Atrazine Stimulates Hemoglobin Accumulation in Daphnia magna: Is it Hormonal or Hypoxic?

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Hemoglobin accumulation in daphnids is an important adaptive response that is regulated by at least two distinct molecular pathways: an endocrine pathway stimulated by terpenoid hormones and an oxygen-sensing pathway involving the hypoxia-inducible factor. We found that the herbicide atrazine elevated hemoglobin levels in Daphnia magna and hypothesized that atrazine induced hemoglobin in daphnids through the hormonal regulatory pathway. This hypothesis was tested by modeling the combined effects of atrazine and the terpenoid hormone mimic pyriproxyfen on hemoglobin mRNA levels assuming the same mechanism of action (concentration addition model) and alternatively, assuming different mechanisms of action (response addition model). Model predictions were then compared to experimental assessments of the combined action of these two chemicals on hemoglobin mRNA levels. Changes in hemoglobin expression were evaluated using real-time RT PCR with primers specific to each of three D magna hemoglobin genes (dhb1, dhb2, and dhb3). Both atrazine and pyriproxyfen significantly elevated levels of the hb2 gene product, while having little effect on hb1 and hb3 gene products. Induction of dhb2 by combinations of atrazine and pyriproxyfen did not conform to the concentration addition predictions. Rather, dhb2 induction by these binary combinations was highly consistent with response addition model predictions. These results indicate that atrazine does not induce hemoglobin through the terpenoid hormone-signaling pathway. Results from this study demonstrate that mixtures modeling can be used to assess a chemical’s mechanism of action and that atrazine likely stimulates hemoglobin accumulation through the oxygen-sensing pathway.

Key Words: cumulative toxicity; terpenoids; mixture modeling; invertebrates; endocrine disruption.

Multiple stressors can converge at a single target gene to elicit a cumulative response. Evaluating the cumulative toxicity of environmental contaminants is a major challenge to the risk assessment process since exposure to environmental chemicals rarely occurs in isolation (Carpenter et al., 1998; USEPA, 2000). The assessment of cumulative toxicity has historically received greatest attention for chemicals that elicit common modes of action since exposure to these individual chemicals may occur at levels below the threshold for toxicity; while in combination, they may exceed the threshold concentration resulting in significant toxicity (USEPA, 1986). The cumulative toxicity of such similar-acting chemicals can be estimated using dose/concentration addition models. The toxic equivalency approach is one such model that is commonly used to estimate the cumulative toxicity of materials that have the same mechanism of toxicity (Safe, 1990; Taioli et al., 2005).

The cumulative toxicity of chemicals with distinct mechanisms of action can be estimated using response addition (also called independent joint action) models. Here, toxicity is not based upon the cumulative dose of the mixture constituents, but rather, is based upon the cumulative response to the mixture constituents (Drescher and Boedeker, 1995; Feron and Groten, 2002; Olmstead and LeBlanc, 2005). The response addition model emanates from probability theory. In effect, response addition represents aggregate probabilities of organismal response to independent chemical exposure events.

These modeling approaches also have the potential to advance our understanding of the mechanism of action of environmental toxicants. For example, a chemical may elicit toxicity through an unknown mechanism of action with overt toxicity that is similar to that of another chemical for which the mechanism of action is known. The mechanism of action of the first chemical can be evaluated by modeling the joint toxicity of various combinations of the two chemicals using both concentration addition and response addition models. Toxicity of these combinations then can be measured and compared to the two sets of model prediction. Conformation to the concentration addition model and not to the response addition model would indicate that both chemicals share the same mechanism of action. Such toxicity evaluations aimed at establishing mechanism of action would be particularly practical and affordable if specific molecular responses are measured using culture cells, tissues, or small invertebrate models to measure toxicity. Modeling exercises such as these could be used to classify chemicals as being similar-acting or dissimilar-acting which...
would then facilitate proper model selection for assessments of cumulative hazard in the risk assessment process.

The herbicide atrazine is a ubiquitous environmental contaminant that is often detected in surface and ground waters (Graziano et al., 2006; Hayes et al., 2003; Squillace et al., 2002). In vertebrates, atrazine can interfere with endocrine signaling by increasing dopamine and reducing norepinephrine concentrations in the hypothalamus, reducing luteinizing hormone and prolactin levels, and possibly increasing the conversion of testosterone to 17β-estradiol (Cooper and Goldman, 1998; Cooper et al., 2000; Sanderson et al., 2000; Stoker et al., 1999). In the crustacean *Daphnia pulecaria*, atrazine was found to increase male sex determination of offspring (Dodson et al., 1999), a terpenoid hormone–regulated process (Olmstead and LeBlanc, 2003). However, subsequent studies with the related species *Daphnia magna* found no evidence for this action of atrazine (Olmstead and LeBlanc, 2003). The terpenoid hormones include juvenile hormone of insects, methyl farnesoate of crustaceans, and retinoic acid of vertebrates that all function as direct modulators of gene activity and as regulators of the activity of other hormones (Chen et al., 1996; Maki et al., 2004; Mu and LeBlanc, 2004).

We serendipitously observed that atrazine stimulated hemoglobin accumulation in the crustacean *D. magna*. Hemoglobin is produced by several genes in daphnids and is, in part, under terpenoid hormone–regulatory control (Rider et al., 2005). Hemoglobin levels also are elevated by low oxygen conditions through the activation of the hypoxia-inducible factor (HIF)/hypoxia response element (HRE)–signaling pathway (Gorr et al., 2004). Due to prior suggestions that atrazine stimulated male sex determination in daphnids (Dodson et al., 1999), we hypothesized that atrazine increased hemoglobin levels via the terpenoid hormone–signaling pathway. We tested this hypothesis by modeling the joint action of atrazine and the terpenoid hormone mimic pyriproxyfen using both concentration addition and response addition paradigms. The induction of hemoglobin mRNA by these binary combinations was then measured and compared to model predictions. This study afforded the dual opportunities to assess the mechanism by which atrazine induces hemoglobin and to evaluate the utility of our proposed approach to assigning mechanisms of toxicity to chemicals using a small invertebrate model.

**MATERIALS AND METHODS**

**Daphnid Culture**

Cultures of the crustacean *D. magna* were originally acquired from U.S. Environmental Protection Agency, Mid-Continent Ecology Division, Duluth, MN, and have been maintained in our laboratory for over 10 years. Daphnid media consisted of deionized water reconstituted to a hardness of ~180 mg/l as CaCO₃ (192 mg/l CaSO₄·H₂O, 192 mg/l NaHCO₃, 120 mg/l MgSO₄, 8.0 mg/l KCl, 1.0 mg/l selenium, and 1.0 mg/l vitamin B₁₂). All daphnid cultures and experiments were housed in incubators set to 20°C with a 16/8-h light/dark cycle. Culture daphnids were maintained at a density of ~50 animals per liter beaker. Culture media was changed three times per week and adults were discarded and replaced with neonates after 3 weeks. Culture daphnids were fed b.i.d. with 2.0 ml (1.4 × 10⁵ cells) of the unicellular green algae *Selenastrum capricornutum* and 1.0 ml (4 mg dry weight) of Tetrafin fish food suspension (Pet International, Chesterfield, New South Wales, Australia) prepared as described previously (Baldwin and LeBlanc, 1994). The *Selenastrum* was cultured in the laboratory using Bold’s basal medium (Nichols, 1973).

**Daphnid Exposures**

Pyriproxyfen and atrazine were acquired from Chem Service, Inc (West Chester, PA). Cobalt chloride and deferoxamine mesylate were purchased from Sigma (St Louis, MO). Exposure concentrations of the chemicals were selected that spanned the range of hemoglobin induction or were evaluated up the maximum concentration tolerated by the organisms. All exposure solutions, including controls, contained 0.01% absolute ethanol, which was used to dissolve the test chemicals, except for deferoxamine mesylate which was dissolved directly in the media.

All chemical exposures were initiated with 6- to 7-day-old female daphnids. Thirty daphnids were exposed in beakers containing 1 l of media. Treatments were replicated two or three times depending upon the experiment. Daphnids were fed 1.0 ml (7 × 10⁷ cells) of algae *S. capricornutum* and 0.5 ml (2 mg dry weight) of Tetrafin fish food suspension at each media change. Beakers were randomly arranged in the incubator to control for position effects. Media was changed every 24 h. Daphnids were collected, rinsed, and snap frozen in liquid nitrogen at designated times for gene expression analyses. Dissolved oxygen concentrations of exposure solutions were measured using a dissolved oxygen meter (YSI, Yellow Springs, OH).

**Real-Time RT PCR**

Frozen tissue samples were 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 was judged by the 260/280 nm absorbance ratio. cDNA was reverse transcribed from 0.5 mg of total RNA with oligo dT primers and the ImProm II Reverse Transcription System (Promega) according to the manufacturer’s instructions. The cDNA was used as template in subsequent PCR reactions.

Oligomeric primers (Table 1) were designed from the nonhomologous untranslated regions of *dbh1*, *dbh2*, and *dbh3* genes and the β-actin gene using Primer Express Software (Applied Biosystems, Foster City, CA). All primers were designed from the nonhomologous untranslated regions of *dbh1*, *dbh2*, and *dbh3* genes and the β-actin gene using Primer Express Software (Applied Biosystems, Foster City, CA). All primers

**TABLE 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dbh1</td>
<td>Forward: 5’ AAATTCAAACGTGGCACTCAA 3’</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ AAGTCTCTCTTGGGAGGGA 3’</td>
<td></td>
</tr>
<tr>
<td>dbh2</td>
<td>Forward: 5’ CCTAGAGCGCAAAATACCTCGT 3’</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ CAGAGAGGGCCAAGAGTGAGC 3’</td>
<td></td>
</tr>
<tr>
<td>dbh3</td>
<td>Forward: 5’ GTTTTGGCGCGTTTTGGGCAAC 3’</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ AGTCTCTATGCTCTTGGGAGGGA 3’</td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>Forward: 5’ CCTAGAGCGCAAAATACCTCGT 3’</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ CAGAGAGGGCCAAGAGTGAGC 3’</td>
<td></td>
</tr>
</tbody>
</table>
were synthesized by Operon Biotechnologies, Inc (Huntsville, AL) and were reconstituted and stored in TE buffer (1 M Tris, 0.5 M EDTA, pH 8.0). The total volume of polymerase chain reactions was 25 ml and consisted of 12.5 ml SYBR Green PCR Master Mix (Applied Biosystems), 0.3 mM each of the forward and reverse primers, 5 ng cDNA, and 8.5 ml of nuclease-free water. All polymerase chain reactions were carried out on an ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA). The PCR temperature profile consisted of one denaturation cycle of 95°C for 10 min, followed by 40 cycles of: 15 s at 95°C, 1 min at 60°C. A corresponding reaction with β-actin primers was carried out for each hemoglobin reaction. The comparative C_T method (2^(-ΔΔC_T)) was used to assess the relative levels of hemoglobin mRNA normalized to mRNA levels of β-actin measured with the same cDNA sample. Validation experiments, as described by the instrument manufacturer, confirmed that the efficiencies of the target (hemoglobin) and endogenous control (β-actin) amplifications were approximately equal (Applied Biosystems).

**Joint Effects Modeling**

**Individual chemical concentration-response analyses.** Concentration-response relationships were characterized for atrazine and pyriproxyfen to derive parameters relevant to the modeling exercises (EC_{50}, ρ, and A_2). Relative hemoglobin levels were plotted against corresponding chemical exposure concentrations and a logistic fit to the data was derived using Origin software (Microcal Software Inc, Northampton, MA) according to the equation:

\[
y = \frac{A_1 - A_2}{1 + \left(\frac{x}{EC_{50}}\right)^\rho} + A_2,
\]

where \(A_1\) and \(A_2\) represent the minimum and maximum transformed hemoglobin levels (i.e., lower and upper asymptotes of the curve), \(x\) is the concentration of the chemical, \(EC_{50}\) is the concentration of the chemical that causes half-maximal hemoglobin induction, and \(\rho\) is the power (Hill slope) of the curve. Hemoglobin mRNA levels measured in daphnid samples following treatments were transformed to percent of maximum to facilitate comparisons among mixtures.

**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 response addition were used to model dhb2 induction. The concentration addition model requires 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 (Equation 1) to solve for concentration in terms of pyriproxyfen units:

\[
C_A = x_0 \times \left(\frac{A_1 - y}{y - A_2}\right)^{1/\rho},
\]

where \(C_A\) is the concentration of atrazine in terms of pyriproxyfen units; \(x_0\) is the concentration of pyriproxyfen that elicits half-maximal response; \(A_1, A_2\), and \(\rho\) represent the lower asymptote, upper asymptote, and power, respectively, of pyriproxyfen’s concentration-response curve, and \(y\) is the response of atrazine calculated from the concentration-response curve for that chemical. Following conversion of atrazine concentrations to pyriproxyfen units, the concentrations of the mixture were 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:

\[
R = \frac{1}{1 + \left(\frac{C_A}{C_{50}}\right)^\rho},
\]

where \(R\) is the response of the mixture, \(x_0\) is the concentration of the reference chemical that elicits half-maximal response, \(C_{50}\) is the concentration of pyriproxyfen, \(C_A\) is concentration of atrazine converted to pyriproxyfen units (Equation 2), and \(\rho\) is the average power associated with the chemicals in the mixture.

Response addition was calculated with the following equation (Olmstead and LeBlanc, 2005):

\[
R = 1 - \prod_{i=1}^{n}(1 - R_i),
\]

where \(R\) represents the response to the mixture and \(R_i\) is the individual response to chemical \(i\).

Modeled predictions were compared to experimentally derived data using a coefficient of determination \(r^2\) [Zar, 1996]). A high coefficient of determination \((\geq 0.70)\) indicates a good fit between modeled and observed data (Rider and LeBlanc, 2005). We hypothesized that if atrazine and pyriproxyfen induced hemoglobin by activating the same regulatory pathway, then the combined effects of the chemicals would be best described by the concentration addition model. Alternatively, if the chemicals induce hemoglobin by different mechanisms, then the response addition model would best describe the joint action of the chemicals.

**RESULTS**

**Hemoglobin mRNA Accumulation with Atrazine Exposure**

Atrazine exposure caused daphnids to develop considerable red coloration of the blood (Fig. 1). Previous studies had shown that this red coloration marks enhanced accumulation of hemoglobin (Rider et al., 2005). Therefore, the time-course for accumulation of the daphnid hemoglobin (dhb) 1, 2, and 3 gene products was evaluated during exposure to 1.0 mg/l atrazine (Fig. 2). dhb1 was constitutively expressed with little (< twofold) change over 60 h of atrazine exposure relative to control levels (Fig. 2A). In contrast, dhb2 mRNA levels

![Fig. 1](https://example.com/fig1.png)

**FIG. 1.** Increased accumulation of hemoglobin with atrazine exposure. Daphnids were unexposed (A) or exposed (B) to 2.0 mg/l atrazine for 48 h. Increased red coloration of the blood, as exemplified in blood-rich, transparent tissues such as the heart and abdominal processes, was evident among atrazine-exposed animals. Such coloration is indicative of hemoglobin induction (Rider et al., 2005).
progressively increased over 48 h exposure to atrazine (Fig. 2B). dhb2 mRNA levels were maximally increased approximately sevenfold in response to atrazine relative to controls. dhb3 mRNA levels were slightly elevated in response to atrazine with maximum elevation occurring at 24 h of exposure (Fig. 2C). In all, dhb1 and dhb3 mRNA levels did not significantly contribute to the overall increase in hemoglobin mRNA levels with atrazine treatment. Among the three gene products evaluated, this increase was due largely to increases in dhb2 mRNA levels. Accordingly, all subsequent evaluations focused exclusively upon dhb2 mRNA.

Joint Effects of Atrazine and Pyriproxyfen on dhb2 mRNA Levels

The joint effects of atrazine and pyriproxyfen on dhb2 induction were modeled and measured in an effort to discern the mechanism by which atrazine induced dhb2. The induction of dhb2 by the individual chemicals was first characterized in order to derive parameters that would be used in the mixtures modeling (Table 2). The logistic equation (Equation 1) provided a good fit to the atrazine ($r^2 = 0.961$, Fig. 3A) and pyriproxyfen ($r^2 = 0.985$, Fig. 3B) concentration-response curves. While both compounds significantly elevated dhb2 in a concentration-related fashion, pyriproxyfen was a much more potent inducer and elicited a greater magnitude of induction at concentrations several orders of magnitude lower that the effective concentration range of atrazine (Fig. 4).

The induction of dhb2 resulting from exposure of daphnids to four mixtures of atrazine and pyriproxyfen was measured and compared to both concentration addition and response addition model predictions (Fig. 5). The measured induction of dhb2 by the binary combinations was consistent with predictions based upon response addition ($r^2 = 0.70$) and not concentration addition ($r^2 < 0.10$). These results indicate that
atrazine does not induce dhb2 via the terpenoid-regulatory pathway, but rather, may induce this gene through the HIF-signaling pathway.

We attempted to test the hypothesis that atrazine induced dhb2 via the HIF pathway by measuring dhb2 induction by chemicals known to activate the HIF pathway in vertebrates, then modeling the combined effects of the HIF-acting compound in combination with atrazine. Both cobalt chloride and deferoxamine mesylate, known inducers of hemoglobin in vertebrates via HIF (Su et al., 2006; Woo et al., 2006), failed to elevate levels of daphnid dhb2 at maximum tolerated exposure concentrations (data not shown). This inactivity of the compounds precluded further evaluation of this hypothesis.

Atrazine exposure experiments were performed in which dissolved oxygen levels in the exposure solutions were measured to determine whether atrazine might induce dhb2 via the HIF pathway indirectly by reducing available oxygen to the animals. Atrazine subtly lowered dissolved oxygen levels of exposure solutions as compared to dissolved oxygen levels of control solutions (Fig. 6).

FIG. 3. dhb2 mRNA induction from exposure to concentrations of atrazine (A) and pyriproxyfen (B). dhb2 mRNA levels were measured by real-time RT PCR following 24-h exposure of daphnids to the respective chemicals. Data points represent the mean (± SD) of three replicate samples. Data were transformed to the percent of maximum response observed for each concentration-response curve.

FIG. 4. Relative potencies of pyriproxyfen (○) and atrazine (■) to induce dhb2 mRNA in daphnids. dhb2 mRNA levels were measured by real-time RT PCR following 24-h exposure to the respective chemicals. Data points represent the mean of three replicate samples. All data were normalized to the maximum induction level measured with pyriproxyfen.

FIG. 5. dhb2 mRNA levels following exposure to mixtures of atrazine and pyriproxyfen compared to concentration addition (dashed line) and response addition (dotted line) model predictions. dhb2 mRNA levels in daphnids were measured by real-time RT PCR at four mixtures of atrazine and pyriproxyfen. Data points represent the mean (± SD) from three replicate samples.

DISCUSSION

The hypothesis was tested that atrazine induces hemoglobin in daphnids through the terpenoid hormone–signaling pathway. This pathway co-regulates both hemoglobin synthesis and male sex determination in daphnids (Rider et al., 2005). Some environmental chemicals have been shown to stimulate this pathway resulting in hemoglobin induction or male sex
Results conclusively demonstrate that atrazine does indeed stimulate hemoglobin synthesis via the dhb2 gene; however, this increase in hemoglobin is not due to the activation of the terpenoid-signaling pathway. These results, coupled with several unsuccessful attempts to stimulate male sex determination in D. magna with atrazine (Olmstead and LeBlanc, 2003, and unpublished data) establish that atrazine does not mimic the action of terpenoid hormones in this species.

An alternative explanation for the induction of dhb2 is that atrazine stimulates hemoglobin production by activating the HIF regulatory pathway. HIF is an oxygen sensor composed of two subunits α and β. The α-subunit is readily degraded under normoxic conditions but is stabilized and accumulates in the cell under hypoxic conditions (Semenza, 2001). The α-subunit then binds to the β-subunit, which is constitutively expressed and available within the cell. This dimer serves as a transcriptional activator that binds the HRE of responsive cells to regulate gene activity (Semenza, 2001). This paradigm for the structure and function of HIF appears to be highly conserved among animals including daphnids (Gorr et al., 2004).

Some chemicals, specifically transition metals and iron chelators, have been shown to stimulate the HIF pathway in vertebrates’ cells (Su et al., 2006; Woo et al., 2006). These materials may interfere with the generation of reactive oxygen species that are the ultimate regulators of HIFα stability (Kietzmann and Gorlach, 2005). We are aware of no significant evidence that atrazine would similarly interfere with the generation of reactive oxygen species. However, we attempted to test this alternative hypothesis for the action of atrazine in mixture experiments with the transition metal cobalt chloride and the iron chelator deferoxamine mesylate. Unfortunately, neither compound activated the HIF pathway as indicated by the lack of hemoglobin induction. The reason for this inactivity is not known although the pharmacokinetics and bioavailability of the materials to target cellular components would certainly differ between cultured cells that respond to these materials and whole daphnids that did not respond.

Dissolved oxygen levels decreased more rapidly between solution changes in the atrazine-containing solutions as compared to controls. Decreased dissolved oxygen levels in atrazine solutions may have been due to increased biological oxygen demand associated with atrazine-mediated death of algal cells provided in the media. Nonetheless, oxygen levels in all solutions remained above 80% of saturation. The slight decrease in dissolved oxygen levels associated with atrazine would not be expected to result in significant induction of hemoglobin in daphnids (Zeis et al., 2004). However, we cannot exclude the possibility that atrazine activates the HIF pathway, at least in part, by reducing O2 that is available to the organisms. This possibility is supported by the observation that atrazine-dependent increases in dhb2 mRNA levels were most prominent during the periods of exposure when dissolved oxygen levels decreased the most (compare dhb2 induction profile in Fig. 2B with dissolved oxygen profile in Fig. 6).

Another goal of this study was to determine whether additive-modeling approaches could be used to assess a chemical’s mechanism of action. Results clearly demonstrate the feasibility of this approach. Using a 24-h assay with a zooplanktonic crustacean species, we were able to determine that atrazine and pyriproxyfen induce hemoglobin levels by different mechanisms. Mixture modeling of the joint action of chemicals using concentration addition and response addition paradigms, accompanied by experimental identification of the appropriate model using cell extracts, whole cells, tissues, or small whole animals can be used to decipher the mechanism of action of a chemical. Empirical effects of the herbicide atrazine (the induction of hemoglobin) were suggestive of a common mechanism of action with the insecticide pyriproxyfen. However, the modeling strategy used provided convincing evidence that these chemicals elicit a common apical effect through different signaling pathways. Thus, best estimates of the cumulative effects of these chemicals in a mixture would require the use of a response addition model. This strategy for assigning mechanism of action could greatly improve the accuracy of assessments of the cumulative toxicity of mixtures in which constituents have uncertain mechanisms of action.
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