach represents a significant improvement over CA and RA models, which require an

assumption of consistent action (either similar or dissimilar, respectively) among constituents. The IA model allows for a more precise assessment of non-interacting mixtures that contain an array of similar and dissimilar chemicals. Many recent studies have successfully modeled mixture toxicity using an IA framework (Teuschler, 2004; Altenburger *et al.*, 2005; Olmstead and LeBlanc, 2005; Rider and LeBlanc, 2005).

Recently, we described an IAI model that was used to accurately predict the toxicity of a ternary mixture displaying significant interactions (Rider and LeBlanc, 2005). The ternary mixture consisted of chemicals with highly specific mechanisms of action: acetylcholinesterase inhibiting organophosphates malathion and parathion and the P450 inhibitor piperonyl butoxide. The current mixture contains both specific-acting organophosphates, and the non-specific polar narcotic chlorophenols. In the previous study, the antagonistic interaction between piperonyl butoxide and the organophosphates was significant and unique to each of the organophosphates (Rider and LeBlanc, 2005). Presently, the synergistic interaction between chlorophenols and organophosphates is more modest. Additionally, interaction terms established in binary exposures with representative chemicals (malathion and 4-chlorophenol) were universally applied to chemicals within the mechanism-based cassette to predict mixture toxicity. These results support the work of Haddad et al. (2001), which found that binary interaction data could be successfully incorporated into higher order PBPK mixture models.

Interactions can be toxicokinetic or toxicodynamic (Andersen and Dennison, 2004). Toxicokinetic interactions occur when one chemical affects the concentration of another chemical at its target site. Toxicodynamic interactions occur when one chemical modifies the

response elicited by another chemical. We attempted to determine whether the present interaction was toxicokinetic or toxicodynamic through the use of mechanistic studies. Based upon the mechanisms of action of the chemicals, we initially hypothesized that chlorophenols were non-specifically binding to lipid membrane components, thereby increasing permeability and allowing the organophosphates greater access to their target site. To the contrary, we demonstrated that chlorophenols did not increase the accumulation or acetylcholinesterase inhibiting activity of organophosphates. Further, we found that organophosphates did not increase the accumulation of chlorophenols. These results suggested that the observed interaction was not toxicokinetic in nature.

Toxicodynamic interactions are difficult to characterize because the response of an organism to a toxicant occurs on multiple organizational levels and the level at which the interaction takes place is unknown. To circumvent this issue, we assessed the toxicodynamic interaction using an energy budget endpoint. This endpoint is integrated enough to accommodate multiple response levels, yet it provides information on the functional target of the interaction. For example, if organophosphates were exacerbating the breakdown of lipid membranes elicited by chlorophenols, we would have expected to see a synergistic effect in the lipid compartment. Analysis of daphnid energy budgets suggested that the chemicals were interacting by synergistically depleting immediate energy stores (carbohydrate content). This finding further supports a toxicodynamic categorization of the interaction because neither chemical class specifically targets carbohydrate stores. Interestingly, organophosphate alone significantly decreased daphnid carbohydrate stores, while having no significant effect on other measured parameters (Fig. 5). 4-Chlorophenol, on the other hand, significantly affected all

parameters, but decreased carbohydrate stores only slightly. These results seem to suggest that organophosphates could be driving the interaction.

Next, we quantified the interaction and incorporated it into an IAI model. As far as we know, this is the first attempt at incorporating a toxicodynamic interaction into a mathematical model of mixture toxicity. The IAI model represented an improvement over simple additive models, but tended to under predict mixture toxicity. This could be due to the placement of the K-function in the equation (Equation 6). As it stands, a modifying term is multiplied to a cassette response. Therefore, if the cassette response is zero, the K-function cannot increase that response value. The multiple-layers of response discussed above could be a confounding element in these calculations. It is possible that toxicodynamic interactions could be intrinsically weak, such as the example presented here, and could be adequately modeled using the IA.

Durkin et al. (1995) established preliminary methods for identifying groups of chemicals that interact in a similar manner (i.e. share interaction patterns). However, significantly more work is needed in order to determine chemical characteristics that confer interaction potential. The present work indicates that specificity of toxic action of constituent chemicals may play a role in determining the likelihood and magnitude of interactions in mixtures. Further study is needed to explore how mechanisms of toxicity relate to mechanisms of interaction. Modeling approaches are useful in this pursuit because they have the potential to allow for high-throughput hypothesis testing with reduced animal and resource use.

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Table 1. Individual chemical concentration-response parameters used in mixture toxicity modeling. The EC50, 95% confidence interval, and power (slope) were calculated by applying a logistic fit to concentration-response data from acute toxicity assays with *Daphnia magna*.

Chemical	EC50 (μM)	95% Confidence Interval (μM)	Power (ρ)
Malathion	0.00722	0.00703-0.00741	13.1
Parathion	0.00811	0.00793-0.00829	18.7
2-Chlorophenol	67.3	63.5-71.1	8.3
4-Chlorophenol	36.0	33.7-38.3	5.5

Table 2. Concentration-response parameters (EC50 and 95% confidence interval) for 5 ratios of 4-chlorophenol to 2-chlorophenol. 2-Chlorophenol is expressed in 4-chlorophenol equivalents.

Ratio	EC50 (μM)	95% Confidence Interval (μM)
1:0	50.0	39.7-60.2
2:1	44.4	39.7-49.1
1:1	46.4	40.1-52.7
1:2	44.5	37.4-51.5
0:1	46.8	37.8-55.8

Table 3. Concentrations of malathion (M), parathion (P), 4-chlorophenol (4-CP), and 2-chlorophenol (2-CP) used in assessing and modeling mixture toxicity. Modeled values were obtained from the Integrated Addition and Interaction model describing a toxicodynamic interaction with organophosphates modifying the toxicity of chlorophenols.

					Response (% immobilization)	
Mixture (#)	M (μM)	P (μM)	4-CP (μM)	2-CP (μM)	Modeled	Measured
1	0.0018	0.0000	8.9	0.0	0	0
2	0.0013	0.0007	9.3	6.4	0	0
3	0.0010	0.0011	9.8	10.0	0	0
4	0.0007	0.0016	10.3	14.0	0	0
5	0.0000	0.0025	10.9	22.1	0	0
6	0.0023	0.0000	11.5	0.0	0	0
7	0.0016	0.0009	12.1	8.2	0	0
8	0.0013	0.0015	12.7	12.9	0	0
9	0.0009	0.0021	13.4	18.1	0	0
10	0.0000	0.0032	14.1	28.6	0	0
11	0.0030	0.0000	14.8	0.0	0	0
12	0.0021	0.0012	15.6	10.6	0	0
13	0.0017	0.0019	16.4	16.7	1	0

14	0.0012	0.0027	17.3	23.4	1	0
15	0.0039	0.0000	19.1	0.0	1	0
16	0.0000	0.0042	18.2	36.9	2	0
17	0.0027	0.0016	20.2	13.7	2	0
18	0.0022	0.0024	21.2	21.5	4	0
19	0.0015	0.0034	22.3	30.3	6	10
20	0.0050	0.0000	24.7	0.0	9	5
21	0.0000	0.0054	23.5	47.7	10	40
22	0.0035	0.0020	26.0	17.7	15	10
23	0.0028	0.0032	27.4	27.9	23	50
24	0.0020	0.0044	28.9	39.1	35	85
25	0.0000	0.0070	30.4	61.7	54	100
26	0.0065	0.0000	32.0	0.0	54	90
27	0.0046	0.0026	33.7	22.8	79	100
28	0.0036	0.0041	35.4	36.0	99	100
29	0.0025	0.0057	37.3	50.5	100	100
30	0.0000	0.0090	39.2	79.7	100	100

Figure Legends

Figure 1. Concentration-response relationships of individual chemicals: malathion (A), parathion (B), 4-chlorophenol (C), and 2-chlorophenol (D). Each data point represents the percentage of immobilized daphnids. Data were fit using a logistic equation.

Figure 2. Comparison of observed results to data generated from simple mixture toxicity models: integrated addition (A), concentration addition (B), and response addition (C). Experimental data (■) were collected from acute toxicity experiments with *Daphnia magna* using 30 formulations (Table 3) of a quaternary mixture of malathion, parathion, 4-chlorophenol, and 2-chlorophenol. The solid line indicates a logistic fit to experimental data. Modeled data is represented by dashed lines.

Figure 3. Accumulation of [^{14}C] chemical by daphnids either in the absence or presence of the actuating chemical. A) Daphnids were exposed to 0.010 μM [^{14}C] malathion alone (white bar) and in combination with 30 μM 4-chlorophenol (striped bars). B) Daphnids were exposed to 30 μM [^{14}C] 4-chlorophenol alone (white bars) and in combination with 0.0060 μM malathion (striped bars) (B).

Figure 4. Acetylcholinesterase activity in daphnids following exposure to malathion alone and with various concentrations of 4-chlorophenol. Treatments with the same letter are not significantly different (ANOVA/Tukey HSD).

Figure 5. Cellular energy allocation of daphnids exposed to malathion and 4-chlorophenol. The amount of energy available in carbohydrate, protein, and lipid compartments and energy consumed was measured in daphnids following exposure to 0.0050 μM malathion, 22 μM 4-chlorophenol, and a combination of the two chemicals. Treatments with the same letter are not significantly different (ANOVA/Tukey HSD).

Figure 6. Relationship between concentration of actuating chemical and degree of interaction. K-functions describe malathion modification of 4-chlorophenol toxicity. K-functions were calculated using equation 5.

Figure 7. Comparison of observed data (■) to predicted values from an Integrated Addition and Interaction model incorporating a toxicodynamic interaction with organophosphates modifying the toxicity of chlorophenols (dotted line). The solid line represents a logistic fit to the observed data. The Integrated Addition model predictions (dashed line) were included for reference. Experimental data were collected from acute toxicity experiments with *Daphnia magna* using 30 formulations (Table 3) of a quaternary mixture of malathion, parathion, 4-chlorophenol, and 2-chlorophenol.

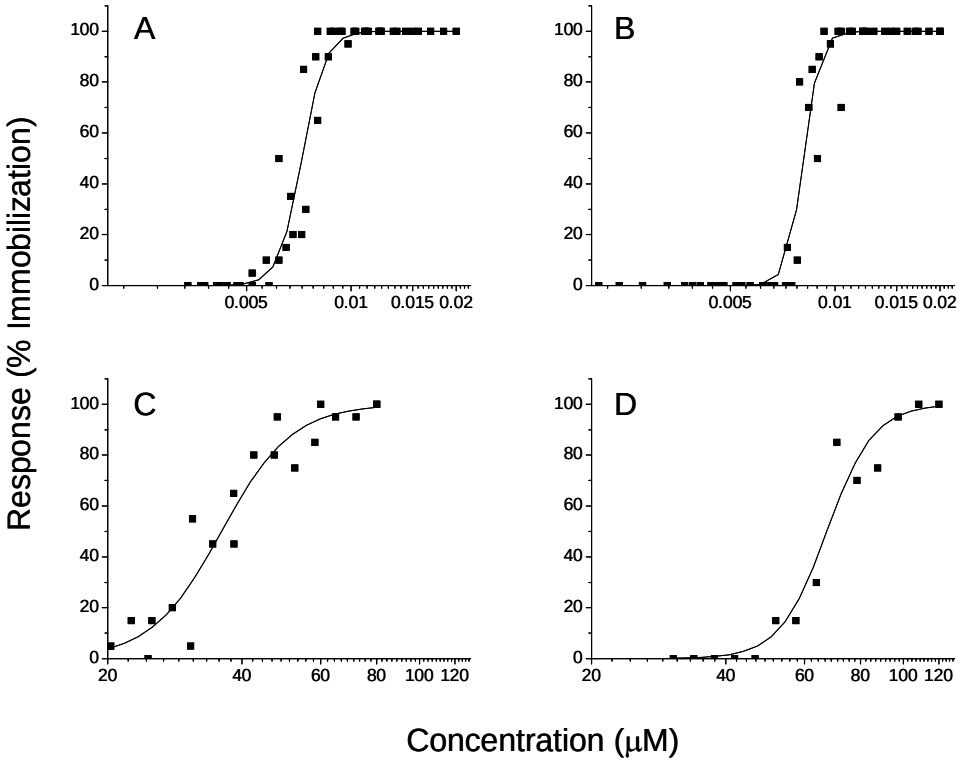
A
B
C
D
Response (% Immobilization)
Concentration (μM)
0
20
40
60
80
100
0.005
0.01
0.015
0.02
120

Figure 1

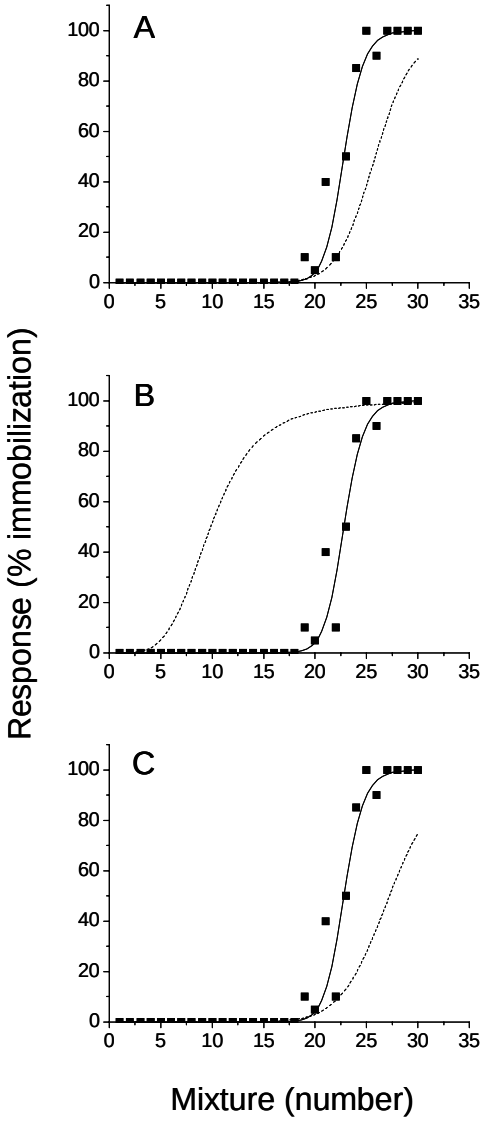
A
B
C
Response (% immobilization)
Mixture (number)
0
20
40
60
80
100
0
5
10
15
20
25
30
35

Figure 2

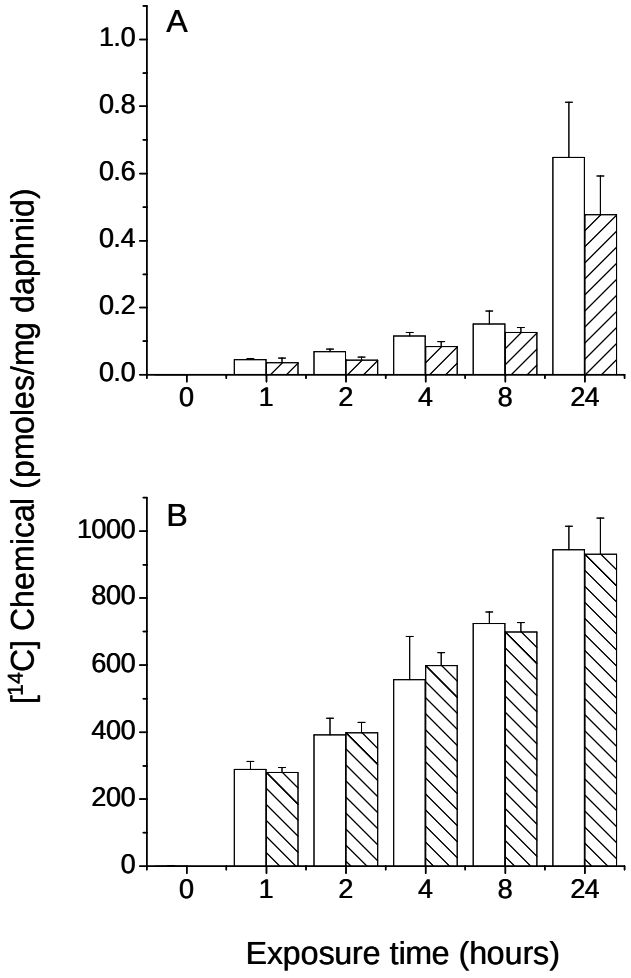
A
B
1.0
0.8
0.6
0.4
0.2
0.0
1000
800
600
400
200
0
0
1
2
4
8
24
[14C] Chemical (pmoles/mg daphnid)
Exposure time (hours)

Figure 3

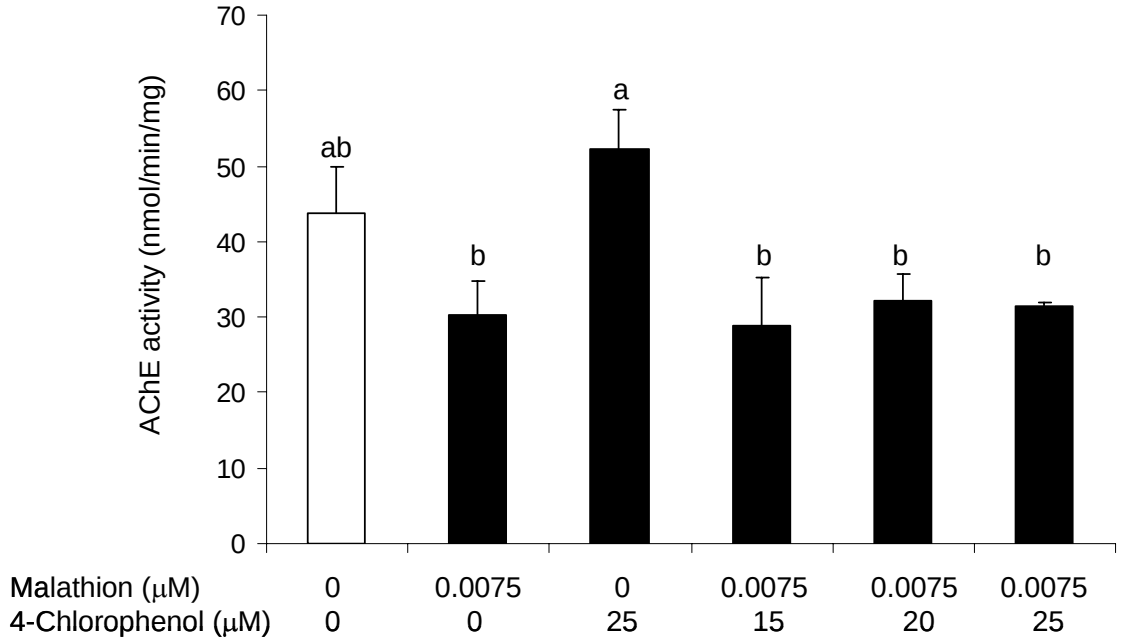
AChE activity (nmol/min/mg)
70
60
50
40
30
20
10
0
ab
b
a
b
b
b
Malathion (μM) 0 0.0075 0 0.0075 0.0075 0.0075
4-Chlorophenol (μM) 0 0 25 15 20 25

Figure 4

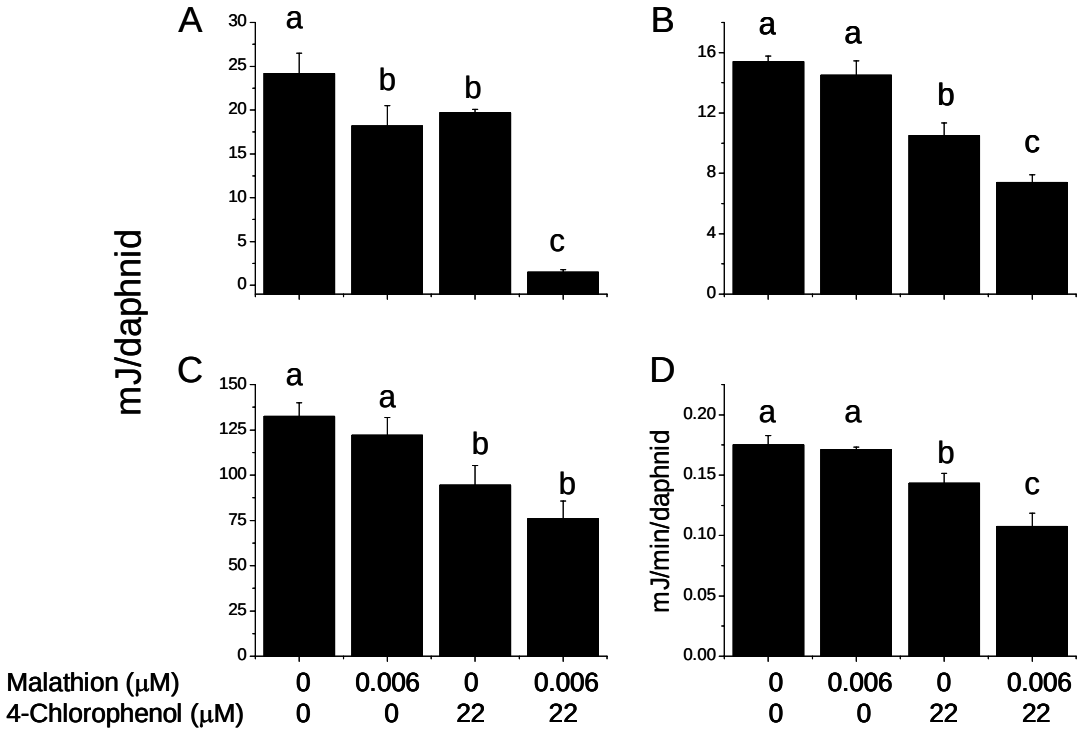
A
B
C
D
a
b
c
mJ/daphnid
mJ/min/daphnid
Malathion (μM)
4-Chlorophenol (μM)
0
0.006
22

Figure 5

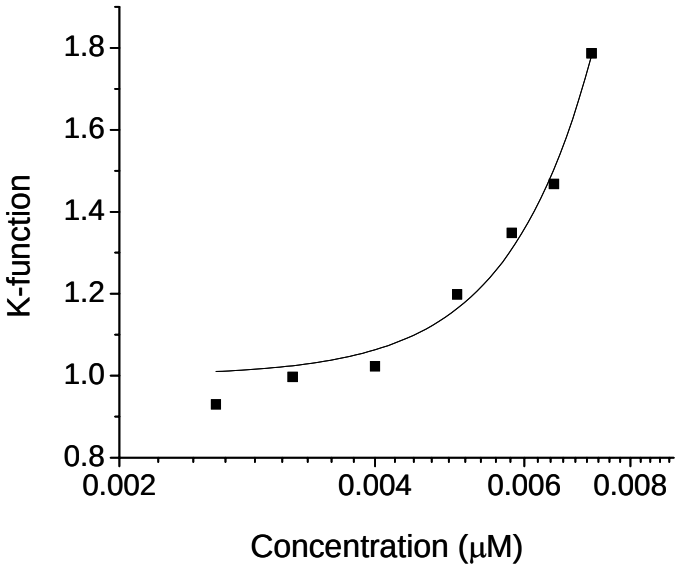
K-function
1.8
1.6
1.4
1.2
1.0
0.8
0.002
0.004
0.006
0.008
Concentration (μM)

Figure 6

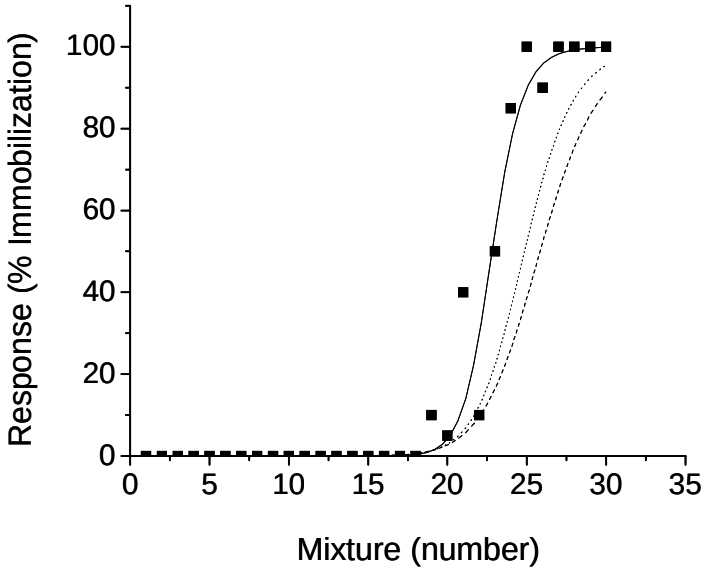
Response (% Immobilization)
100
80
60
40
20
0
0
5
10
15
20
25
30
35
Mixture (number)

Figure 7

Stress Signaling: Co-regulation of hemoglobin and male sex determination through a terpenoid signaling pathway in a crustacean

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ABSTRACT

Environmental signals can activate neuro-endocrine cascades that regulate various physiological processes. In the present study, we demonstrate that two responses to environmental stress signaling in the crustacean *Daphnia magna* - hemoglobin accumulation and male offspring production – are co-elevated by the crustacean terpenoid hormone methyl farnesoate and several synthetic analogs. Potency of the hormones with respect to the induction of both hemoglobin and male offspring was highly correlated suggesting that both processes are regulated by the same terpenoid signaling pathway. Six clones of the *D. pulex/pulicaria* species complex that were previously characterized as unable to produce male offspring and five clones that were capable of producing males were evaluated for both hemoglobin induction and male offspring production in response to methyl farnesoate. Four of the five male-producing clones produced both hemoglobin and male offspring in response to the hormone. Five of the six obligate parthenogenic clones produced neither hemoglobin nor males in response to the hormone. These results provide additional evidence that both physiological processes are regulated by the same signaling pathway. Furthermore, results indicate that the non-male producing clones are largely defective in some methyl farnesoate signaling component downstream from methyl farnesoate synthesis but upstream from the genes regulated by the hormone. A likely candidate for the site of the defect is the methyl farnesoate receptor. As a consequence of this defect, non-male producing clones have lost their responsiveness to environmental signals that are transduced by this endocrine pathway. This defect in signaling would likely enhance population growth in stable environments due to the elimination of males from the population, assuming that other processes critical to population growth are not also compromised by this defect.

Key words: Cladocera, Juvenoid, Endocrine disruption, Evolution, Nuclear receptor

INTRODUCTION

Environmental signals regulate a variety of key processes in animal physiology. In many genera, environmental signals such as changes in day length or temperature signal the onset or termination of reproduction (Steger and Bartke, 1996), thus ensuring that reproduction occurs at a time that maximizes the survival of the offspring (Bronson, 1985). These environmental signals typically stimulate the release of neuro-endocrine signaling molecules that initiate endocrine cascades culminating in physiological responses to the environmental cue. A well characterized neuro-endocrine response to environmental signaling is the detection of photoperiod changes by the pineal gland and its regulation of hypothalamic-pituitary cascades via the action of melatonin in vertebrates (Steger and Bartke, 1996). Similar photoperiod-initiated neuro-endocrine cascades have been identified in insects, though the organs, hormones, and regulated events differ significantly from those characterized in vertebrates (Horie et al., 2000; Yamashita, 1996).

The terpenoid hormone methyl farnesoate is emerging as a major hormone in crustaceans that is responsible for transducing environmental signals. Methyl farnesoate is synthesized by the mandibular organ of Decapod crustaceans (Laufer et al., 1987) and its secretion is negatively regulated by members of the crustacean hyperglycemic hormone (CHH) family of neuropeptides that are synthesized by the X-organ/sinus gland complex (Liu and Laufer, 1996). Methyl farnesoate is structurally similar to juvenoid hormones of insects and retinoid hormones of vertebrates and its activity is likely mediated through interaction with a nuclear receptor.

Methyl farnesoate has been associated with a variety of physiological processes in crustaceans related to reproduction including testicular maturation (Kalavathy et al., 1999), ovarian development (Reddy and Ramamurthi, 1998), vitellogenesis (Vogel and Borst, 1989), and mating behavior (Laufer et al., 1993). Reproductive maturation in crustaceans also is subject to various environmental signals (Benzie, 1997). Whether some of these signals are transduced within the organism by methyl farnesoate is not currently known.

The freshwater crustacean *Daphnia magna* utilizes two reproductive strategies that are regulated by environmental signals. Daphnids typically reproduce asexually by parthenogenesis (Hebert, 1978; Lynch and Gabriel, 1983). Diploid female offspring that are genetically identical to their mother are generally produced during asexual reproduction. Asexual reproduction provides for rapid expansion of the population, in a favorable environment. In response to environmental signals including a drop in food quantity or quality, a decrease in photoperiod, and crowding (Carvalho and Hughes, 1983; Klevien et al., 1992; Stross and Hill, 1968; Stross and Hill, 1968b) daphnids enter a sexual reproductive phase by producing males and sexually-responsive females. These animals mate; the fertilized, diapause eggs are encased in a protective ephippium; and these eggs are released into the environment. These diapause eggs can resist desiccation or freezing and can hatch decades following release (Hebert, 1978). In addition, the hydrophobic ephippium facilitates dispersal of the eggs through air currents or adherence to migrating species.

We recently discovered that methyl farnesoate is a male sex determinant in daphnids (Olmstead and LeBlanc, 2002). Exposure of maternal organisms with maturing oocytes in the

ovaries to methyl farnesoate causes the oocytes to develop into males. This discovery suggests that a methyl farnesoate signaling cascade is responsible for transducing environmental signals that are responsible for the switch from asexual to sexual reproduction. In a search for environmental signals that stimulate male offspring production via the methyl farnesoate signaling pathway, we observed that females, stimulated to produce male offspring, often develop a distinct copper color. We speculated that this color change may represent increased hemoglobin accumulation in these organisms (Hoshi et al., 1977). In the present study, we tested the hypothesis that male offspring and hemoglobin production are co-regulated by the same signaling pathway. Results demonstrate the existence of an environmental stress response in daphnids that is mediated by terpenoid hormones. Results also identify potential means for identifying the terpenoid receptors of arthropods and provide insight into mechanisms responsible for the development of asexual populations of daphnids.

MATERIALS AND METHODS

Daphnids

Daphnia magna used in these experiments have been cultured at North Carolina State University for over 10 years and were originally derived from laboratory stocks maintained at the US Environmental Protection Agency laboratory, Duluth, MN. Clones of the *D. pulex/ pulicaria* species complex were provided by Dr. Jeffry Dudycha, Indiana University, USA. Several of these strains were never observed to produce male offspring and were judged to be obligate parthenogens (J. Dudycha, personal communication). All daphnids were cultured and experimentally maintained in deionized water reconstituted with 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 mg/L selenium and 1.0 mg/L vitamin B_{12}.

D. magna and *D. pulex/pulicaria* cultures were maintained at a density of ~50 and ~200 brood daphnids/L culture medium, respectively. Culture medium was renewed and offspring were discarded three times weekly. Brood daphnids were discarded after 3 weeks in the culture and replaced with neonatal organisms. Cultured *D. magna* were fed twice daily with 1.0 mL (~4 mg dry weight) of Tetrafin® fish food suspension (Pet International, Chesterfill, New South Wales, Australia) and 2.0 mL (1.4×10^8 cells) of a suspension of unicellular green algae, *Selenastrum capricornutum*. *D. pulex/pulicaria* cultures received the same food mixture but at half the provision rate. The algae were cultured in Bold's basal medium. Culture and experimental solutions were maintained at 20°C under a 16 hr photoperiod. These culture conditions maintained the daphnids in the parthenogenic reproductive phase with virtually no males produced.

Hormone treatment

Daphnids were exposed to the terpenoid hormone methyl farnesoate (Echelon Biosciences, Salt Lake City, UT), and the analogs pyriproxyfen (Chem Service, West Chester, PA), methoprene (Chem Service), and fenoxycarb (Chem Service). This group of compounds is referred to as terpenoid hormones in this study based upon common activity to the terpene methyl farnesoate, but recognizing that pyriproxyfen and fenoxycarb lack isoprene units characteristic of terpenes. Daphnids (juveniles for hemoglobin induction and gravid adults for male sex determination) were isolated from the cultures and individually housed in 50 mL beakers containing 40 mL of culture media. For each experiment, 5-10 daphnids were exposed to the desired concentration of chemical and 5-10 daphnids were exposed to media containing no chemical. Daphnids were provided food twice daily ($1.4 \text{ X } 10^7$ cells of algae and 50 μL fish

food suspension for *D. magna,* 5.6X 10^6 cells of algae and 20 μL fish food suspension for *D. pulex/pulicaria*). Daphnids were maintained under the same conditions as described for culturing. Hemoglobin levels were measured after 48 hrs.

For male offspring production, daphnids were maintained under these conditions and were transferred to new media every 2-3 days until the release of the third brood of offspring. Sex of the individual offspring in this brood was determined. Third broods were evaluated to ensure that the maternal organisms had been exposed to the hormone during the critical period of ovarian oocyte maturation when sex of the offspring is determined (Olmstead and LeBlanc, 2002). Methyl farnesoate was dissolved in methanol and the analogs were dissolved in ethanol for delivery to the daphnid media. Carrier solvent was also added to the control media at the same concentration present in the respective hormone treatment and concentrations in the final solutions never exceeded 0.02%.

Hemoglobin analyses

Spectrophotometry: Hemoglobin content of individual *D. magna* was determined according to Van Dam et al. (van Dam et al., 1995) with modifications. Individual adult daphnids were homogenized in 600 μL of distilled water by sonication for 5 seconds using a Vitra-Cell™ hand-held sonicator (Sonics & Materials Inc. Danbury, CT, USA). Particles were pelleted by centrifugation at 10,000 *g*. An aliquot (400 μL) of the supernatant was used to measure hemoglobin content. The remainder of the supernatant was used to measure protein content (Bradford, 1976). Standard solutions of bovine hemoglobin (Sigma, St. Louis, MO; 1.0 to 60 μg hemoglobin in 400 μL) were used to generate standard curves. Twenty-four μL of 0.10% KCN

was added to each 400 µL sample. Samples were incubated for 5 min. at room temperature, then absorbance was measured at 380, 415, and 440 nm. Absorbances at 380 and 440 nm were used to discern background absorbance flanking the absorbance peak (415 nm) of oxygenated hemoglobin. Absorbance due to hemoglobin was calculated as: $E_{415}-((E_{380} + E_{440})/2)$. Hemoglobin absorbance values were converted to µg hemoglobin using the standard curve. Final hemoglobin values associated with individual daphnids were either normalized to the protein content of the homogenate or were presented as µg hemoglobin per daphnid.

Hemoglobin color scoring: The small size of *D. pulex/pulicaria* and juvenile *D. magna* precluded analyses of hemoglobin levels in individual animals. Therefore, a colorimetric procedure was devised and validated using adult *D. magna* as a means of estimating hemoglobin content of individuals that were too small for direct spectrophotometric analyses. Color of daphnids as related to hemoglobin content was numerically scored using a narrative description of the color associated with the gonadal/visceral region and respiratory appendages (Fig. 1A) in combination with direct comparison to a color-gradient card which facilitated judgment of the intensity of color. The color gradient card was computer generated (Photoshop, Adobe, San Jose CA) and depicted shades of copper that encompassed the range exhibited by the daphnids. The transparency of daphnids allowed for direct microscopic color scoring of individuals without harming the organisms. Thus, changes in relative hemoglobin levels could be monitored in the same organism over time. For method validation, daphnids were scored for level of coloration using a dissecting microscope (10X magnification), then hemoglobin content of the individual daphnids was quantified by spectrophotometry. The relationship between the two measures of hemoglobin was determined (Figs. 1B,C). Coefficient of variation between different technicians

scoring the same daphnids was <15%. Coefficient of variation of standard curves that described the relationship between color score and true hemoglobin level (as μg/daphnid) deduced by different technicians using different animals was <35% with respect to both slope and X intercept. This approach was considered semi-quantitative as variables such as size of the organisms would likely impact the scoring interpretation. This approach was only used to judge relative differences in hemoglobin levels in relation to hormone treatment. The method proved highly effective for assessing relative hemoglobin in individuals for such comparisons within these limitations.

Electrophoresis: Increases in pigmented protein (i.e., hemoglobin) with pyriproxyfen treatment was assessed following native protein polyacrylamide gel electrophoresis. Seventeen to 20 adult *D. magna* were homogenized as described above following exposure to either 3 nM pyriproxyfen or carrier solvent without pyriproxyfen. Protein content of the supernatant was determined (Bradford, 1976) and a volume of supernatant containing 350 μg protein was subjected to electrophoretic separation according to Laemmli (1970) with the following modifications. Electrophoresis was performed using a Mighty Small^{tm} electrophoresis apparatus (Bio-Rad, Hercules, CA) with a 5% running gel and a 4% stacking gel. Both gels were prepared at a pH of 8.8. Sodium dodecyl sulfate was not added to any of the gels or buffers. Electrophoresis was performed for 30 min. at 4^{o}C and 200 volts. Following electrophoresis, gel images were digitized with a digital imager (model 640BU, Acer CM, City of Industry, CA, USA).

mRNA analyses: Hemoglobin mRNA levels were quantified by real-time RT-PCR. Thirty control or hormone-exposed daphnids were crushed to a fine powder with a mortar - containing liquid nitrogen - and pestle. RNA was isolated from the powdered preparation using the SV Total RNA Isolation System (Promega, Madison, WI). The RNA yield was determined by absorbance at 260 nm and purity was measured by the 260/280 nm ratio. Integrity of RNA was confirmed by agarose/formaldehyde gel electrophoresis (Sambrook et al., 1989). RNA was converted to cDNA using the Promega ImProm-II™ Reverse Transcription System with oligo (dT) primers.

The sequences for *D. magna* hemoglobin and actin genes were accessed through GenBank. Primers were designed for the highly specific non-homologous untranslated region (UTR) of hemoglobin *hb2* gene. The primers were designed using ABI Primer Express Software (Applied Biosystems, Foster City, CA) and the primer option with the lowest penalty (26.5) was selected. The hemoglobin primers were: forward 5′ TCCTCTGACGACCTGGACTCAT 3′, reverse 5′ CCATTAGCCGAGGTTGAAATTG 3′. Constitutively expressed actin was used as a control for the reaction and to normalize hemoglobin results among samples (forward 5′ CCTGAGCGCAAATACTCCGT 3′, and reverse 5′ CAGAGAGGCCAAGATGGAGC 3′). All primers were acquired from Qiagen (Valencia, CA) and were reconstituted in TE buffer (1M Tris, 0.5 M EDTA, pH 8.0). The hemoglobin *hb2* gene product was selected for analyses because this gene is primarily responsible for the increase in hemoglobin mRNA in response to terpenoid signaling (manuscript in preparation).

Real-time RT-PCR was carried out using the ABI PRISM® 7000 Sequence Detection System with SYBR® Green PCR Mastermix on MicroAmp® Optical 96-well Reaction Plates equipped with ABI PRISM™ Optical Adhesive Covers (Applied Biosystems). The following default parameters were used: 1 cycle of 50°C for 2 min; 1 cycle of 95°C for 10 min; 40 cycles of 95°C for 15 s followed by 60°C for 1 min; 1 cycle of 4°C for ∞.

Dissociation curves were routinely generated with amplification products using the protocol provided by the instrument manufacturer. A single melting peak was consistently present following each amplification, indicating that only a single product was amplified in each well. Relative hemoglobin mRNA levels in the starting samples were discerned by the threshold cycle (Ct). The Ct is the PCR cycle at which a statistically significant increase in amplification product is detected (Bustin, 2000). Relative hemoglobin mRNA levels in the samples were determined by dividing the Ct derived with hemoglobin primers by the Ct derived with actin primers.

Sequence identity of the PCR product also was established. Amplification product was generated for sequencing using a Bio-Rad iCycler with the same cDNA and primers that were used in real-time RT-PCR. Amplification was performed using Promega PCR Master Mix with the same thermal profile as was used for real-time RT-PCR. A single amplification product was detected following electrophoresis in a 3.5% agarose gel. This product was excised from the gel and sequenced (Seqwright, Houston, TX). The derived sequence was entered into an NCBI nucleotide Blast search. Significant matches were made with "*Daphnia magna* dhb2 mRNA for hemoglobin" (E value = 2e-18) and "*Daphnia magna* hemoglobin gene cluster (*dhb3*, *dhb1* and

dhb2 genes)" (E value = 4e-17), accession numbers AB021136 and AB021134 respectively. No significant homology was determined between the amplification product and any other gene product.

Male sex determination

Neonatal male and female daphnids were distinguished based upon the longer first antennae of males as determined under 10X magnification (Olmstead and LeBlanc, 2000). Neonatal daphnids identified as male using this criterion mature into completely differentiated males that exhibit normal male reproductive behaviors (personal observations).

Statistics

Significant differences were evaluated using ANOVA and Dunnett's multiple comparison test when comparing several treatments to a control. Paired comparisons were evaluated using Student's *t* test. Significance was established at $p \leq 0.05$ and all analyses were performed using JMP statistical software (SAS Institute, Cary, NC).

RESULTS

Increased hemoglobin accumulation with terpenoid hormone treatment We had previously demonstrated that male offspring production is under the regulatory control of a terpenoid signaling pathway (Olmstead and LeBlanc, 2002; Olmstead and LeBlanc, 2003). Experiments were performed to determine whether hemoglobin is similarly regulated by terpenoid hormones. Hemoglobin levels were significantly elevated from exposure of daphnids to the crustacean

terpenoid hormone methyl farnesoate (Fig. 2A) and several synthetic analogs of the hormone (Figs. 2B-D). Relative potency of the terpenoids to elevate hemoglobin levels and induce male offspring production was highly correlated (Fig. 3). The parallel potencies of the different terpenoids in regulating both processes suggest that a common terpenoid receptor mediates these two signaling pathways.

The increase in hemoglobin levels with terpenoid treatment was further evaluated using the potent inducer pyriproxyfen (Fig. 4). Exposure of daphnids to pyriproxyfen significantly elevated levels of pigment (Fig. 4A) that was specifically associated with an electrophoretically distinct protein (Fig. 4B) that shared spectral characteristics with hemoglobin (Fig. 4C). Pyriproxyfen also elevated levels of hemoglobin hb2 mRNA as measured by real-time RT-PCR (Fig. 4C). Taken together, these results demonstrate that the increase in pigmentation in response to terpenoid hormones represents elevated hemoglobin levels. Elevated hemoglobin levels were evident within the first 24 hours of exposure to pyriproxyfen and levels continued to increase linearly over at least the next 24 hours (Fig. 5).

Responsiveness of daphnid clones to terpenoid hormones Eleven clonal populations of the *D. pulex*/*pulicaria* species complex, along with our laboratory stock of *D. magna*, were evaluated for responsiveness to methyl farnesoate with respect to hemoglobin accumulation and male sex determination. Five populations of *D. pulex/pulicaria* were observed in culture to produce males and 6 were considered non-male producers based upon extensive observation. Methyl farnesoate treatment elevated hemoglobin levels in all five clones of male producers, along with the laboratory stock of *D. magna* (Table 1). All but one clone (MP5) also produced male offspring

in response to methyl farnesoate (Table 1). In contrast, none of the non-male producing clones produced male offspring in response to methyl farnesoate and only one clone (NP1) produced hemoglobin in response to the hormone. The general co-responsiveness of hemoglobin accumulation and male sex determination among the various clones to methyl farnesoate provides further evidence that these processes are regulated by a common signaling pathway. Clones NP2 through NP6 are apparently defective in some component of this pathway. In contrast, the induction of hemoglobin but not male offspring production by methyl farnesoate with clones MP5 and NP1 suggests that these clones are defective in sex determining genes downstream from the methyl farnesoate signaling pathway.

DISCUSSION

We have described a novel endocrine signaling pathway that regulates two distinct, and seemingly unrelated, physiological processes – hemoglobin production and male sex determination. Both processes are known to be responsive to specific, distinct environmental signals. The regulation of these processes by a common endocrine pathway suggests that both hemoglobin induction and male sex determination also may occur in response to some common environmental signals.

Hemoglobin levels are typically elevated in daphnids in response to low dissolved oxygen (Kimura et al., 1999; Kobayashi and Hoshi, 1982) through the action of the hypoxia signaling pathway (Bunn and Poyton, 1996; Nambu et al., 1996). Hypoxia inducible factor (HIF) is a transcriptional activator that responds to hypoxia by binding to hypoxia response elements (HRE) located in the promoter region of responsive genes (Bunn and Poyton, 1996). The

hypoxia-activated hemoglobin *hb2* gene of *Daphnia magna* is flanked by several HREs that are able to confer robustly induced and HIF-dependent gene transcription in response to low oxygen levels (Gorr et al., submitted). The induction of hemoglobin confers increased tolerance of the daphnids to low environmental dissolved oxygen levels allowing the organisms to survive this stress (Pirow et al., 2001).

Male offspring are produced by daphnids in response to environmental stressors such as reduced food and crowding (Hobaek and Larsson, 1990; Olmstead and LeBlanc, 2001). Other environmental signals, such as changes in photoperiod, are also known to stimulate the production of male offspring (Stross and Hill, 1968). Environmental signals that stimulate male offspring production are typically associated with the onset of seasonal conditions (i.e. pond desiccation or freezing) that will prove inhospitable to the population. The introduction of males into the population allows for sexual reproduction which is typically associated with the generation of drought or freezing resistant diapause eggs. This reproductive strategy allows for survival of the population during periods of adversity.

The environmental signal to produce male offspring is transduced, within the organism, by a terpenoid signaling pathway (Fig. 6) (Olmstead and LeBlanc, 2002; Tatarazako et al., 2003). Results of the present study demonstrate that hemoglobin levels are regulated by this same terpenoid signaling pathway in addition to the hypoxia/HIF signaling pathway. Male sex determination required only a pulse of methyl farnesoate during a critical period of ovarian oocyte maturation (Olmstead and LeBlanc, 2002). In contrast, hemoglobin levels increase with increasing duration of elevated hormone levels (Fig. 5). Thus, environmental factors that

stimulate a sustained elevation in hormone levels would likely impact both sex determination and hemoglobin levels, while transient increases in hormone levels would significantly impact only sex determination. Taken together, environmental factors may exist that impact hemoglobin synthesis only (sustained low oxygen), male offspring production only (those causing a terpenoid hormone pulse), or both processes (those causing a sustained increase in terpenoid hormone). Studies are underway to identify putative environmental signals that co-stimulate both processes.

Zeis et al. (2004) recently reported that hemoglobin concentration in daphnids increased with increasing temperature. Mitchell (2001) noted a low incidence of sexually ambiguous offspring when daphnids were reared at 30^{o}C. Elevated temperature may prove to be an environmental signal that stimulates both hemoglobin induction and male sex determination through the common signaling pathway. Preliminary experiments in our laboratory support this premise. However, elevated temperature may stimulate different signaling pathways resulting in multiple outcomes. For example, oxygen saturation decreases with increasing water temperature which may stimulate hemoglobin production via the hypoxia signaling pathway. Increased temperature may also adversely impact the uptake or assimilation of nutrients resulting in male production via the terpenoid signaling pathway.

The minimum components to the putative terpenoid signaling pathway described in this study would consist of the hormone (i.e., methyl farnesoate), it's receptor (i.e., methyl farnesoate receptor) and response elements on responsive genes (i.e., hemoglobin genes, sex determining genes) (Fig. 6). Components upstream of the responsive genes would be common components

to the pathway. Five of the six non-male producing clones of daphnids evaluated produced neither male offspring nor hemoglobin in response to methyl farnesoate treatment. This complete lack of responsiveness suggests that these clones are deficient in some common component of the signaling pathway. Since methyl farnesoate was provided to the organisms, hormone was not deficient in the treated organisms. Thus the likely common component that was deficient in these organisms is the methyl farnesoate receptor. In contrast, one of the evaluated clones produced hemoglobin in response to methyl farnesoate but did not produce males. This would suggest that the common components to the signaling pathway are intact but that this clone is deficient in some terminal sex-determining genes. These variously deficient clones may prove highly useful in future studies aimed at identifying the methyl farnesoate receptor as well as genes involved in sex determination.

The regulation of male offspring production by methyl farnesoate implies that daphnids possess sex determining genes, such as *sex-1* in *C. elegans* (Carmi et al., 1998), *dsx* in *Drosophila* (Yi and Zarkower, 1999), or *csd* in the honeybee (Beye et al., 2003). Some of the sex-determining genes of daphnids, along with the methyl farnesoate-responsive hemoglobin genes, may possess cis-acting regulatory elements that interact with the putative methyl farnesoate receptor. We have identified a functional cis element in the promoter of the hemoglobin *hb2* gene of *D. magna* that binds nuclear factor(s) present in methyl farnesoate-treated daphnids (manuscript in preparation). This element resembles binding motifs of several mammalian orphan receptors, most notably NGFI-B (Wilson et al., 1991), SF-1 (Wilson et al., 1993), and RZR (Carlberg et al., 1994) proteins, all of which bind as monomers to their target DNA.

The co-regulation of hemoglobin levels and male offspring production by a common signaling pathway suggests some survival advantage to this phenomenon. Daphnids commonly inhabit temporary ponds (Dudycha, 2004) and environmental signals that forewarn the complete desiccation of the habitat during summer may stimulate the terpenoid signaling pathway. Thus, this early signal of impending habitat loss would provide sufficient time for entry of the population into the sexual phase of the reproductive cycle. Desiccation-resistant diapause eggs would result from the sexual reproduction allowing for survival of the population. Increased hemoglobin may occur in response to this signal to meet the respiratory requirement of sexual reproduction in a habitat experiencing decreasing oxygen-carrying capacity due to increasing temperature. Future studies may reveal that the methyl farnesoate signaling pathway induces specific hemoglobin subunits that have increased oxygen affinity in elevated temperature environments (Lamkemeyer et al., 2003).

Lastly, results of the present study provide insight into the evolution of non-male producing populations of daphnids. As cyclic parthenogens, daphnid populations can benefit from rapid population growth during periods of environmental stasis, yet can resort to sexual reproduction to survive periods of environmental change. Costs of sexual reproduction to the population are significant since males contribute no offspring to the population and sexual reproduction produces few offspring relative to the numbers produced clonally (Innes et al., 2000; Korpelainen, 1992). The benefit of sexual reproduction is genetic exchange among individuals coupled to diapause and dispersion (Hebert, 1978; Rispe and Pierre, 1998). Sexual reproduction may be crucial to the survival of populations inhabiting environments that are periodically

rendered inhospitable due to complete desiccation, freezing, etc. A mutation that disables the terpenoid stress signaling pathway, such as a mutation in the methyl farnesoate receptor gene, in a population of daphnids inhabiting a marginally-variable environment (i.e., a habitat where environmental signals stimulate sexual reproduction, but where sexual reproduction/diapause egg production is not necessary for the survival of the population) would be selected for as these mutant daphnids would not pay the costs associated with male production. Such a marginally-variable environment may exist, for example where water temperatures become significantly elevated but the habitat never completely desiccates. Considering that all of the methyl farnesoate analogs used in this study have commercial application as insecticides raises questions as to whether the introduction of such chemicals into the environment could result in the establishment of artificial marginal environments causing genetic drift from cyclic parthenogenic to non-male producing parthenogenic populations.

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FIGURE LEGEND

Fig. 1 Colorimetric scoring of hemoglobin content of daphnids. **A.** Criteria used to assign a color score to individual daphnids. Animals that were scored 9 were place on a color grid of progressively darker shades of copper and a score was assigned based upon the best color match with the grid. Example relationships between color score assigned to daphnids and hemoglobin content normalized to the soluble protein content of the daphnids (**B**) and as total hemoglobin per daphnid (**C**). Error bars represent standard errors (n=2-4).

Fig. 2. Hemoglobin levels in juvenile daphnids (*D. magna*) following treatment with the crustacean terpenoid hormone methyl farnesoate (**A**) and three synthetic analogs (**B-D**). Hemoglobin levels were assessed colorimetrically after 48 hours exposure to the terpenoids. Ten daphnids were exposed to each treatment. Error bars represent standard errors. An asterisk denotes a significant ($p \leq 0.05$, ANOVA, Dunnetts *t* test) difference from the respective control (0 nM).

Fig. 3. Relationship between the concentrations of methyl farnesoate and analogs that elevated hemoglobin to 70% of the maximum level (X axis) and concentrations that caused a 50% incidence of male broods of offspring (Y axis) in *D. magna*. Male offspring assessments were reported previously (Olmstead and LeBlanc, 2003). The 70% of maximal induction was used as the descriptor of relative potency for hemoglobin levels because this value could be interpolated from all of the concentration-reponse relationships depicted in Fig. 2. Correlation coefficient (r^2) = 0.999.

Fig. 4. Induction of hemoglobin in daphnids (*Daphnia magna*) exposed to 0 or 3 nM pyriproxyfen. **A.** Control and exposed daphnids. **B.** Electrophoretically-separated pigmented protein from individual control and exposed daphnids. **C.** Hemoglobin protein levels (μg/mg protein) in daphnids by absorbance at 415 nm (dark bars) and relative hb2 mRNA levels, normalized to levels of actin transcripts, measured by real-time rtPCR (light bars). Hemoglobin protein levels represent the mean and standard error of four samples each prepared from 17-20 individuals. Hemoglobin mRNA levels represent the mean and standard error of 2 samples each prepared from 30 individuals. An asterisk denotes a significant ($p \leq 0.05$) difference between control and pyriproxyfen–treated daphnids (Student's *t* test).

Fig. 5. Hemoglobin levels in juvenile daphnids (*D. magna*) during exposure to 0.93 nM pyriproxyfen (circles) and concurrently maintained controls (squares). Data are presented as the mean and standard error of 10 daphnids. Hemoglobin levels were measured colorimetrically.

Fig. 6. Diagrammatic representation of the putative juvenoid signaling pathway.

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Table 1. Responsiveness of male producing (MP) and non-male producing (NP) clones of daphnids to methyl farnesoate. Daphnids were exposed to either 0 or 220 nM methyl farnesoate and evaluated for both male offspring and hemoglobin production as described in the Materials and Methods. Data are presented as mean ± se (n=5-10).

Species	Clone	Relative Hemoglobin[a]		Male Offspring (%)	
		Untreated	MF-treated	Untreated	MF-treated
Male producers[b]					
magna	NCSU1	5.1±0	11±1*	0±0	100±0*
pulex	MP1	6.1±0.8	17±1.9*	2±2	100±0*
pulex	MP2	5.6±0.5	8.6±0.6*	0±0	100±0*
hybrid[c]	MP3	7.4±1.5	25±2.4*	0±0	52±29*
pulicaria	MP4	2.5±0	5.6±0.5*	0±0	34±25*
pulicaria	MP5	5.3±0.4	16±0.9*	0±0	0±0
Non-male producers[b]					
pulex	NP1	5.6±0.5	19±1*	0±0	0±0
pulex	NP2	5.1±0	5.1±0	0±0	0±0
pulex	NP3	4.1±0.6	5.1±3.8	0±0	0±0
hybrid[c]	NP4	3.0±0.5	4.1±0.6	0±0	0±0
hybrid[c]	NP5	4.6±0.5	4.6±0.5	0±0	0±0
pulex	NP6	14±2	14±2	0±0	0±0

[a]Hemoglobin levels were estimated colorimetrically and reported values (μg/daphnid) were derived from a standard curve prepared with *D. magna.*

[b]Designations are based upon the observed presence or absence of males in cultures maintained under environmental conditions that favor sexual reproduction.

[c]Hybrids were produced from controlled crosses of *D. pulex* and *D. pulicaria* (J. Dudycha, Indiana University, personal communication).

An asterisk denotes a significant increase as compared to the respective control ($p \leq 0.05$).

A. **Hemoglobin Color Score**

1. No discernable color.
2. Slight yellow color in gonadal/visceral region.
3. Distinct yellow color in gonadal/visceral region.
4. Same as 3, but with a few pockets of light brown.
5. Same as 4, but with copper colored streaks among the thoracic appendages.
6. Predominantly light brown in the gonadal/visceral region with some yellow pockets; copper colored streaks among the thoracic appendages.
7. Predominantly light brown in the gonadal/visceral region with some copper colored pockets; copper colored streaks among the thoracic appendages.
8. Roughly equal distribution of light brown and copper color in the gonadal/visceral region, copper colored streaks among the thoracic appendages.

9+. More copper-colored regions in the gonadal/visceral region than light brown colored regions, copper colored streaks among the thoracic appendages.

Animals scored a 9+ are placed on the color chart and final score (≥9) is based upon the color shade of the darkest region in the gonadal/visceral region.

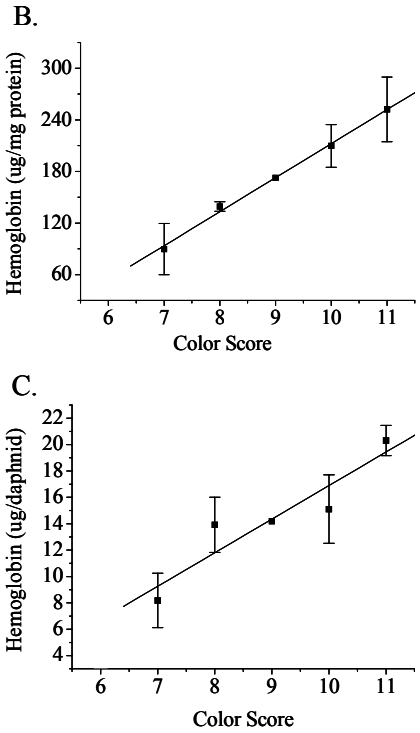

Figure 1

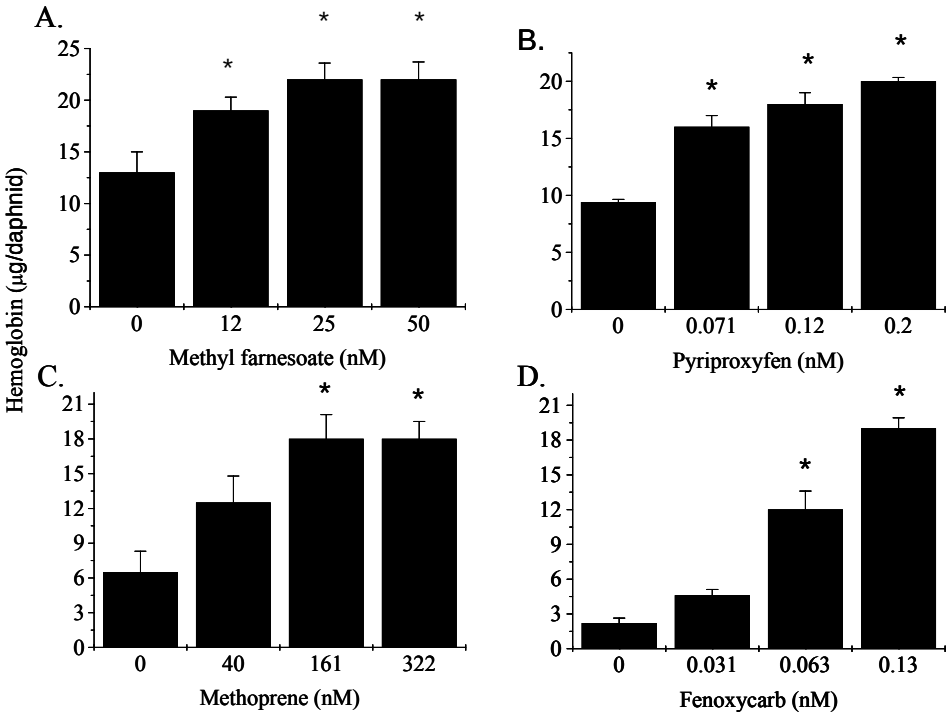
A.
B.
C.
D.
Hemoglobin (µg/daphnid)
Methyl farnesoate (nM)
Pyriproxyfen (nM)
Methoprene (nM)
Fenoxycarb (nM)
0
12
25
50
0.071
0.12
0.2
40
161
322
0.031
0.063
0.13

Figure 2

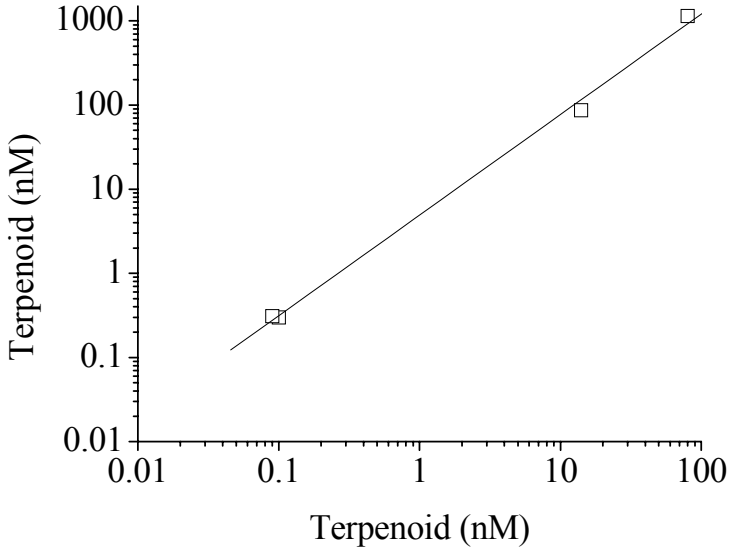

Figure 3

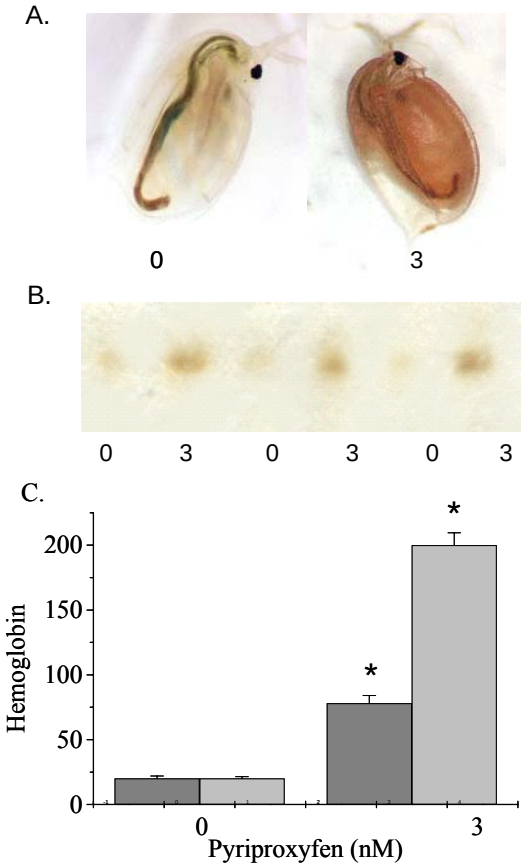

Figure 4

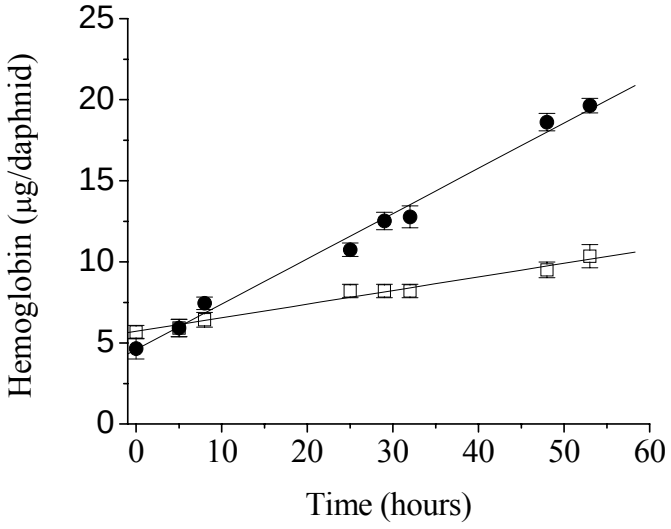
Hemoglobin (µg/daphnid)
25
20
15
10
5
0
0
10
20
30
40
50
60
Time (hours)

Figure 5

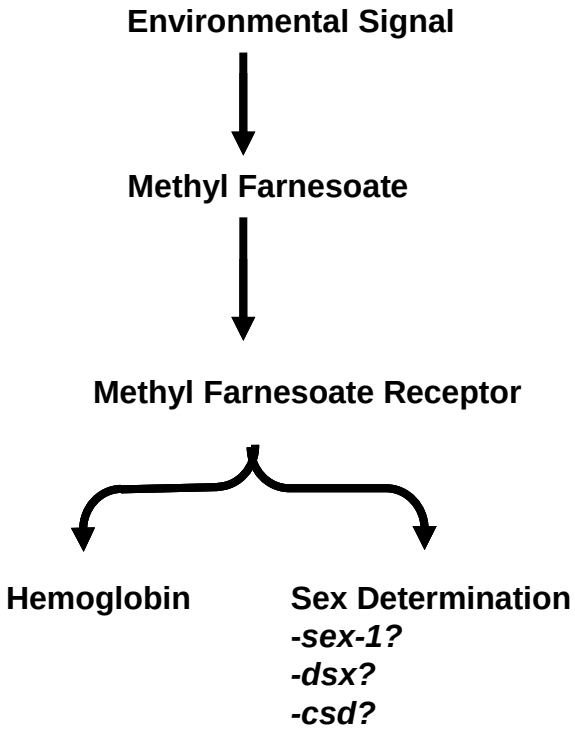
Environmental Signal
Methyl Farnesoate
Methyl Farnesoate Receptor
Hemoglobin
Sex Determination
-sex-1?
-dsx?
-csd?

Figure 6

A candidate juvenoid hormone receptor cis-element in the *Daphnia magna* hb2 hemoglobin gene promoter

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ABSTRACT

Hemoglobin levels are significantly elevated in the crustacean *Daphnia magna* by juvenoid hormones. The present study was undertaken to identify the specific globin (hb) genes that are induced by juvenoids and to identify putative juvenoid response elements (JREs) that may mediate this induction. Gene product of globin 2 (hb2), but not globin 1 and globin 3, was robustly elevated following juvenoid treatment of daphnids. A candidate JRE, located in the promoter of hb2, bound activated factor(s) in response to juvenoid treatment of daphnids. This hormone induced protein:JRE interaction was robust when daphnids were reared at high oxygen tension but was inhibited when daphnids were reared under low pO_2, implying that hypoxia might act to disrupt juvenoid-mediated endocrine signaling. The candidate JRE consists of a steroid/retinoid-response element-like half-site adjacent to a 5' AT-rich extension and thus bears resemblance to response elements that bind monomeric nuclear receptors. The induction of hb2 mRNA levels by juvenoid treatment occurred rapidly (within 4 hrs of exposure) and was not attenuated by treatment of daphnids with cycloheximide. In contrast, cycloheximide treatment did block hormone-mediated elevations in hemoglobin protein levels. Thus, induction of hb2 by juvenoids was not dependent upon the synthesis of secondary transcription factors that bound the JRE but was likely due to activation of the gene directly by the juvenoid-receptor complex. Affinity pull-down experiments with nuclear proteins extracted from juvenoid-treated daphnids using the JRE as bait yielded a 52 kDa candidate for a monomeric nuclear receptor in *D. magna* that may mediate the regulatory activity of juvenoids.

methyl farnesoate, juvenoid, crustacean, response element, sex determination

INTRODUCTION

Daphnids are small, freshwater crustaceans that are key components to most freshwater ecosystems. Daphnids produce mostly female offspring and reproduce asexually through most of their lifecycle [1-3]. However, in response to specific environmental stress signals such as shortened photoperiod, crowding, or lack of food, daphnid females produce male offspring. These males can mate with receptive females that carry haploid eggs which require fertilization to develop. Following fertilization, diploid embryos are encased in a protective ephippium and can exist in a diapause state. Endowed with an astonishing tolerance toward freezing, desiccation or anoxia, these embryos are able to hatch even after decades of dormancy [4]. The sexual phase of the daphnid's reproductive cycle, therefore, provides not only for genetic recombination but also for survival through environmental adversity [1].

The endocrine factors, which relay these environmental signals during oocyte maturation for male sex determination, were determined in our previous work to include the juvenoid hormone methyl farnesoate and various synthetic analogs, but not 20-hydroxyecdysone or related steroids [5,6]. More recently, we demonstrated that juvenoids also elevated hemoglobin levels in maternal daphnids and that production of males and hemoglobin are co-regulated by a common juvenoid signaling pathway [7].

Methyl farnesoate, the unepoxidated form of juvenile hormone III (JHIII), is a major sesquiterpenoid hormone of crustaceans with functions similar to those of juvenile hormones in insects [8]. In crustaceans, methyl farnesoate has been measured in over 30 species including decapods (crabs, shrimp, etc.), cirripedes (barnacles), and branchiopods (brine shrimp) [9]. The

functional characterization of methyl farnesoate in crustaceans has been largely restricted to classical observational endocrine approaches involving hormone administration or ablation. Using such approaches, methyl farnesoate has been implicated in reproductive maturation [10,11], larval morphogenesis [12-14], adult male reproductive behavior [15-17] and sex determination [5,6]. Several phenoxyphenoxy-based compounds, such as fenoxycarb and pyriproxyfen (see Fig. 1), can mimic the action of the natural hormone methyl farnesoate with orders of magnitude greater potencies. For example, methyl farnesoate and pyriproxyfen stimulate male offspring production with EC50 values of 87 nM and 0.31 nM, respectively [6,7]. Similarly, pyriproxyfen and structurally related phenoxyphenoxy derivatives also actively stimulate a diverse set of developmental and genetic responses in insects [18-23], including the induction of known juvenile hormone (JH) target genes, e.g. vitellogenin in locusts [21].

The precise mechanism by which methyl farnesoate regulates physiological functions or transduces stress responses is not known since a nuclear receptor for juvenile hormones has not been identified in crustaceans or with certainty in insects. Considering the structural similarities between methyl farnesoate and JHIII (Fig. 1), these hormones likely elicit regulatory activities through similar processes. Several lines of evidence indicate that JHIII functions as a ligand to an enigmatic DNA binding factor that activates genes equipped with an appropriate juvenoid response element (JRE). In *Drosophila*, the retinoid X receptor homolog ultraspiracle (Usp) [24-28] and the basic-helix-loop-helix/PAS transcription factor Met [29,30] are viewed as candidates for mediating JH-dependent effects on target genes. However, it is questionable whether Usp functions as a physiological JH receptor (JhR) in flies considering the high micromolar concentrations of JHIII required to activate Usp [31] and the consistent failure to find any JH-

mediated activation of JRE-reporter constructs in Usp expressing fly cells [32,33]. Similarly, reports on the exclusive nuclear localization of Met, even in tissues not targeted by JH, raises questions regarding this protein's proposed role as a JhR [34]. While several receptors may contribute in various capacities to this signaling pathway, the definitive identification of a nuclear JHR (insects) or a methyl farnesoate receptor (MfR) in crustaceans remains elusive.

We recently demonstrated that hypoxia-mediated transactivation of hb2, one of the four globin genes in *D. magna*, requires the binding of hypoxia inducible transcription factor (HIF) to hypoxia response elements (HREs) located within its promoter (phb2) [35]. To initiate equivalent work on the induction of hb2 expression through the juvenoid-mediated interaction between cis-elements and trans-acting factors, we now report the characterization of a JRE within the hb2 promoter of *D. magna*. We also explore the relationship between hypoxic and juvenoid stimuli converging upon hb2. Finally, by using an immobilized version of the JRE, we present first evidence of a specifically pulled-down nuclear binding factor that may represent the methyl farnesoate receptor.

MATERIALS AND METHODS

Animal culture and exposure to hypoxia or hormones Daphnids were maintained at North Carolina State University (NCSU animals) [7] and at Brigham and Women's Hospital (BWH animals) [35] using husbandry as cited. Hormone treatments (36 h exposures unless otherwise indicated) were performed at NCSU as reported previously [7]. Normoxia/hormone vs. hypoxia/hormone double exposures were carried out at BWH by using approximately 1000

daphnids in a three-arm, 1 liter flask flow-through system throughout the hypoxic challenge. The hypoxic in-flow gas was composed of computer generated mixtures of O_2 and N_2 (Environics Series 200, Tolland, CT). The pO_2 of the animal milieu was controlled through an oxygen/temperature sensor (CellOx 325, WTW Measurement Systems, Woburn, MA) and gradually adjusted (within 3-6 hrs) to a final concentration between 2-3% oxygen. Normoxic animals were kept in 1 liter of either air-exposed or air-bubbled water. These normoxic or hypoxic cultures each were simultaneously exposed to 400 nM methyl farnesoate (Echelon Biosciences, Salt Lake City, UT) or 3.0 nM pyriproxyfen (Chem Service, West Chester, PA; Crescent Chemical Company, Islandia, NY), dissolved in absolute ethanol (see [6,7] for concentration-response curves between juvenoid-concentration and induction of male daphnids or total hemoglobin). All solutions to which daphnids were exposed, including controls, contained 0.002% ethanol. Normoxic and control animals were occasionally bubbled with air to evaluate potential confounding physical effects of bubbling (i.e., volatilization of hormone, turbulence to daphnids) on the differential hormone responses reported below between normoxic and hypoxic daphnid cultures. Therefore, three experimental treatments of normoxia/ethanol, normoxia/juvenoid and hypoxia/juvenoid were created. These treatments were carried out for 36 hrs at room temperature.

Semi-quantitative RT-PCR Changes in expression of hb1, hb2, and hb3 genes following exposure to the juvenoid pyriproxyfen were monitored using a semi-quantitative RT-PCR approach. One week-old daphnids were exposed to 0 or 3.0 nM pyriproxyfen, in the presence or absence of 0.36 μM cycloheximide (Sigma), as described for specific experiments. Exposure to each treatment was performed in three 1 liter beakers (50 daphnids/l of media). Following exposure, 25-30

daphnids were randomly removed from each beaker, transferred to a mortar filled with liquid nitrogen, and ground to a fine powder with a pestle for RNA isolation. RNA was isolated from approximately 30 mg of each sample with the SV Total RNA Isolation kit (Promega, Madison, WI) according to the manufacturer's instructions. The quantity of RNA was determined by absorbance at 260 nm and purity by the 260/280 nm ratio. RNA was reverse transcribed to cDNA with oligo (dT) primers using the Promega ImProm-II™ Reverse Transcription System.

The primers for hb1, hb2, and hb3 used in PCR were designed to specifically amplify each of the three *D. magna* hemoglobin cDNA's which are known in their entirety [36]. Hb4 cDNA was not evaluated in this study since only its C-terminal coding sequence and 3' untranslated region have been characterized to date (Dr. H. Yamagata, pers. comm.). Actin served as a control for loading and amplification. Primer sequences used to amplify actin and the hemoglobin cDNA's are provided in Table 1. All primers were synthesized by Qiagen (Valencia, CA) and were reconstituted and stored in TE buffer. The total volume of polymerase chain reactions was 50 μl and consisted of 25 μl PCR Master Mix (Promega), 0.1 μM each of the upstream and downstream primers, 1 μl cDNA, and 22 μl of nuclease-free water. All polymerase chain reactions were carried out on a PTC-100™ Programmable Thermal Controller (Bio-Rad, Las Vegas, NV). The PCR temperature profile consisted of one denaturation cycle of 95 °C for 2 minutes, followed by a variable number of cycles of: 1 minute at 95 °C, 1 minute of annealing, and 1 minute of extension at 72 °C. A corresponding reaction with actin primers was carried out for each hemoglobin reaction. Amplification of all products was initially performed at varying numbers of cycles to the point where each subsequent amplification cycle doubled the product amount. This number of cycles was then used to estimate the relative amount of mRNA in each

sample. Resulting PCR products were resolved on 2% agarose gels and stained with ethidium bromide. Gels were visualized and the density of each band was quantified using Scion imaging software (available online at www.scioncorp.com). Relative hemoglobin was calculated by dividing the hemoglobin band density by the actin band density for each sample. PCR products were purified and sequenced (Seqwright, Houston, TX). Sequenced fragments were entered into Blast nucleotide searches to confirm the identity of the PCR products.

Hemoglobin measurements Total hemoglobin levels in daphnids were measured in some experiments as we have described previously [7]. Briefly, daphnids were homogenized in distilled water and the post 10,000 *g* supernatant was used to measure hemoglobin by absorbance at 415 mM following the addition of KCN to a final concentration of 0.006%.

Electrophoretic mobility shift assays (EMSA) Following treatment, as indicated in the text, daphnids were harvested for the extraction of whole cell proteins for use in electrophoretic mobility shift assays. Sifted animals were washed in ice-cold 1x phosphate buffered saline and quick frozen in liquid nitrogen. Subsequently, daphnids were homogenized and further processed through: a) wash-centrifugation cycles in hypotonic buffer and b) extraction of whole cell protein during Dounce homogenization of tissue pellets in hypertonic buffer as detailed previously [35,37]. Protein content of the extracts was determined by protein-dye binding using commercially available reagents (BioRad, St Louis, MO). Extracts were kept frozen at -80 °C until used in the assays. For EMSA's, whole cell protein (20-25 μg) was incubated in binding buffer (15 mM Tris-HCl pH7.5, 75 mM KCl, 1 mM $MgCl_2$, 1.05 mM EDTA, 5.25 mM DTT, 0.5 mM sodium vanadate, 10% glycerol, 0.03% Igepal CA-630) with [γ-^{32}P]ATP labeled

oligonucleotides (~1 ng, "probe") as summarized in Table 2. Poly(dI-dC) (1 μg) was used in these binding reactions to minimize nonspecific binding to the probe. DNA:protein complexes were resolved from free probe by electrophoretic separation in native 5% polyacrylamide gels. Heterologous supershift experiments were performed using monoclonal anti-Usp and anti-EcR-common-region (DDA2.7; AG10.2) antibodies that are directed against conserved epitopes in some of *Drosophila*'s candidate JhR's (see above). Anti-Usp and anti-EcR antibodies were generously provided by Drs. Bass (EMBL Heidelberg, Germany) and Thummel (University of Utah, Salt Lake City, USA), respectively. Relative signal intensities of gel shifted bands between juvenoid-exposed vs control or between normoxia/juvenoid vs hypoxia/juvenoid samples were quantified by background-subtracted phosphoimaging densitometry (ImageQuant, Mac Version 1.2, Amersham Biosciences, Piscataway, NJ, USA).

Pull-down assays Nuclear proteins were prepared from daphnids as generally described previously [35] for use in pull-down assays. In brief, daphnids were sifted, washed with ice-cold phosphate-buffered saline and quick-frozen in liquid nitrogen. Daphnids were subsequently homogenized and tissue pellets were resuspended in three volumes of hypotonic buffer, then homogenized with a Dounce homogenizer, and centrifuged at 3,000 rpm for 5 min. The organellar (nuclear) pellet was mixed for 30 min in 3 volumes of hypertonic extraction buffer (20 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 420 mM KCl, and 20% glycerol) to dissociate proteins and DNA. The mixture was centrifuged at 14,000 rpm for 30 min and the supernatant was dialyzed against one change of buffer containing 5 mM Tris-HCl (pH 7.8), 25 mM KCl, 50 μM EDTA, 5% glycerol, 250 μM sodium vanadate, 125 μM dithiothreitol. Buffers were supplemented with Complete-Mini-EDTA-free™ protease inhibitor cocktail tablets (Roche,

Indianapolis, IN) to minimize sample proteolysis. All steps were carried out either on ice or at 4ºC.

The -247 wild-type and m3 mutant biotinylated probes were designed as illustrated in Table 2. Oligonucleotides were synthesized, biotinylated at the sense strand 5' end, purified by polyacrylamide gel electrophoresis, and annealed in equimolar amounts of both strands (Integrated DNA Technologies, Coralville, IA). The -247 or m3 affinity beads were processed and pull-down assays performed as described [38]. In brief, Dynabeads M-280 Streptavidin (Dynal, Inc., Lake Success, N. Y.) were washed three times in phosphate-buffered saline (pH 7.4) containing 0.1% bovine serum albumin and two times with Tris-EDTA containing 1 M NaCl. Between each wash, beads were pulled down using a magnetic particle concentrator (Dynal, Inc.). Double-stranded, biotinylated DNA probe (10 pmol) was added to the beads (250 μg), and the mixture was incubated for 40 min at room temperature under constant mixing. The beads were then washed three times in Tris-EDTA with 1 M NaCl and two times in 1x binding buffer (10 mM Tris (pH 7.5), 50 mM KCl, 1mM $MgCl_2$, 1 mM EDTA, 5.5 mM DTT, 5% glycerol, 0.03% Nonidet P-40).

Nuclear protein extract (35 μg) was preincubated for 5 min in 1x binding buffer, 2.5 μg of poly(dI-dC), and 1 mM sodium orthovanadate (Sigma) in a total volume of 100 μl. Dynabeads M-280 Streptavidin (250 μg) bound to probe (10 pmol) was then added to the mixture and incubated an additional 20 min at room temperature. Proteins bound to the probe were pulled down with a magnetic particle concentrator. The beads were washed once in 0.5x binding buffer (5 mM Tris (pH 7.5), 25 mM KCl, 0.95 mM EDTA, 5 mM DTT, 0.03% Nonidet P-40)

containing 0.5 μg of poly(dI-dC) per ml, and subsequently pulled down with a magnetic particle concentrator. Beads were resuspended in 1x NuPAGE sample buffer (Invitrogen, Carlsbad, CA), incubated for 5 min at 100°C, and resolved by SDS-PAGE. Electrophoresis was performed at 200 V for 50 min, using a 4-20% NuPAGE Novex Bis-Tris gradient gel (Invitrogen). Protein bands were visualized by silver staining (Silver Stain Plus, Bio-Rad).

RESULTS

Juvenoid-mediated hemoglobin gene induction The expression of the globin genes hb1, hb2 and hb3 in response to pyriproxyfen was evaluated in an effort to identify the sources of the increased hemoglobin accumulation in response to juvenoids by daphnids. The primers used for product amplification (see Table 1) successfully targeted the intended globin gene products with each amplicon having greatest identity (95 to 100 %) to its respective cDNA. The hb1 and hb3 mRNA levels were marginally elevated (1.7-fold) in response to pyriproxyfen exposure (Fig. 2a, c). In contrast, hb2 mRNA levels were increased nearly 8-fold in response to the same treatment (Fig. 2b). Clearly, the hb2 gene is highly responsive to the regulatory activity of pyriproxyfen and is the primary contributor, among these genes, to the increased accumulation of hemoglobin following juvenoid treatment. Comparable results were observed among daphnids treated with methyl farnesoate (data not shown).

Discovery of a methyl farnesoate receptor candidate cis-element Having established that the terpenoid methyl farnesoate and certain phenoxyphenoxy-based analogs (i.e., pyriproxyfen, fenoxycarb, see Fig. 1) activate *D. magna*'s globin hb2 gene independent of pO_2 fluctuations, we sought to identify response element(s) associated with the hb2 gene as possible target sites of the

crustacean nuclear juvenoid receptor. Since *D. magna*'s genome contains at least four globin genes in the arrangement $^{5'}$hb4-hb3-hb1-hb2$^{3'}$ [36,39], we conducted a computer search for candidate juvenoid response elements (JREs) within the 5' flank of hb2 (i.e., the hb1 – hb2 intergenic DNA region (1-2igDNA)). Functional, HIF-binding hypoxia response elements (HREs) have recently been characterized [35] within the 1-2igDNA residing hb2 promoter (phb2). The JRE query we used in our current search for 1-2igDNA juvenoid regulatory sites shared similarity to elements described by Wyatt and colleagues for juvenile hormone (JH)-inducible genes, such as vitellogenin or jhp21, in locusts [21,40-42]. This locust motif loosely conforms to two direct repeat half-sites separated by four nucleotide spacers. Its consensus reads: $^{5'}A/_G{}^A/_G{}^A/_G$GTTnnnn$^A/_T{}^A/_C$C$^A/_T$N$^{3'}$ with half-sites in capital letters, lower case n = spacer nucleotides, N = any nucleotide. The JRE search within 1-2igDNA revealed one candidate motif: $^{5'}$GAAGTTcatcTCCAC$^{3'}$ (half-sites: capitalized, spacer: lower case) at position -229 relative to the hb2 transcription start site (TSS; note that all sequence distances and probe names reported herein are given in reference to the number of nucleotides between the furthest 5' base of the sequence and the TSS) [39]. Next, we designed a 60mer DNA oligonucleotide (JRE_{60}), which spanned the candidate motif, and additional 19 to 25mer DNA oligonucleotides to distinguish defined regions around the -229 site (see Table 2) as radiolabeled probes in subsequent electrophoretic mobility shift assays (EMSAs). Fig. 3a depicts the 1-2igDNA with its characterized HREs [35] and shows the phb2 region that was evaluated for juvenoid-induced DNA-binding activity. Also shown are the oligonucleotide probes that were used to screen for DNA-binding activity in whole cell extracts following exposure of daphnids to either methyl farnesoate or pyriproxyfen under normoxic or hypoxic pO_2. Hormone and pO_2 exposure of, and

protein extraction from, whole animals was necessitated by the lack of a continuous *Daphnia* cell line (see [35] for failed efforts to culture and immortalize primary cells from *Daphnia* embryos).

Among the first generation probes used in these EMSAs (green, Fig. 3a), only probes -247 and JRE_{60} yielded reproducible gel shifts derived from pyriproxyfen-treated daphnids (Fig. 3b right panel). Furthermore, gel shifts with both probes -247 and JRE_{60} were attenuated when daphnids were treated with pyriproxyfen under low pO_2 (Fig 3b, compare normoxic (N) vs. hypoxic (H) pO_2 exposure in lanes 10+11, 18+19; and see below). Binding activity also was evaluated using a 33mer DR12 probe (USP_{33}) containing the AGGTCA half-site direct repeat of an ecdysone response element that is known to bind both Usp, one of *Drosophila*'s nuclear receptor candidates for juvenile hormone III (JHIII, Fig. 1) and HR38 alone as well as the Usp:EcR and Usp:HR38 heterodimers [43,44]. However, neither this probe, nor the one containing the locust-like JRE at -229 (Fig. 3a, probe -233) resulted in detectable gel shifts (Fig. 3b, lane 12+13; 20+21), suggesting that putative JRE's previously identified in locust [21,40-42] and *Drosophila* [43,44] are not recognized by factors involved in juvenoid signaling in daphnids. Along similar lines, attempts to supershift the -247 bound complex with antibodies directed against *Drosophila*'s Usp or EcR protein repeatedly failed (data not shown), further underscoring the notion that sufficiently divergent or even novel *Daphnia* factor(s) recognize the -247 sequence.

Subsequent analyses with probe -247 (Fig. 3b left panel) revealed that whole cell extracts derived from pyriproxyfen-treated daphnids consistently conferred a 2-3 fold enhanced gel shift as compared to control daphnids treated only with ethanol solvent (Fig. 3b, lanes 1 vs 3; 4 vs 5

and 6). Exposure of daphnids to methyl farnesoate also augmented the gel shift measured with probe -247 (Fig. 3b, lane 1 vs 2). Pyriproxyfen was significantly more effective than methyl farnesoate at stimulating this protein:DNA interaction (Fig. 3b, lane 2 vs 3) which is consistent with the differential bioassay potency among juvenoids (Fig. 1) and the susceptibility of methyl farnesoate to inactivating biotransformations within the intact organisms [45]. An appreciable level of binding to probe -247 occurred with control extracts (Fig. 3b, lanes 1, 4), suggesting the presence of endogenous ligand (e.g., methyl farnesoate) within the daphnids.

Cross-talk between hypoxia and juvenoid signaling pathways Considering the wealth of functional HREs within phb2 (Fig. 3a and [35]) that contribute to conferring hb2 induction in response to hypoxia, experiments were conducted to determine whether hypoxia influences the juvenoid-dependent -247 binding activity. Using whole cell extracts that were derived from pyriproxyfen-treated daphnids reared under normoxic or hypoxic conditions, we found that culture for 36 h at 2-3% oxygen reproducibly inhibited pyriproxyfen-induced DNA binding (Fig. 3b, lanes 5 and 6 vs 7; 10 vs 11). Accordingly, all follow-up experiments were performed under normoxic pO_2. In addition the JRE_{60} probe yielded a novel gel shift that occurred under normoxic, but not hypoxic, conditions (Fig. 3b, lanes 18 (asterisk) vs 19).

The JRE resembles a target site for monomeric nuclear receptors The -247 sequence lacks the two half-site constituency that is characteristic of most binding sites for *dimeric* nuclear hormone receptors. Instead, -247 does contain a core $^{5'}$TGGTTA$^{3'}$ that is preceded on the 5' end by an AT-rich extension ($^{5'}$TTTTTATTTTT$^{3'}$; Fig. 4a). This core sequence bears resemblance to the consensus half-site AGGTCA, found in many steroid and retinoid response elements, and to the

functional JRE half-site in locusts ($^{5'}$AGGTTC$^{3'}$) (61). Association of similar half-sites with a 5' AT-rich extension is characteristic of binding sites targeted by *monomeric* mammalian nuclear receptors such as NGFI-B, RORα1 and FTZ-F1, as well as *Drosophila*'s NGFI-B homolog HR38 [46-49].

The sequence within probe -247 was more definitively evaluated using five mutant oligonucleotides (designated m1 through m5) consisting of triple base substitutions of various regions of the -247 probe (Fig. 4a). Binding of the pyriproxyfen-induced complex to all mutant -247 probes was markedly reduced relative to the unaltered 'wildtype' sequence (Fig. 4c). The fact that probes m1, m2, m3 exhibited the least binding activity (7-18% of wildtype binding, Fig. 4c), along with the lesser perturbance of -247-binding by alterations within the core (m4, m5), is consistent with the requirement for an intact AT-rich extension to maximize high-affinity contacts. A 20-fold excess of -247 wildtype cold competitor oligonucleotide over -247 radiolabeled probe abrogated the formation of the -247 complex, thus confirming the specificity of this interaction (Fig, 4c, lane 8). In contrast, probes m1-m3 competitor oligonucleotides were least effective at disrupting binding to the wildtype -247 probe when equivalently used in 20-fold excess over -247 probe (Fig. 4c, lane 8 vs. 9-11). The near-loss of any binding (Fig. 4c, lane 4: 7% residual binding) or competition (Fig. 4c, lane 11) with probe m3 marks the AT-rich region immediately upstream to the core sequence, in striking resemblance to the DNA binding by NGFI-B [50,51], as most critical for maximal association with the pyriproxyfen-induced complex. The requirement of an AT-extension at the 5' end of the JRE half-site is further inferred from analyses of the hb1 and 3 promoter regions (phb1, phb3). These phb1 and phb3 sequences contain the following motifs as sole candidates for the JRE (the core half-site is

underlined and compromised AT-rich region is in lower case; 5' position for phb1 motif: -186, phb3 motif: -266):

phb2: 5′TTTTTATTTTTCTGGTTA3′;

phb1: 5′AAAcTAgggTTTTGGTTA3′;

phb3: 5′AAAcTccTTTTCTGGTTC3′.

These compromised AT extensions may be responsible for the marginal transactivation of the hb1 and hb3genes by juvenoids (Fig. 2).

Truncated -247 derivatives (Fig. 4b, "new probes" in Fig. 3a; sequences in Table 2), which were devoid of either the entire (T_{10}) AT-extension (probe -258, not to be confused with -258 HRE, see Fig. 3a) or retained only half of the extension (T_5, probe -247.2) also resulted in a much reduced and smeared association with the complex under normoxic oxygen tensions while maintaining interference of binding by hypoxic exposure (Fig. 4c, lanes 16-19 vs lane 14). Because the AT-extension-only probe -242 (no TGGTTA core, Fig. 4b) revealed roughly half of the wildtype capacity to bind this endocrine factor (Fig. 4c, lanes 20 vs 14), an approximately even split in affinity to the -247 sequence between core and extension can be surmised. This even distribution of binding affinity is consistent with that observed by Meinke et al. [50] for the NGFI-B recognition site.

Hb2 mRNA induction by juvenoids does not require de novo protein synthesis Increased JRE binding following juvenoid treatment of daphnids may result from a ligand-induced activation of existing transcription factors, which then directly bind to the JRE. Alternatively, juvenoid treatment may result in the receptor-mediated induction of secondary transcription factors that

bind the JRE and stimulate hb2 transcription. Induction of hb2 mRNA in response to pyriproxifen in the presence and absence of the translation inhibitor cycloheximide was therefore investigated. Pyriproxyfen treatment significantly elevated hb2 mRNA levels in the absence or presence of cycloheximide (Fig. 5b). In contrast, hemoglobin protein levels were elevated only when daphnids were exposed to pyriproxyfen in the absence of cycloheximide (Fig. 5a). Thus, cycloheximide treatment successfully blocked new protein synthesis, but had no effect on the induction of hb2 mRNA. The exposure time required for pyriproxyfen to induce hb2 mRNA levels also was investigated, since the induction of hb2 would be rapid if this transactivation was the direct consequence of pyriproxyfen binding to an existing receptor. Elevation of hb2 mRNA levels was discernable after as little as 2 hrs exposure of daphnids to pyriproxyfen (Fig. 6). Comparable rapid induction also occurred with methyl farnesoate exposure (data not shown). We conclude that juvenoids activate existing juvenoid receptor that directly binds the hb2 JRE to stimulate transcription.

Visualization of a candidate methyl farnesoate receptor Specific binding of a juvenoid-activated nuclear factor to the -247 nucleotide sequence suggested that this sequence could be used as immobilized bait to capture its protein complement (juvenoid receptor). Electrophoretic separation and silver staining visualization of proteins pulled down by the -247 probe affixed to magnetic beads yielded a 52 kDa protein from pyriproxyfen-treated animals (Fig. 7, lane 3) that was neither detected in untreated daphnids (lane 2), nor in reaction with the mutant bait (m3/-247 probe; lanes 4, 5). Several additional proteins were pulled down non-specifically as assessed by their lack of discriminate binding to wild-type and mutant bait and their lack of response to pyriproxyfen-treatment (e.g., nsA, nsB bands). Thus, the 52 kDa protein exhibits specific JRE

recognition and pyriproxyfen induction which are two important characteristics of a putative juvenoid receptor. In addition, these results point to a strategy for the future isolation and characterization of this protein.

DISCUSSION

This study identifies a candidate response element (JRE) for the daphnid juvenoid or methyl farnesoate receptor (MfR) within the hb2 promoter region at position -230, suggesting that transcriptional activation of the hb2 gene by juvenoids is mediated by a receptor (i.e., MfR) occupying this cis element. The MfR:JRE interaction was clearly potentiated by animal exposure to nanomolar concentrations of the putative MfR ligands pyriproxyfen and methyl farnesoate. Pyriproxyfen was far more effective in stimulating binding to the JRE as compared to the natural hormone methyl farnesoate. This potency difference is consistent with the superior biological potency of pyriproxyfen to stimulate male sex determination in daphnids and globin transcripts (see above and [6,7]). Such a super-stimulated MfR function in the presence of pyriproxyfen, a phenoxyphenoxy compound, bears resemblance to the ligand-selectivities of the lipid-sensing PPAR factors in mammals [52,53].

The daphnid JRE consists of a core steroid/retinoid-type half-site flanked on the 5' end by an AT extension. This structure conforms to that of a response element for a monomeric nuclear receptor. For example, the mammalian orphan receptor NGFI-B binds to its response element $^{5'}$AAAAGGTCA$^{3'}$ [48] as a monomer by means of separate contacts to the AGGTCA core and the 5' AAA extension [50]. The latter interaction allows for a novel interface between the minor groove of the 5' adenine bases and the receptor's C-terminal extension of its DNA binding

domain that is key in imparting high binding affinity [50,51]. In regard to potential JREs in insects, a recently identified 30 bp juvenoid response region upstream of the JH esterase gene from the spruce budworm was found to consist of two half-sites separated by a 4-nucleotide spacer [54]. However, one of the AGATTA half-sites is flanked on the 5' end by an AT-rich sequence whose deletion also resulted in a significantly reduced competition with the binding activity to the wildtype sequence, thereby implying conserved binding mechanisms between crustacean and insect juvenile hormone nuclear receptors. Based upon the structural similarities of the candidate phb2 JRE to known cis-elements of monomeric receptors, which all consist of steroid/retinoid element-like half-sites together with 5' AT-rich extended sequences for high-affinity receptor interaction with its target DNA, we tentatively conclude that the daphnid nuclear juvenoid receptor binds as a monomer to probe -247.

The fact that the MfR:JRE complexation is inhibited by hypoxia implies that low oxygen tensions might potentially disrupt some endocrine functions in daphnids. Aquatic environments world wide are faced with low oxygen tensions due to eutrophication [55]. Oxygen deprivation has been shown to interfere with endocrine signaling in fish resulting in reduced fecundity, developmental delays, and malformations [56,57]. Kubo et al. [58] observed that bisphenol A inhibits hypoxic responses of cultured cells by enhancing the degradation of the regulatory HIF subunit, HIF-1α. We recently demonstrated that, while bisphenol A has no intrinsic juvenoid activity in daphnids, it potentiates the ability of methyl farnesoate to stimulate male sex determination of offspring [59]. These observations in combination with results from the present study suggest that bisphenol A may enhance the activity of methyl farnesoate by reducing the

occupancy of HIF/HRE complexes that otherwise attenuate responsiveness of adjacent JREs to methyl farnesoate-dependent receptor binding.

We hypothesize that the proximity between the functional -258 HRE [35] and -230 JRE motifs (only 10 bases apart, Fig. 3a) in phb2 could be a central feature for a mutually exclusive co-regulation of this gene by hypoxic/HIF or juvenoid/MfR mediated signaling pathways. We propose that under normoxic conditions, expression of the hb2 gene is predominantly under the regulatory control of methyl farnesoate or related juvenoid hormone(s). As oxygen levels begin to decline, hb2 might progressively fall under a HIF/HRE regime, at the expense of its hormonal responsiveness. Such a 'sliding response' between two stimuli likely provides, in analogy to *Chironomus* larvae [60,61], for physiologically and environmentally adjustable hemoglobin levels. This would allow *Daphnia* to accommodate increased metabolic requirements and oxygen homeostasis during critical periods of reproduction [62] as well as during periods of environmental stress [35]. If hypoxia (HIF) indeed acts to disrupt methyl farnesoate-driven induction of hemoglobin gene expression, the same inhibition might be found for the production of male offspring and sexual reproduction in *D. magna*, since globin genes and sex-determining genes likely represent different effector targets within the same methyl farnesoate/MfR pathway [7]. Support for this hypothesis is found in the earlier works of Loudon [63,64] who observed a direct correlation between the incidence of male offspring developing from eggs and environmental oxygen tensions for the beetle *Tenebrio molitor* (i.e., progressive hypoxia yielded less and less males). Future work needs to explore the ecologically relevant impact of low oxygen tensions on juvenoid-mediated gene regulation with regard to metabolic (e.g. O_2

transport; hypometabolism) or reproductive (e.g. sex determination; reproductive diapause) control circuits in these model arthropods.

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Table 1. Sequences and amplification conditions used to evaluate induction of the hb1, hb2, hb3, and actin mRNA by RT-PCR following exposure of daphnids to pyriproxyfen.

cDNA	Forward primer	Reverse primer	Amplicon size (bp)	Number of cycles
hb1	5′ AACAAATTGCTCTGGTTGCC 3′	5′ GACATAAAGGTTTTTGAGTGCCA 3′	286	26
hb2	5′ TTACCACCAGTGTCACCACTGTC 3′	5′ CGGCGAACTTGCTGAACTTCTTT 3′	186	29
hb3	5′ ACCACCGTTTCCGCCGAC 3′	5′ TTGGTCAAAAGCTCGGCTTGG 3′	203	44
Actin	5′ AAAAGGAAATCACCGCCCTTG 3′	5′ CTGGGAAGTGAAAACGTCAAGAGAG 3′	342	24-26

Table 2. Oligonucleotide probes used in electrophoretic mobility shift assays. Wildtype (wt) and mutant (m) -247 probes are indicated; half-sites of putative insect JRE in probes -233, -219 and JRE_{60}, as well as of Usp/HR38 binding sites in probe USP_{33} are underlined; phb2 denotes globin 2 (hb2) promoter of *Daphnia magna*, with probe position given as 5'/3' distances in nucleotides to hb2 transcription start site.

Probe name	Probe sequence: 5'-3' orientation, single strand only	Comment/Reference
-266	TGGTTAAAAATGTTTGTACGTGATT	phb2 at -242/-266
-233	TGTGGAGATGAACTTCTTTT	phb2 at -214/-233; contains putative JRE (55, 59-61)
-219	GGTTGTTTGCTTTCTGTGGA	phb2 at -200/-219
-209	CTGTCGTCTGGTTGTTTGC	phb2 at -191/-209
JRE_{60}	CTGTCGTCTGGTTGTTTGCTTTCTGTGGAGATGAACTTC TTTTTATTTTTCTGGTTAAAA	phb2 at -191/-250
USP_{33}	GCGAAAGGTCAAGAGGCCAAAGAAGGTCAGGAA	Drosophila ng gene 33mer, binds Usp and Usp:HR38 (5, 7)
wt-247	TCTTTTTATTTTTCTGGTTA	phb2 at -228/-247, contains JRE (this paper)
m1/-247	TCTgggTATTTTTCTGGTTA	1. mutant of -247: T3 → G3 in lower case
m2/-247	TCTTTTgggTTTTCTGGTTA	2. mutant of -247: TAT → G3 in lower case

m3/-247	TCTTTTTATgggTCTGGTTA	3. mutant of -247: T3 → G3 in lower case
m4/-247	TCTTTTTATTTTaaaGGTTA	4. mutant of -247: TCT → A3 in lower case
m5/-247	TCTTTTTATTTTTCTaaaTA	5. mutant of -247: GGT → A3 in lower case
-247.2	…ATTTTTCTGGTTA…	1. truncation of -247: T_5 + core, flanked by MluI and KpnI sites (…)
-242	TTCTTTTTATTTTTCT	2. truncation of -247: T_{10} only
-258	TCTGGTTAAAAATGTTTGT	3. truncation of -247: core only

FIGURE LEGENDS

Fig. 1. **Chemical structure and relative potency of natural juvenile hormones (juvenile hormone III, methyl farnesoate), terpenoid analogs (methoprene) and phenoxyphenoxy-based analogs (pyriproxyfen, fenoxycarb).** Potencies reflect the ability of the chemicals to stimulate male offspring production in daphnids relative to methyl farnesoate. Values were derived by dividing the EC50 values for methyl farnesoate by the EC50 value for the juvenoid ([5-7,65]; and unpublished data).

Fig. 2. **Daphnid hemoglobin mRNA levels, normalized to actin, in response to 36 h exposure to 0 and 3.0 nM pyriproxyfen and measured by semi-quantitative RT-PCR.** Primers used to detect globin gene product specifically amplified sequences derived from the hb1 (**a**), hb2 (**b**) and hb3 (**c**) genes. Bars represent the mean + standard deviation of three replicate treatments.

Fig. 3. **Juvenoid response element (JRE) in the hb2 promoter of *D. magna*.**
a) Schematics of the hb1-hb2 intergenic DNA with hypoxia response elements (HREs), the transcription start site (TSS) and the first three exons (red blocks) of hb2 indicated. DNA between positions -275/-191 is highlighted to visualize the phb2 region around the computationally retrieved JRE candidate $^{5'}$*gaagtt*catc*tccac*$^{3'}$ (half-sites in italics) at position -229. First generation EMSA oligonucleotides (green), used to screen the highlighted DNA for methyl farnesoate- and pyriproxifen-inducible gel shifts are depicted underneath in alignment to the sequence (see Table 2 for sequences). The oligonucleotides are named in

accordance to the position of their 5' nucleotide relative to the TSS (23). Also depicted, with capitalized bases, are the functional HRE at -258 (11) and the JRE at -230 that was identified in this study. Second generation EMSA oligonucleotides ("new probes", yellow), all being truncation derivatives of probe -247, are shown at the bottom of the graphics in alignment to the sequence.

b) EMSA screen for juvenoid-induced DNA binding activity using 20-25 μg of whole cell protein per lane from normoxic (N) and hypoxic (H) *D. magna* cultures, which had been exposed for 36 h to 3nM pyriproxyfen ("PP") (lanes 8-21). Oligonucleotide EMSA probes are indicated above each N/H pair of lanes and can be identified in Fig. 3a. Lanes 1-7 summarize the juvenoid-induced DNA binding activities obtained with probe -247 in response to 36 h exposure to either 400 nM methyl farnesoate ("MF" lane 2) or 3 nM pyriproxyfen ("PP" lanes 3, 5-7) when compared with exposure to 0.002% ethanol (controls, "C" lanes 1+4) under either normoxic ("N" lanes 1-6) or hypoxic ("H" lane 7) conditions. Results shown in lanes 1-7 were reproduced in 2-4 independent exposures.

Fig. 4. **Requirement of -247 bases for binding of pyriproxyfen (PP)-induced complex**.

a) Series of -247 mutants ("m1" – "m5") containing triple-base mutations (capitalized alterations to wildtype sequence) in the JRE. The -247 wildtype sequence ("wt") is shown with core (underlined) and 5' AT extension (italics). The 5' to 3' sequences read from right to left to facilitate identification of -247 bases within bottom strand in Fig. 3a.

b) Truncation derivatives of the -247 probe containing the "core" half-site ($^{5'}$TGGTTA$^{3'}$) and/or complete ($^{5'}$TTTTTATTTTT$^{3'}$ = T_{10}) or half-preserved (T_5) 5' extension ("5'AT") regions. Truncated oligonucleotides are illustrated in Fig. 3a (see Table 2 for sequences).

c) EMSA screen of wildtype -247 oligonucleotide (wt, lane 1) and -247 triple-base mutants (m1-m5) (lanes 2-6), of -247 probe (lane 7) in competition (competitor: +) with 20-fold excess of wildtype (lane 8) or m1-m5 -247 oligonucleotides (lanes 9-13), and of -247 truncation derivatives (lanes 16-21) relative to -247 wildtype probe (lanes 14+15, see text for discussion). Whole cell protein (25 μg), extracted from normoxic (N) or hypoxic (H) daphnid cultures, that were also treated for 36 h with 3 nM pyriproxyfen (+ PP), was used as shown above lanes. Binding of the pyriproxyfen-induced complex from normoxic animals by the five mutant probes (lanes 2-6) and the truncation derivatives (lanes 16, 18, 20) relative to the -247 wildtype probe (100%; lanes 1, 14), as determined by densitometric analyses of phosphoimaged gel shift signals, are indicated underneath the respective lanes.

Fig. 5 **Hemoglobin protein (a) and hb2 mRNA (b) levels following exposure to pyriproxyfen (PP, 3.0 nM), cycloheximide (CHX, 0.36 μM) individually or in combination.** Data are presented as the mean and standard deviation of three replicate treatments each consisting of 3 (protein) or 30 (mRNA) adult daphnids. An asterisk denotes a significant ($p<0.01$) difference from the control (ANOVA, Dunnett's test).

Fig. 6 **Hemoglobin hb2 mRNA levels during exposure of daphnids to 3.0 nM pyriproxyfen.** The hb2 mRNA levels among pyriproxyfen-exposed animals are expressed relative to hb2 mRNA levels measured in control daphnids that were sampled at the same time period. Data are presented as mean $\pm$ standard deviation of two replicate treatments with each treatment consisting of 25 adult daphnids. Significance in elevation in hb2 mRNA as compared to control levels at time 0 are denoted by p values (ANOVA and Dunnett's test).

Fig. 7 **Silver-stained SDS-PAGE separation of proteins precipitated from nuclear extracts by the -247 wildtype sequence ("wt") and a -247 mutant sequence ("mut").** Lane 1: molecular weight markers, lanes 2, 3: proteins from untreated (lane 2) and pyriproxyfen (pp)-treated (lane 3) daphnids pulled-down with the *wt* -247 bait; lanes 4, 5: proteins from pp-treated (lane 4) and untreated (lane 5) daphnids precipitated by the *mut* m3 -247 bait. Proteins were separated using a 4-20% gradient gel. MfR denotes the location of the putative juvenoid receptor, "nsA" and "nsB" denote the locations of two non-specifically isolated proteins. Note the non-variant staining of bands nsA and nsB as indication for approximately equal loading across samples (lanes 2-5). These same results were obtained in two completely separate experiments.

Fig. 1

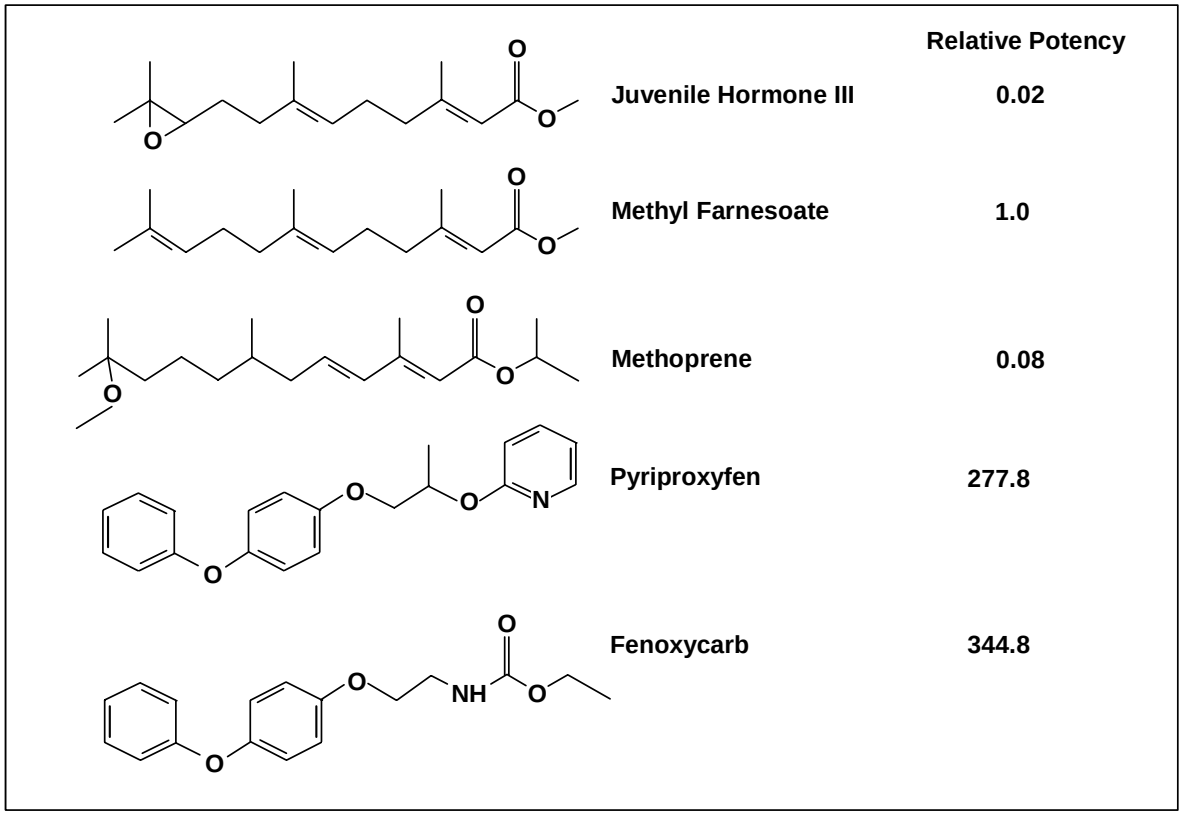

Relative Potency
Juvenile Hormone III
0.02
Methyl Farnesoate
1.0
Methoprene
0.08
Pyriproxyfen
277.8
Fenoxycarb
344.8

Fig. 2

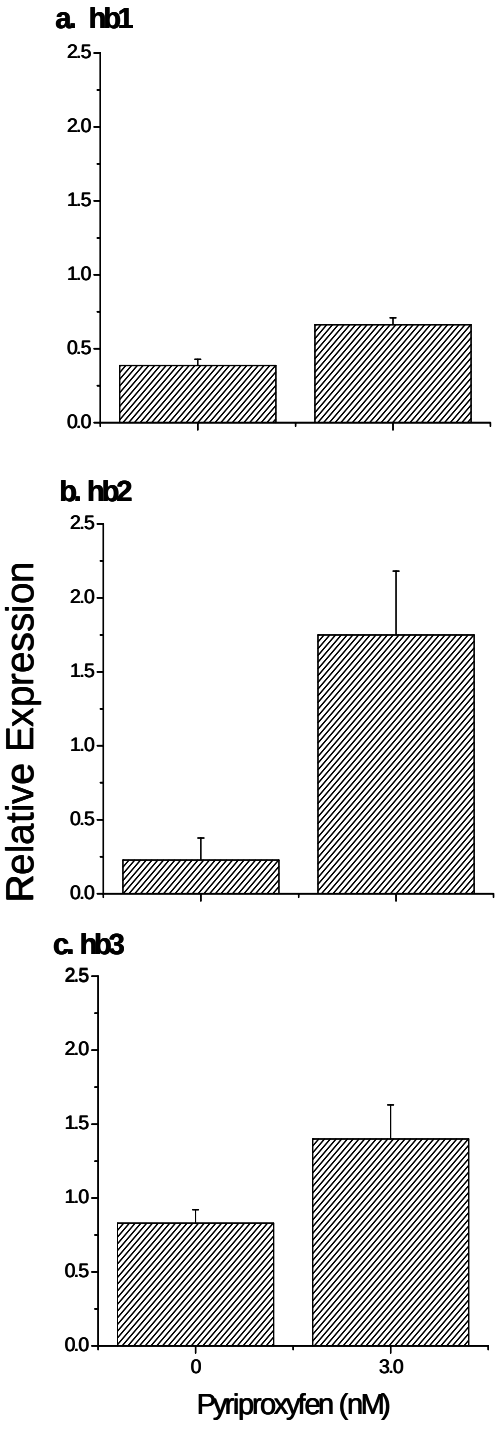
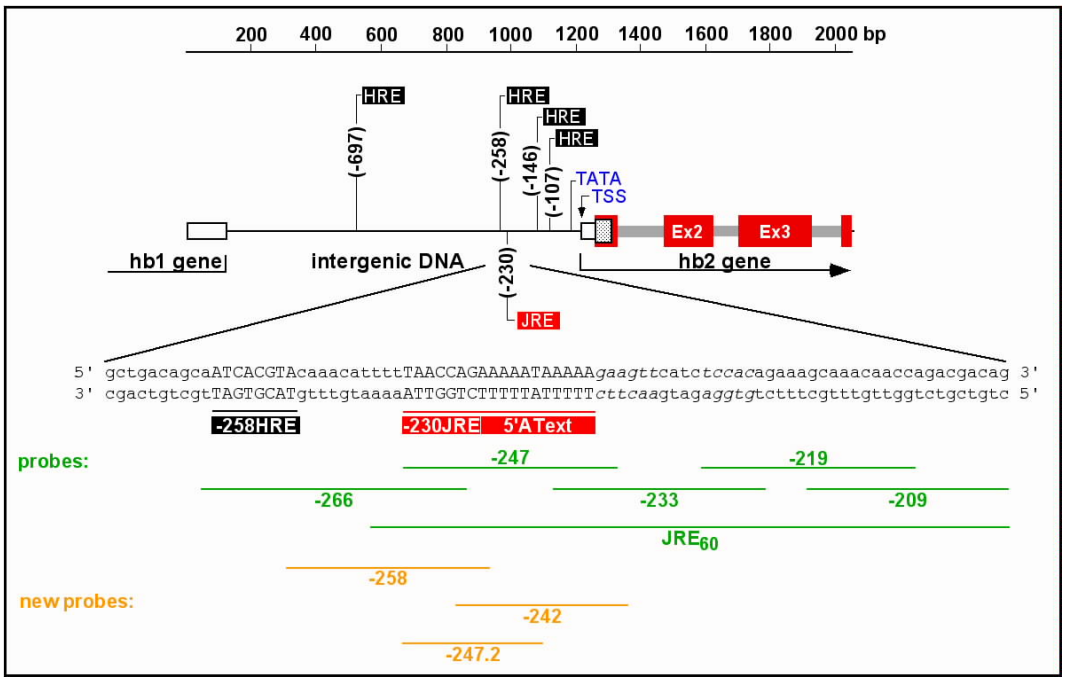

a. hb1
2.5
2.0
1.5
1.0
0.5
0.0
b. hb2
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
c. hb3
2.5
2.0
1.5
1.0
0.5
0.0
0
3.0
Pyriproxyfen (nM)

Fig. 3

a)


200 400 600 800 1000 1200 1400 1600 1800 2000 bp
HRE
HRE
HRE
HRE
(-697)
(-258)
(-146)
(-107)
TATA
TSS
Ex2
Ex3
hb1 gene
intergenic DNA
hb2 gene
(-230)
JRE
5' gctgacagcaATCACGTAcaaacattttTAACCAGAAAAATAAAAAgaagttcatctccacagaaagcaaacaaccagacgacag 3'
3' cgactgtcgtTAGTGCATgtttgtaaaaATTGGTCTTTTTATTTTTcttcaagtagaggtgtctttcgtttgttggtctgctgtc 5'
-258HRE
-230JRE
5'AText
probes:
-247
-219
-266
-233
-209
JRE60
-258
new probes:
-242
-247.2

b)

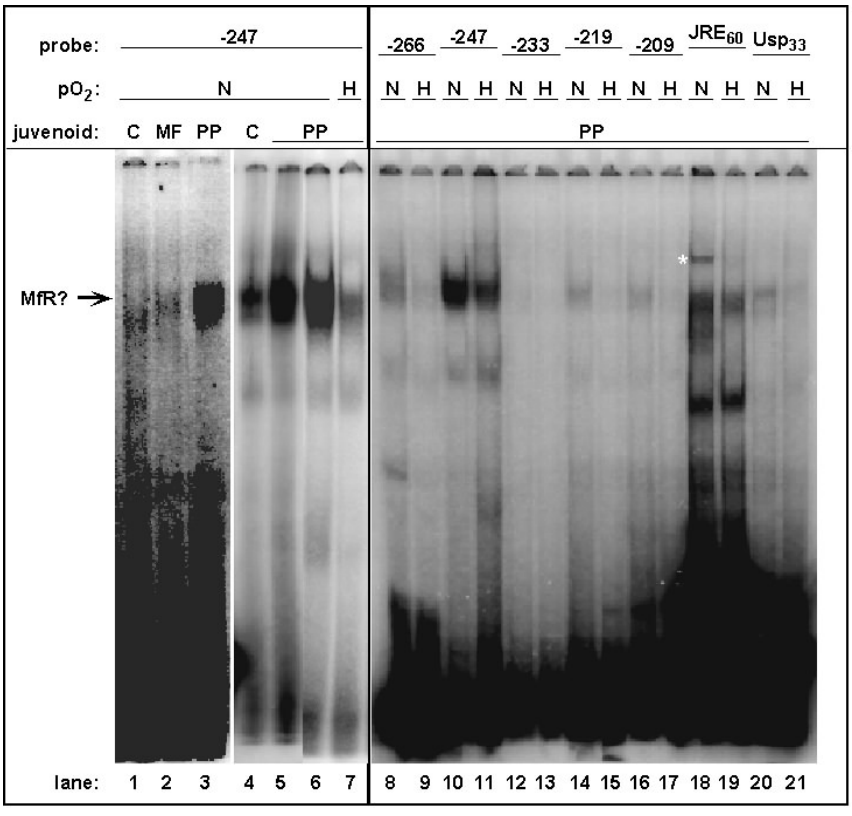

probe:
-247
-266
-247
-233
-219
-209
JRE60
Usp33
pO2:
N
H
juvenoid:
C MF PP C PP
PP
MfR?
lane:
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

Fig. 4

a)

```
wt/-247: 3' ATTGGTCTTTTTTATTTTTCT 5'
m1/-247: 3' --------------GGG---- 5'
m2/-247: 3' ------------GGG------ 5'
m3/-247: 3' ---------GGG---------- 5'
m4/-247: 3' -----AAA------------- 5'
m5/-247: 3' --AAA---------------- 5'
```

b)

Probe	core TGGTTA	extens. 5' AT
-247	+	T_{10}
-242	–	T_{10}
-258	+	–
-247.2	+	T_5

c)

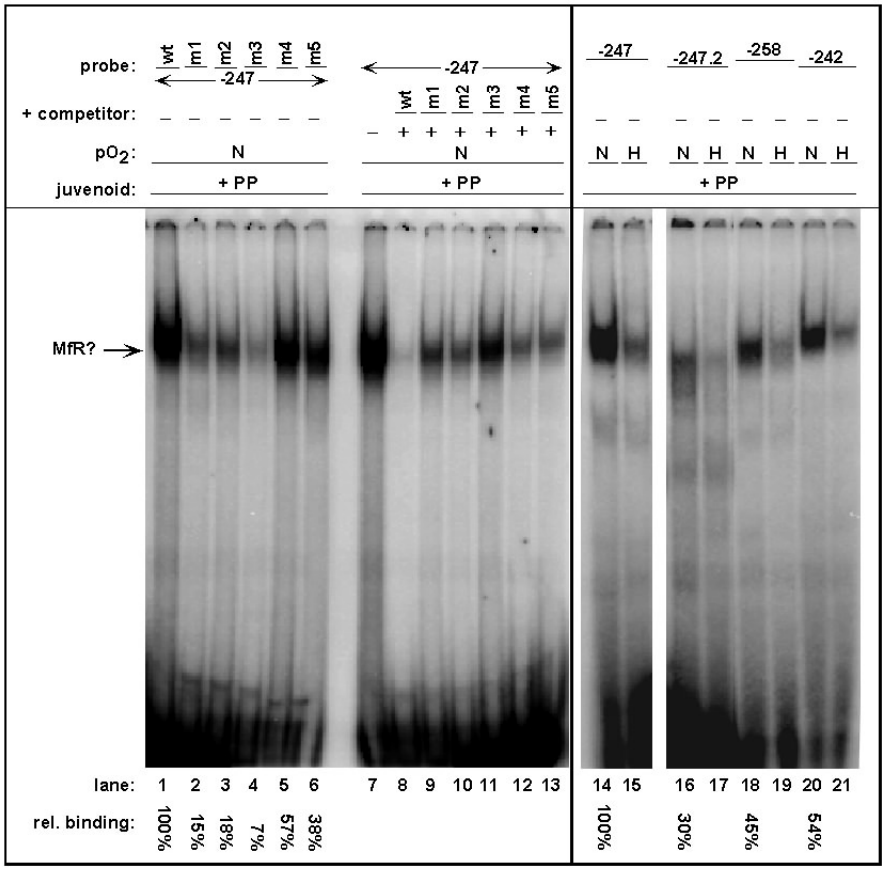


Fig. 5

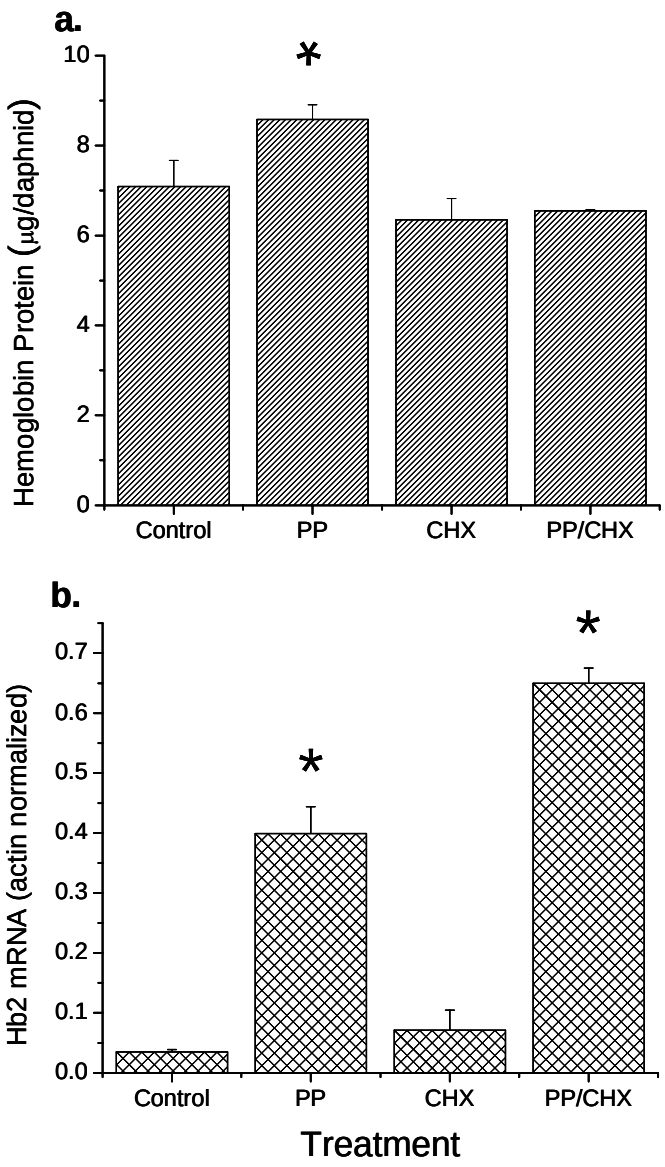

a.
Hemoglobin Protein (µg/daphnid)
10
8
6
4
2
0
*
Control
PP
CHX
PP/CHX
b.
Hb2 mRNA (actin normalized)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
Control
PP
CHX
PP/CHX
Treatment

Fig. 6

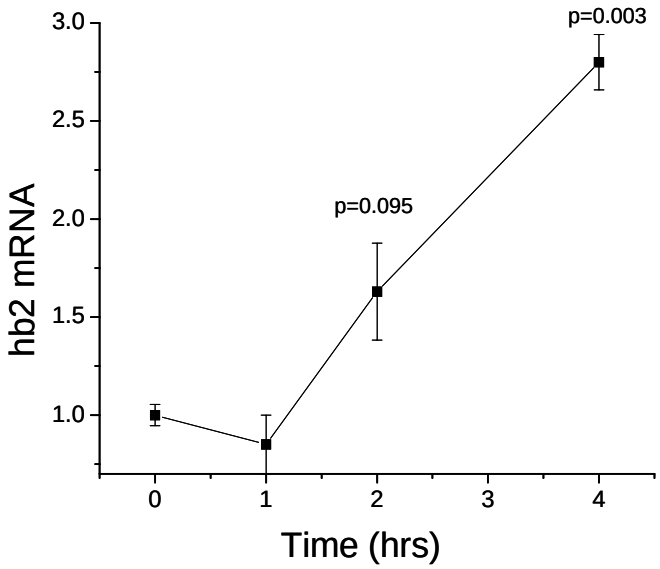
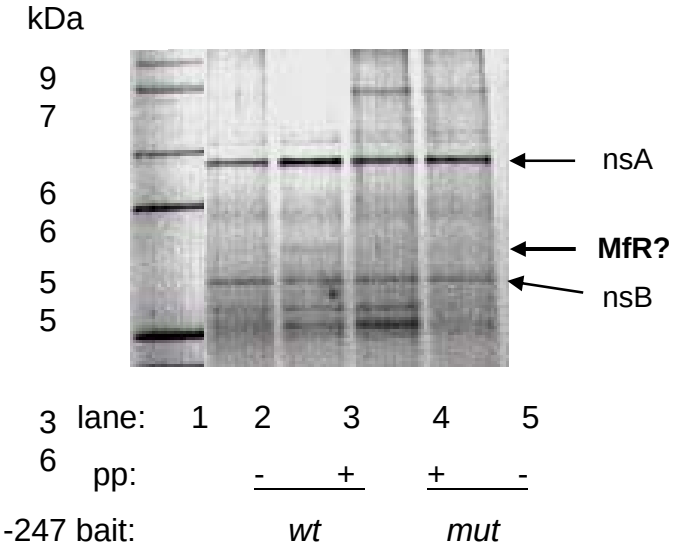


Fig. 7



Atrazine Stimulates Stress-Responsive Hemoglobin Induction in *Daphnia magna*: Is it Hormonal or Hypoxic?

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Abstract

Hemoglobin accumulation in daphnids is an important adaptive response that is stimulated by two distinct molecular pathways: 1) a hypoxia-mediated pathway via hypoxia inducible factor (HIF) binding to cis-acting hypoxia response elements (HREs) and 2) an endocrine pathway stimulated by juvenoid hormones interacting with a juvenoid response element (JRE). We found that atrazine induced expression of hemoglobin in *Daphnia magna* despite having no clear relationship to either hypoxia or juvenoid hormones. We modeled and measured the joint action of atrazine and the juvenoid hormone pyriproxyfen to determine whether these compounds induced dhb2 gene expression according to concentration addition or independent joint action. Conformation of the action of the two chemicals to concentration addition would indicate that atrazine functions as a juvenoid hormone to induce dhb2. Conformation of the action of the two chemicals to a model for independent joint action would imply that atrazine induces dhb2 through the HIF or some other non-juvenoid signaling pathway. Atrazine-mediated changes in hemoglobin expression were evaluated using real-time RT PCR with primers specific to each of the three sequenced *D. magna* hemoglobin genes (dhb1, dhb2, and dhb3). Atrazine significantly induced dhb2 gene expression (7-fold), while having little effect on the expression of dhb1 and dhb3 (<2-fold). Among these 3 genes, dhb2 is also most susceptible to the action of hypoxia and juvenoids. The combined action of atrazine and pyriproxyfen mixtures on dhb2 expression was measured and compared to effects predicted using concentration addition and independent joint action models. Induction of dhb2 by the chemical mixtures did not significantly deviate from model predictions based upon independent joint action but did significantly vary from concentration addition model predictions. Results from this study

demonstrate that molecular interactions among chemicals conform to standard mixture modeling approaches and that atrazine induces hb2 gene expression through a pathway that does not involve the JRE.

Key words: insect growth regulators, juvenoids, mixture modeling, invertebrates, endocrine disruption

Introduction

Multiple stressors can converge at a single target gene to elicit an adaptive response (Cypser and Johnson, 2002; Morgan and Snell, 2002). For example, in the microcrustacean, *Daphnia magna*, the hemoglobin 2 gene (dhb2) is induced in hypoxic conditions via the binding of hypoxia inducible factor (HIF) to hypoxia response elements (HRE) (Gorr *et al.*, 2004) and by an endocrine pathway involving the interaction of juvenoid hormones with a juvenoid response element (JRE) (Rider *et al.*, 2005; Gorr *et al.*, submitted). The plasticity of hemoglobin induction reflects its importance as an adaptive response. Indeed, hemoglobin accumulation in daphnids correlates with increased longevity, fecundity, and general physiological activity (Fox *et al.*, 1951; Koh *et al.*, 1997).

Hemoglobin expression in daphnids is a useful biomarker in assessing the health of aquatic ecosystems because it is specifically targeted by both physical (i.e. low dissolved oxygen as a result of anthropogenic eutrophication) and chemical stressors (i.e. invertebrate endocrine disruptors such as insect growth regulating insecticides). In a recent survey of U.S. groundwater quality, 47% of samples contained mixtures of compounds (Squillace *et al.*, 2002). The three most common mixture components identified were deethylatrazine (an atrazine degradation product), atrazine, and anthropogenic nitrate (Squillace *et al.*, 2002). Limited evidence suggests that atrazine and nitrate interact synergistically (Porter *et al.*, 1999). Additionally, atrazine has been associated with endocrine activity in an invertebrate microcrustacean (Dodson *et al.*, 1999).

Cumulative effects of distinct stressors on gene expression should correspond to predictions from mixture toxicity models based on concepts of additivity. Concentration addition would apply to chemicals that regulate gene expression via a common response element. Whereas; the independent joint action model would describe the combined action of chemicals that induce gene expression via different response elements. (see Cassee *et al.*, 1998 for model descriptions). Therefore, mixtures models can be used to provide insight in the mechanisms by which chemicals induce gene expression.

The initial objective of this study was to characterize atrazine mediated hemoglobin induction. The induction of dhb2 by combinations of atrazine and the juvenoid hormone pyriproxyfen was then measured and modeled in order to determine whether atrazine induced hb2 through the juvenoid response element..

Materials and Methods

Daphnid culture

The microcrustacean daphnid *Daphnia magna* was used in all experiments. Daphnids were originally acquired from US Environmental Protection Agency, Mid-Continent Ecology Division - Duluth, MN and have been maintained in our laboratory for over ten years. 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}). All daphnid cultures and experiments were housed in incubators set to 20° C with a 16 h-8 h light-dark cycle. Culture daphnids were maintained at a density of ~50 animals per 1 L beaker. Cultures were changed three times per week and adults were discarded and replaced

with neonates after three weeks. Culture daphnids were fed twice a day with 2.0 mL (1.4×10^8 cells) of the unicellular green algae *Selenastrum capricornutum* and 1.0 mL (4 mg dry weight) of Tetrafin® fish food suspension (Pet International, Chesterfill, New South Wales, Australia) prepared as described previously (Baldwin and LeBlanc, 1994). The *Selenastrum* was cultured in the laboratory using Bold's basal medium.

Test chemicals and concentration selection

Pyriproxyfen (PS 2110) and atrazine (PS 380) were acquired from Chem Service, Inc. (West Chester, PA). The purity of chemicals was ≥98 %. Exposure concentrations of the chemicals were selected that spanned the entire range from minimum to maximum induction. These exposure levels were not overtly toxic to the daphnids. Each chemical was present in the greatest mixtures level at their respective EC_{04} concentrations. Lower mixture levels used to assess gene expression consisted of dilutions of this highest concentration. All exposure solutions, including controls, contained 0.01% absolute ethanol, which was used to dissolve the test chemicals.

Real-time RT PCR

Daphnid exposure Six day-old female daphnids were exposed to individual chemicals and combinations for 24 hours. Exposure to each treatment was performed in three 1 liter beakers (30 daphnids/l of media). Daphnids were transferred to cryogenic tubes, rinsed, and snap frozen in liquid nitrogen following exposure.

RNA isolation Previously frozen sample tissue was crushed to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was isolated from approximately 30 mg of

each sample using the SV Total RNA Isolation kit (Promega, Madison, WI) according to the manufacturer's instructions. The quantity of RNA was determined by absorbance at 260 nm and purity by the 260/280 nm ratio.

Reverse transcription The ImProm II Reverse Transcription System (Promega) was used to reverse transcribe 0.5 μg of total mRNA with oligo dT primers according to the manufacturer's instructions.

Real time PCR Primers (Table 1) were designed from the non-homologous untranslated regions of hb1, hb2, and hb3 and from the actin gene using Primer Express Software (Applied Biosystems, Foster City, CA). All primers were synthesized by Operon Biotechnologies, Inc (Huntsville, AL) and were reconstituted and stored in TE buffer. The total volume of polymerase chain reactions was 25 μl and consisted of 12.5 μl SYBR® Green PCR Master Mix (Applied Biosystems), 0.3 μM each of the forward and reverse primers, 5 ng cDNA, and 8.5 μl of nuclease-free water. All polymerase chain reactions were carried out on an ABI Prism® 7000 Sequence Detection system. The PCR temperature profile consisted of one denaturation cycle of 95 °C for 10 minutes, followed by 40 cycles of: 15 second at 95 °C, 1 minute at 60°C. A corresponding reaction with actin primers was carried out for each hemoglobin reaction. Relative hemoglobin 2 mRNA was calculated using the $2^{-\Delta\Delta C_T}$ equation for measuring relative gene expression. Real-time PCR reactions were followed by dissociation curve analyses, which confirmed that a single product with the appropriate melting temperature was amplified in each well.

Atrazine-induced hemoglobin expression pattern

Approximately twenty, 7-day old daphnids were exposed to either 1.0 mg/L atrazine or ethanol carrier in 1L of media contained in 1L beakers. Each treatment consisted of two beakers per time point. Daphnids were fed 1.0 mL (7×10^7 cells) of the unicellular green algae *Selenastrum capricornutum* and 0.5 mL (2 mg dry weight) of Tetrafin® fish food suspension. Beakers were randomly arranged in the incubator to control for position effects. Media was changed every 24 hours. Daphnids were collected, rinsed, and snap frozen in liquid nitrogen following 0, 12, 24, 36, 48, and 60 hours of exposure. RNA Isolation, reverse transcription, and real-time PCR were performed as described above. Dissolved oxygen to exposure solutions was measured using a YSI dissolve oxygen meter (Yellow Springs, OH). Significant differences were detected between control and atrazine-treated groups using analysis of variance (ANOVA) and Tukey HSD tests (JMP software; SAS, Cary, NC).

Joint effects modeling

Individual chemical toxicity measurements The concentration-response relationships were characterized for each individual chemical to be used in mixture modeling. Relative dhb2 induction values were converted to percent of maximal response. To do this, relative hemoglobin induction values were plotted against corresponding chemical exposure concentrations and a logistic fit to the data was derived using the following equation:

$$y = \frac{A_1 - A_2}{1 + \left(\frac{x_i}{x_0}\right)^{\rho}} + A_2 \qquad \text{Equation 1}$$

where A_1 and A_2 represent the lower and upper asymptotes of the curve, x_i is the concentration of chemical i, x_0 is the concentration of chemical i at the center of the curve, and ρ is the slope of the curve. The upper asymptote (A_2) was taken as the maximal response. This value was then used to convert relative hemoglobin induction values to percent of maximum.

Additivity models The induction of dhb2 by binary mixtures of atrazine and pyriproxyfen was experimentally determined and compared to additive model predictions. Concepts of concentration addition and independent joint action were used to model dhb2 induction. Concentration addition models require that the concentration of chemicals within a mixture be converted to relative units of a designated reference chemical within the mixture. Pyriproxyfen was selected as the reference chemical in these binary mixtures and atrazine concentrations were converted to pyriproxyfen units by rearranging the logistic equation to solve for concentration in terms of the reference chemical:

$$x_r = x_0 \times \sqrt[\rho]{\frac{(A_1 - y)}{(y - A_2)}} \qquad \text{Equation 3}$$

where x_r is the concentration of the test chemical (atrazine) in terms of the reference chemical (pyriproxyfen); x_0 is the concentration of the reference chemical that elicits half maximal response; $A1$, $A2$, and ρ represent the lower asymptote, upper asymptote, and slope, respectively, of the reference chemical concentration curve, and y is the response of the test chemical calculated from the concentration-response curve of that chemical. Following conversion of atrazine concentrations to pyriproxyfen units, the concentration of the mixture was derived as the sum of the chemical concentrations in pyriproxyfen units. The

concentration addition equation from Olmstead and LeBlanc (2005), with minor modification, was used to model the combined action of the chemicals:

$$R = \frac{1}{1 + \left(\frac{x_0}{x_m} \right)^{\rho'}} \qquad \text{Equation 4}$$

where R is the response of the mixture, x_0 is the concentration of the reference chemical that elicits half maximal response, x_m is total mixture concentration in terms of the reference chemical, and ρ' is the average slope of the chemicals in the mixture.

Response addition was calculated with the following equation:

$$R = 1 - \prod_{i=1}^{n} (1 - R_i) \qquad \text{Equation 5}$$

where R represents the response to the mixture and R_i is the response to chemical i.

Modeled predictions were compared to experimentally determined data using a coefficient of determination (r^2; Zar, 1996). A high coefficient of determination would indicate a good fit between modeled and observed data.

Results

Pattern of hemoglobin mRNA expression following atrazine exposure

The time course of induction of the three *D. magna* hemoglobin genes during atrazine exposure was assessed (Figure 1). Dhb1 was constitutively expressed with little induction (<2-fold) in response to atrazine (Figure 1A). In contrast, dhb2 mRNA levels were elevated approximately 7-fold in response to atrazine over the time course of exposure (Figure 1B).

Dhb3 mRNA levels were slightly elevated in atrazine treated daphnids at the 24 and 48 hour time points (Figure 1C).

Hypoxic conditions can stimulate hemoglobin induction via the HRE. We therefore measured dissolved oxygen levels in exposure solutions to determine whether the induction in dhb2 by atrazine may relate to differences in oxygen tension between control and atrazine solutions. Dissolved oxygen levels were comparable between control and atrazine solutions throughout the exposure period (Figure 2; $P<0.05$; ANOVA/Tukey HSD) and were never below 80% of saturation. Therefore, the increase in dhb2 expression was not due to a reduction in dissolved oxygen caused by atrazine.

Joint effects of atrazine and pyriproxyfen on hb2 mRNA levels

The joint effects of atrazine and the juvenoid hormone pyriproxyfen on dhb2 induction were measured, and modeled according to both concentration addition and independent joint action. First, the induction concentration-response curves for the individual chemicals were experimentally determined (Figure 3). The logistic equation (Equation 1) provided a good fit to atrazine ($r^2 = 0.961$) and pyriproxyfen ($r^2 = 0.985$) data (Figures 3A and 3B, respectively). The maximum dhb2 inductions elicited by each of the chemicals were compared (Figure 4) in order to convert atrazine into terms of the reference chemical, pyriproxyfen. The necessary modeling parameters (Table 2) were then derived from these scaled concentration-response curves (Figure 4). Pyriproxyfen was a much more potent inducer of dhb2 with effects observed at exposure concentrations several orders of magnitude lower than those required by atrazine (Table 2 and Figure 4). In addition, the

maximal induction observed with atrazine was only 7% of maximal induction observed with pyriproxyfen (Table2 and Figure 4).

Comparison of measured dhb2 gene induction by the binary mixtures with model predictions revealed that the combined effects of the chemicals were consistent with independent joint action ($r^2 = 0.70$) and not concentration addition ($r^2 < 0.1$) (Figure 5). These results indicate that atrazine does not induce dhb2 gene expression via the juvenoid signaling pathway.

Discussion

Hemoglobin expression in *D. magna* is dependant upon both ambient oxygen conditions (Kobayashi *et al.*, 1988; Zeis *et al.*, 2003) and the hormonal milieu of the organism. Daphnids with higher hemoglobin titers exhibit increased filtration and ventilation rates, as well as improved survival and fecundity in low oxygen conditions (Fox, 1951; Pirow *et al.*, 2001). Therefore, hemoglobin expression represents an integrated response that has implications for population level effects (Koh *et al.*, 1997).

In the present study, we found that the environmentally ubiquitous herbicide atrazine induced hemoglobin in daphnids. The profile of induction of three daphnid hemoglobin genes (dhb1, dhb2, and dhb3) by atrazine was similar to that observed with hypoxia (Zeis *et al.*, 2003) and juvenoid hormone treatment (Rider *et al.*, 2005). Under these three regulatory scenarios the stimulus induces dhb1 minimally, dhb2 significantly, and dhb3 inconsistently.

The profile of induction of the hemoglobin genes by atrazine was therefore not diagnostic of the mechanism of this action of the herbicide.

Atrazine is a triazine herbicide that elicits toxicity to plants by disrupting photosynthesis. Specifically, atrazine and other photosysem II herbicides, bind to an electron acceptor (Q_B protein), thereby blocking electron transport and subsequent ATP production in chloroplasts (Oettmeier, 1999). Atrazine is generally considered to be of low toxicity to animals because of its plant-specific mode of toxicity. Acute responses to the toxicity of atrazine typically are measured at daily dosages or environmental exposure concentrations in the high parts per million (Solomon *et al.*, 1996).

The observed induction of the dhb2 gene by atrazine could be mediated by the juvenoid response element located in the promoter region of this gene. Atrazine has not been reported to elicit juvenile hormone-like activities in insects. Dodson et al. (1999) reported that atrazine stimulated the production of male offspring in daphnids (*Daphnia pulicaria*). Male sex determination in daphnids is regulated by juvenoid hormones (Olmstead and LeBlanc, 2002). However, Olmstead and LeBlanc (2003) were unable to replicate this observation. Extensive attempts in our laboratory to stimulate male offspring production in daphnids exposed to atrazine under various conditions have proven unsuccessful. Atrazine, therefore, does not appear to be a junvenoid hormone mimic in daphnids. Consistent with this conclusion, results of the present study indicate that the induction of dhb2 by atrazine does not involve gene regulation by the juvenoid response element.

An alternative mechanism by which atrazine induces the dhb2 gene would be via the hypoxia signaling pathway. The promoter region of the dhb2 gene contains several hypoxia response elements (HRE) that bind hypoxia inducible factor (HIF) in response to low oxygen conditions (Gorr *et al.,* 2004). This binding of HIF to HRE results in increased dhb2 gene expression. Some chemical treatments (i.e. cobalt chloride) mimic hypoxia and stimulate the HIF/HRE signaling pathway in mammalian systems (Maxwell and Salnikow, 2004; Vengellur, et al., 2005), though no precedent exists for atrazine eliciting such an effect. Atrazine also could stimulate the HIF/HRE signaling pathway by decreasing ambient oxygen levels perhaps by killing algal cells, which are provided to the daphnids for food. This possibility is unlikely, as dissolved oxygen concentrations remained $\geq$80% of saturation in all treatments which would likely preclude activation of the hypoxia signaling pathway. Finally, atrazine might induce cellular hypoxia by uncoupling mitochondrial oxidative phosphorylation as this biochemical process shares similarity to ATP production in chloroplasts. We are aware of no precedent for such effects of atrazine but future studies should consider this possibility. Results of the present study indicate that the hypoxia signaling pathway is a likely mechanism by which atrazine induces dhb2 gene expression. However, we cannot exclude the possibility that atrazine induces the dhb2 gene via regulatory mechanisms that have not yet been discerned.

Evidence suggests that atrazine does elicit toxicological effects through mechanisms that are not yet understood. Cooper et al. (2000) have reported that atrazine interferes with hypothalamic control of pituitary LH secretion in rats. Thomas et al. (2005) has reported similar effects in the Atlantic croaker in response to hypoxia. Taken together, these

observations suggest that atrazine may elicit effects in the brain of vertebrates indicative of cellular hypoxia. The mechanism through which this occurs may be the same mechanism responsible for the induction of dhb2.

Hayes et al. (2002, 2003) has reported that atrazine interferes with gonadal development and other sex specific traits in anurans. These effects have been reported at low ppb exposure concentrations that have resisted replication by other investigators (Carr *et al.,* 2003). However, effects similar to those reported by Hayes have been reported at higher atrazine exposure concentrations (Carr *et al.,* 2003; Tavera-Mendoza *et al.,* 2002). The mechanism by which atrazine elicits these developmental effects has not yet been discerned. Developmental abnormalities in anurans have been associated with hypoxia (Mills and Barnhart, 1999). However, we are aware of no studies in which the developmental effects specifically associated with atrazine have also been observed with hypoxia. Nonetheless, developmental abnormalities in anurans associated with atrazine may involve cellular hypoxia, perhaps with environmental anoxia as a co-variable that is responsible for inconsistencies in observed effects among labs.

Results from the present study demonstrate that chemical interactions at the molecular level can be modeled using conventional additivity approaches. This is significant since chemical mixture modeling of effects at defined gene targets along with in vitro (i.e. reporter genes) confirmation of model predictions would greatly expand the database on the combined action of environmental chemicals. Chemicals exist in the environment (Kolpin *et al.* 2000; Kolpin *et al.* 2002; Stackelberg *et al.* 2001) and in organisms (Carpenter *et al.*

1998) as complex mixtures. Modeling of defined actions of such mixtures would refine hazard characterizations of such mixtures and would allow for predictions of chemical combinations, which might prove hazardous in the whole organism.

Finally, as demonstrated in this study, molecular modeling of the joint action of chemicals can be used as a tool to decipher the mode of action of a chemical. Empirical effects of atrazine (the induction of hemoglobin) were suggestive of juvenoid activity associated with the chemical. However, the modeling strategy used provided convincing evidence that this effect was not juvenoid related. The hypoxia signaling pathway was implicated, by default, as the mechanism of action. Future modeling studies, designed after the present study, may definitively establish the involvement of the hypoxia signaling pathway in the action of atrazine. Alternatively, these modeling experiments may establish the existence of an atrazine-stimulated signaling pathway yet to be discovered.

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Table 1. Primers used in real-time PCR amplification of the three hemoglobins (dhb1, dhb2, and dhb3) with known sequences and the housekeeping gene β-actin. Primers were designed from sequences contained in the untranslated region of each of the three hemoglobin genes using Primer Express software (Applied Biosystems).

Gene	Primer sequence	Amplicon size (bp)
dhb1	Forward: 5' AAATTCAAACGTGGCACTCAAA 3' Reverse: 5' AAGTCCTCGTTGGGAGGGA 3'	51
dhb2	Forward: 5' CCTGAGCGCAAATACTCCGT 3' Reverse: 5' CAGAGAGGCCAAGATGGAGC 3'	51
dhb3	Forward: 5' GTTTTGGCCGTTTTTGTCAAC 3' Reverse: 5' AGTTTCTTAGTCCTTGCGTGGG 3'	51
Actin	Forward: 5' CCTGAGCGCAAATACTCCGT 3' Reverse: 5' CAGAGAGGCCAAGATGGAGC 3'	51

Table 2. Concentration response parameters of atrazine and pyriproxyfen used in mixture modeling. Parameters include lower asymptote or minimum response (A_1), upper asymptote or maximum response (A_2), concentration eliciting half maximal effect (x_0), and slope of the concentration response curve (ρ). Parameters were derived from a logistic fit to data from individual chemicals relative to the reference chemical pyriproxyfen. Pyriproxyfen asymptote values were set at 0 and 100 and atrazine asymptote values were relative to these set values. x_0 values are as μg/l.

	Chemicals	
Parameters	Pyriproxyfen	Atrazine
A1	0	0
A2	100	7.48
X_0	0.149	210
P	3.12	1.41

Fig. 1 **Hemoglobin induction from atrazine exposure.** Dhb1 (**A**), dhb2 (**B**), and dhb3 (**C**) mRNA levels among control (○) and 1 mg/L atrazine-treated (■) daphnids were measured by real-time RT-PCR analysis. Values of mRNA were normalized to β-actin and calculated relative to the time zero control. Each data point represents the mean (± standard deviation) from two samples. Asterisks denote significant deviation of treated values from corresponding controls ($P<0.05$; ANOVA, Tukey HSD).

Fig. 2 **Dissolved oxygen levels in control and atrazine solutions during the induction time-course.** Dissolved oxygen was measured in control (○) and atrazine (■) solutions at time points corresponding with mRNA assessment depicted in Figure 1. Symbols represent the mean (± standard deviation) from two replicate samples. Arrows indicate media renewal. No significant differences in dissolved oxygen levels were detected between control and atrazine solutions ($P>0.05$; ANOVA, Tukey HSD).

Fig. 3 **Relationship between atrazine (A) and pyriproxyfen (B) exposure concentration and Dhb2 mRNA induction in daphnids.** Dhb2 mRNA levels were measured by real-time RT PCR following 24-hours exposure to each chemical. Data points represent the mean (± standard deviation) of three replicate samples.

Fig. 4 **Observed dhb2 mRNA induction from exposure to mixtures of atrazine and pyriproxyfen in comparison to concentration addition (dashed line) and independent joint action (dotted line) model predictions.** Dhb2 mRNA levels in daphnids were measured by real-time RT PCR at four dilutions of an atrazine/ pyriproxyfen mixture with

each chemical present at concentrations that elicit 4% of the maximum pyriproxyfen response. Data points represent the mean (± standard deviation) from three replicate samples.

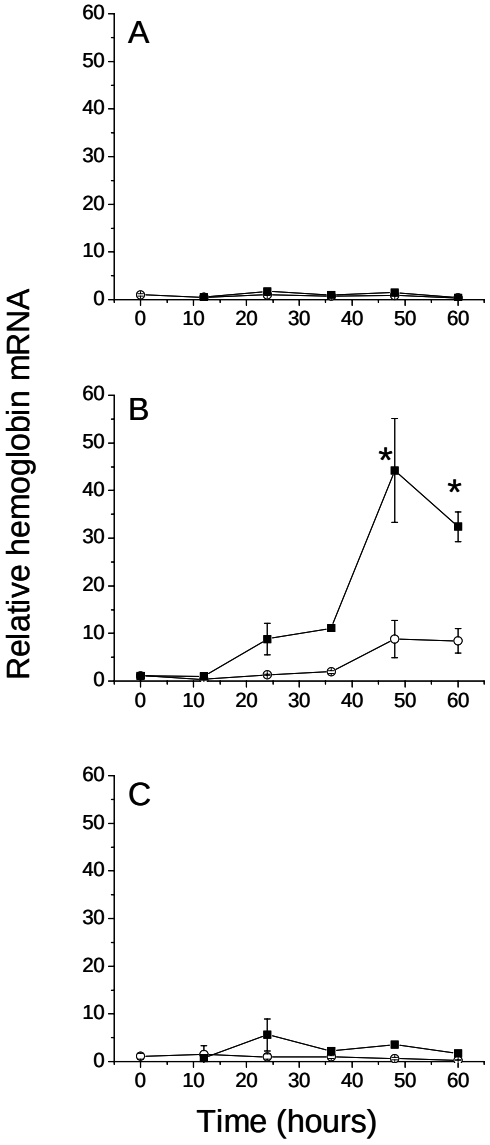

Figure 1

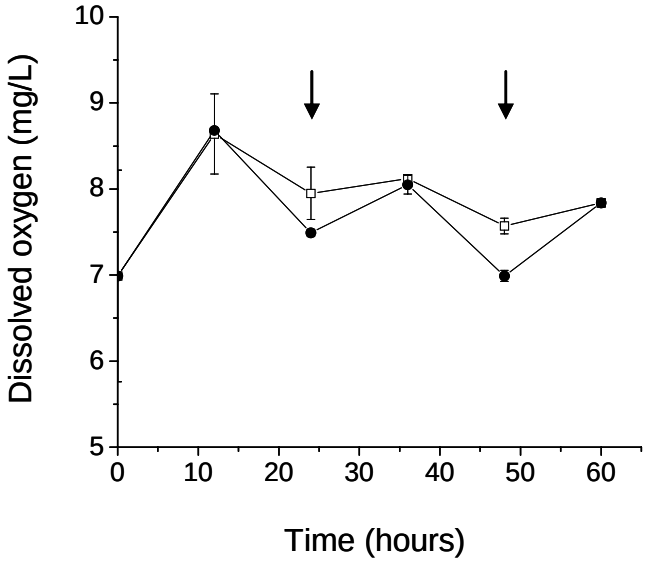

Figure 2

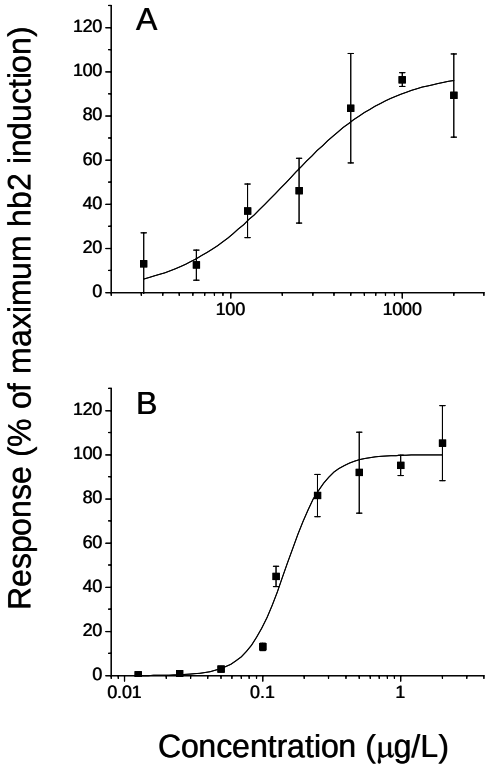
A
B
Response (% of maximum hb2 induction)
Concentration (μg/L)
0
20
40
60
80
100
120
100
1000
0.01
0.1
1

Figure 3

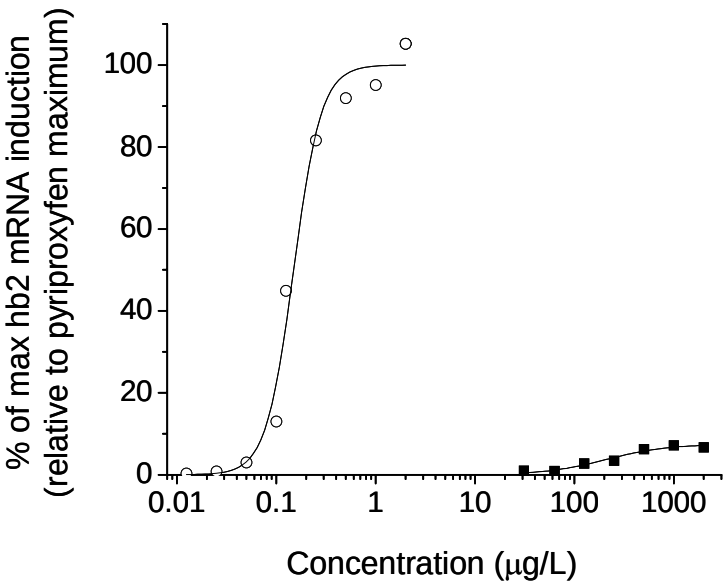
% of max hb2 mRNA induction
(relative to pyriproxyfen maximum)
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Concentration (μg/L)

Figure 4

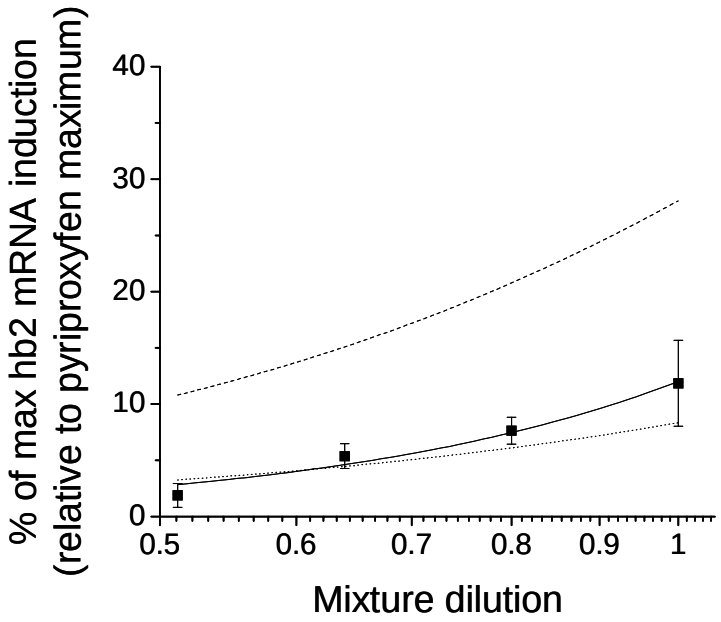
% of max hb2 mRNA induction
(relative to pyriproxyfen maximum)
40
30
20
10
0
0.5
0.6
0.7
0.8
0.9
1
Mixture dilution

Figure 5