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Regulation and dysregulation of vitellogenin mRNA accumulation in daphnids (*Daphnia magna*)

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Abstract

The induction of vitellogenin in oviparous vertebrates has become the gold standard biomarker of exposure to estrogenic chemicals in the environment. This biomarker of estrogen exposure also has been used in arthropods, however, little is known of the factors that regulate the expression of vitellogenin in these organisms. We investigated changes in accumulation of mRNA products of the vitellogenin gene Vtg2 in daphnids (*Daphnia magna*) exposed to a diverse array of chemicals. We further evaluated the involvement of hormonal factors in the regulation of vitellogenin expression that may be targets of xenobiotic chemicals. Expression of the Vtg2 gene was highly responsive to exposure to various chemicals with an expression range spanning approximately four orders of magnitude. Chemicals causing the greatest induction were piperonyl butoxide, chlordane, 4-nonylphenol, cadmium, and chloroform. Among these, only 4-nonylphenol is recognized to be estrogenic. Exposure to several chemicals also suppressed Vtg2 mRNA levels, as much as 100-fold. Suppressive chemicals included cyproterone acetate, acetone, triclosan, and atrazine. Exposure to the estrogens diethylstilbestrol and bisphenol A had little effect on vitellogenin mRNA levels further substantiating that these genes are not induced by estrogen exposure. Exposure to the potent ecdysteroids 20-hydroxyecdysone and ponasterone A revealed that Vtg2 was subject to strong suppressive control by these hormones. Vtg2 mRNA levels were not significantly affected from exposure to several juvenoid hormones. Results indicate that ecdysteroids are suppressors of vitellogenin gene expression and that vitellogenin mRNA levels can be elevated or suppressed in daphnids by xenobiotics that elicit antiecdysteroidal or ecdysteroidal activity, respectively. Importantly, daphnid Vtg2 is not elevated in response to estrogenic activity.

Keywords

vitellogenin; water flea; biomarker; ecdysteroids; estrogens; endocrine disruption

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Introduction

The induction of vitellogenin in oviparous vertebrates has gained prominence as a biomarker of exposure to estrogenic chemicals in the environment (Denslow, 1999; Heppell et al., 1995; Palmer et al., 1998). Vitellogenin, the precursor to the yolk protein vitellin, is normally produced in the liver of females in response to 17β-estradiol stimulation. The vitellogenin is secreted into the blood and is transported to the ovaries where it is incorporated into maturing oocytes. Exposure of non-reproductive females or males to estrogenic compounds causes the aberrant production of vitellogenin which accumulates in the blood. These elevated blood levels of vitellogenin can be readily detected by immunoassays (Parks et al., 1999; Specker and Anderson, 1994). Increased accumulation of vitellogenin mRNA levels in the liver also can serve as a biomarker of exposure to estrogenic chemicals (Korte et al., 2000). The use of vitellogenin induction as a biomarker of exposure to estrogenic compounds has been successfully used in fish (Folmar et al., 1996; Zhang et al., 2005), amphibians (Palmer et al., 1998), and reptiles (Palmer and Palmer, 1995).

The use of vitellogenin or vitellogenin-like protein induction as a biomarker of exposure of arthropods to estrogenic compounds also has been reported (Matozzo et al., 2007). Protein induction with exposure to 17β-estradiol has been reported in crustacean species including barnacle (Billinghurst et al., 2000), shrimp (Huang et al., 2006), and prawn (Yano and Hoshino, 2006). Induction of vitellogenin by the xenoestrogen 4-nonylphenol in arthropods also has been reported in barnacles (Billinghurst et al., 2000), mysid shrimp (Ghekiere et al., 2006), prawn (Sanders et al., 2005), and midges (Hahn et al., 2002). Such observations, in conjunction with reports that estrogens stimulate female reproductive maturation in crustaceans (Anderson et al., 1999; Sapolsky et al., 1984; Sarojini et al., 1986; Sarojini et al., 1990), have led to suggestions that vitellogenin induction in arthropods can serve as a viable biomarker of exposure to estrogenic compounds in arthropods as well as in vertebrates (Depledge and Billinghurst, 1999; Matozzo et al., 2007). However, the premise that vitellogenin in arthropods is positively regulated by estrogens is equivocal.

Among protostome invertebrates, the estrogen receptors appears to have evolved only among the lophotrozoans (Baker, 2008) and this invertebrate estrogen receptor does not require estrogen binding for activity (Kajiwara et al., 2006; Keay et al., 2006). An estrogen receptor was not found in the fully sequenced *Daphnia pulex* genome (Thomson et al., 2009) and estrogens have no recognized function in insects (DeLoof and Huybrechts, 1998). Rather, much of the regulatory functions associated with estrogens in vertebrates can be attributed to ecdysteroid and juvenoid hormones in arthropods (De Loof and Huybrechts, 1998; Laufer and Biggers, 2001). Taken together, these observations argue against vitellogenin induction in arthropods serving as a biomarker of exposure to estrogenic chemicals in arthropods.

Vitellogenin induction in crustaceans has been reported for chemicals having no known estrogenic activity such as copper, zinc (Martin-Diaz et al., 2005), and fipronil (Volz and Chandler, 2004). Thus, arthropod vitellogenin may indeed be susceptible to exposure to environmental chemicals; but, the activity responsible for this induction may be estrogen-independent. Indeed, juvenoid and ecdysteroid hormones orchestrate vitellogenin synthesis in insects with juvenoids typically inducing vitellogenesis and ecdysteroids have either a stimulatory or suppressive effect, depending upon the species (Barchuk et al., 2002; Comas et al., 1999; Gunawardene et al., 2002; Handler and Postlethwait, 2005). Limited evidence suggests that juvenoids stimulate vitellogenesis in decapods crustaceans (Laufer et al., 1998; Rodriguez et al., 2002) but suppress vitellogeneis in branchiopod crustaceans (Tokishita et al., 2006).

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Daphnids (Crustacea, Branchiopoda, Cladocera) are extensively used as a test species in ecotoxicology. Vitellogenin in daphnids is the product of at least two genes designated Vtg1 and Vtg2 (Tokishita et al., 2006). In preliminary experiments, we observed that Vtg2 mRNA levels were considerably more responsive to chemical exposure as compared to Vtg1 mRNA levels. We therefore evaluated the expression of the Vtg2 gene of *Daphnia magna* to gain insight into: 1) the responsiveness of the gene to exposure to various environmental chemicals, and, 2) the hormonal regulation of the gene. Results both advance our understanding of the strengths and limitations of this potential biomarker of xenobioltic exposure, as well as, increase our understanding of the endogenous factors that regulate vitellogenesis in cladocerans.

Materials and Methods

Daphnids

Daphnids (*Daphnia magna*) used in this study were obtained from cultures maintained in our laboratory for over 17 years. Daphnids were reared in media reconstituted from deionized water as described previously (Wang et al., 2007). Cultured daphnids were maintained at a density of 40 adults per 1000 ml of media and were fed twice daily with 2.0 ml (1.4×10^8 cells) of a suspension of unicellular green algae, *Pseudokirchneriella subcapitata* and 1.0 ml (~4 mg dry weight) of Tetrafin™ fish food suspension (Tetra Holding Inc., Blacksburg, VA, USA) per 1000 ml of media. All daphnids were housed in incubators set to 20°C with a 16/8 hour light/dark cycle. Daphnids used in the experiments reproduced exclusively by parthenogenesis and were all female.

Chemical exposures

Daphnids were initially exposed to twenty-five chemicals to screen various functional classes of chemicals for effects on daphnid Vtg2 mRNA levels. Functional classes included estrogens, androgens, retinoids, juvenoids, uncouplers of oxidative phosphorylation, GABA receptor blockers, narcotics, and cytochrome P450 inhibitors. Young adult female daphnids (7–14 days old) that had molted within the previous 12 hours were used in these experiments. Daphnids were exposed to a single concentration of the chemical which represented the highest concentration used in preliminary experiments that elicited no discernible adverse response by the exposed organisms (Table 1). Ten daphnids per treatment were individually exposed to the chemicals in 50 ml beakers containing 40 ml of media. Chemicals were dissolved in deionized water or ethanol for delivery to the exposure media (same media as used for culturing). Carrier concentrations in exposure media never exceeded 0.05% (v/v). For each experiment, control daphnids were exposed to the same concentration of ethanol as the treated animals. Daphnids were transferred daily to fresh media containing the exposure chemical and food (0.7×10^7 cells of *P. subcapitata*). Animals were exposed to the chemicals for a total of 72 hours.

At the end of the exposures, animals were placed in RNALater™ (Qiagen, Valencia, CA, USA) solution in groups (2 replicates consisting of 5 animals each) and stored at 4°C overnight then maintained at −80 °C until processed. At processing, RNALater™ solution was removed and replaced with Promega™ lysis buffer (Madison, WI, USA). Samples were homogenized with a dounce homogenizer and RNA was isolated using the SV Total RNA Isolation System (Promega). RNA yield was determined by absorbance at 260 nm and its purity was measured by the 260/280 nm absorbance ratio with a Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Montchanin, DE). RNA was reverse transcribed to cDNA with oligo dT primers using the ImProm-II Reverse Transcription System (Promega).

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Vtg analyses

D. magna Vtg2 mRNA levels were measured using real-time RT PCR. Oligonucleotide primers used were the same as described and used by Tokishita et al. (2006). EcRb primers were derived from the mRNA sequence described by Kato et al. (2007). HR3 and E75 primer sequences used were described previously by Hannas and LeBlanc (2010). Hb1 and Hb2 primers were designed from the nonhomologous untranslated regions of the respective genes (Kimura et al., 1999) using Primer Express Software (Applied Biosystems, Foster City, CA). Primer sequences are described in Table 1. Actin (accession #AJ292554) and GAPDH (accession #AJ292555) cDNA were also amplified and used in the normalization of transcripts. Quantitative real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using default parameters. Amplification mixtures consisted of 12.5 μl SYBR Green PCR Master Mix (Applied Biosystems), 300 nM primers, 20 ng template cDNA in a total volume of 25 μl. Primer concentrations were optimized following the manufacturer's recommendations. The reaction mixtures were first kept at 95°C for 5 minutes, followed by 40 cycles with each cycle consisting of a temperature of 95°C for 5 sec followed by 60°C for 1 min. After the PCR reactions, the melting temperature of PCR product was determined using the dissociation protocol provided by the instrument manufacturer. A single melting peak was detected for all samples indicating no amplification of non-target DNA. Furthermore, only a single amplification product was detected following electrophoresis in a 2% agarose gel and staining with ethidium bromide. The comparative C_T method ($2^{-\Delta\Delta CT}$) was used to assess the relative levels of mRNA. Validation experiments, as described by the instrument manufacturer, confirmed that the efficiencies of the target and endogenous controls (actin and GAPDH) amplifications were approximately equal.

Vitellogenin expression profiling over a molt cycle

Molt-synchronized daphnids were generated as described previously (Hannas and LeBlanc, 2010). Briefly, ~300 nine to ten-day old daphnids were individually placed in 50 ml beakers and monitored continuously (≤every two hours) for molting. Upon molting, the daphnid was designated as being at time 0 in its molt cycle and the animal was targeted for sampling at either time 0, 12, 24, 36, 48, and 60 hours. This process continued until 30 animals were targeted for sampling at each time point. Once selected for the experiment, daphnids were individually maintained in 40 ml solutions containing the chemical under evaluation at the desired concentration. Animals were provided food daily (0.7×10^7 cells of *P. subcapitata*, 50 μl fish food suspension). At sampling, animals were placed in RNALater™ solution in groups (3 replicates consisting of ~10 animals per replicate for each time point) and stored at 4°C overnight then maintained at −80°C until processed. At processing, total RNA was isolated and cDNA was prepared as described above.

Statistics

Significant difference between two means was evaluated using Student's t test. Significant differences among several means were assessed using ANOVA and Tukey's Multiple Comparison Test. Equality of variances and normality were established using Levene's test and the Shapiro-Wik test, respectively.

Results

Modulation of vitellogenin mRNA levels with chemical exposure

Twenty-five chemicals were screened for their ability to modulate Vtg2 mRNA levels in *D. magna.* Vtg2 levels were both elevated and suppressed in response to chemical exposure with mRNA levels spanning nearly four orders of magnitude (Fig. 1). Among the most

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potent inducers of Vtg2 mRNA levels were piperonyl butoxide (20.7-fold elevation), chlordane (14.5-fold elevation), 4-nonylphenol (10.8-fold), and cadmium (6.6-fold elevation). Among these only 4-nonyphenol is recognized to have estrogenic activity. The estrogens diethylstilbestrol and bisphenol A had little effect on Vtg2 levels (1.6 and 1.4-fold elevation, respectively). Several chemicals suppressed Vtg2 expression (Fig. 1). Most notable were cyproterone acetate (0.0046-fold expression), acetone (0.071-fold expression), triclosan (0.22-fold expression), and atrazine (0.32-fold expression). Half of the chemicals evaluated (52%) had little impact (relative expression levels >0.5<2.0-fold) on Vtg2 mRNA levels.

These initial experiments were designed to screen various chemicals for their potential to modulate vitellogenin mRNA levels in daphnids. Animals used in these experiments underwent gross screening for consistency in the molt/reproductive cycle (see methods) but results could be confounded by inter-animal differences in vitellogenin levels as related to molt/reproductive stage differences among individuals. Therefore, more definitive experiments were performed with the most potent stimulator of vitellogenin mRNA levels (piperonyl butoxide) and the most potent suppressor of vitellogenin mRNA levels (cyproterone acetate). These experiments were performed with individuals that were precisely molt-synchronized, vitellogenin mRNA levels were measured at various points along a time-course of exposure, and replication was increased. Vtg2 mRNA levels varied significantly over the molt/reproductive cycle of untreated (control) daphnids (Fig. 2A). Most notably, Vtg2 mRNA levels climbed between 12 and 24 hours after the previous molt, and then dropped precipitously between 24 and 48 hours after the previous molt. Exposure to piperonyl butoxide blocked the post 24-hour decline in mRNA levels resulting in significantly elevated levels of Vtg2 mRNA levels relative to controls 48 hours after the previous molt (Fig. 2A). Cyproterone acetate severely suppressed the mid-cycle elevation in Vtg2 mRNA levels (Fig. 2B).

Regulation of vitellogenin mRNA levels by ecdysteroids and juvenoids

Since estrogens appeared to have little role in regulating daphnid vitellogenin mRNA levels, the role of the ecdysteroids and juvenoids in vitellogenin expression was next evaluated. These hormones are major regulators of processes relating to development, growth, and reproduction in arthropods. Exposure of daphnids to the ecdysteroids 20-hydroxyecdysone and ponasterone A for 72 hours decreased Vtg2 mRNA levels (Fig. 3). In an effort to establish the effectiveness of the ecdysteroid treatment, mRNA levels were also measured for the nuclear receptors HR3 and E75, which are elevated in daphnids in response to ecdysteroids (Hannas and LeBlanc, 2010) and EcRb which is suppressed 72 hours after ecdysteroid treatment (LeBlanc, 2008). Both HR3 and E75 mRNA levels were elevated in response to 20-hydroxyecdysone and ponasterone A, though the design of the experiment (animals not molt-synchronized, animal tissues combined reducing the number of experimental units) precluded establishing statistical significance to the increases. Both ecdysteroids also significantly suppressed EcRb mRNA levels, thus confirming the effectiveness of the ecdysteroid treatment regimen. The apparent suppressive effect of ecdysteroids on Vtg2 mRNA levels was confirmed using molt- synchronized daphnids. Continuous exposure of molt-synchronized daphnids to 20-hydroxyecdysone through the first 48 hours after molting progressively suppressed Vtg2 mRNA levels (Fig. 4).

Finally, the ability of the juvenoids fenoxycarb and methyl farnesoate to modulate vitellogenin mRNA levels in daphnids was evaluated. In addition, the effectiveness of treatment was confirmed by evaluating hemoglobin Hb1 and Hb2 mRNA levels with juvenoid treatment. Hemoglobin in daphnids is elevated by juvenoid hormones (Oda et al., 2005; Olmstead and LeBlanc, 2002; Rider et al., 2005). Exposure of daphnids to fenoxycarb for 72 hrs elevated hemoglobin Hb1 and Hb2 mRNA levels but had no significant effect on

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Vtg2 levels (Fig. 5). The effect of methyl farnesoate was more definitively evaluated in a time course exposure using molt-synchronized daphnids. Again, both Hb1 and Hb2 mRNA levels were elevated by the juvenoid, but Vtg2 levels were unchanged (Fig. 6).

Taken together, evaluation of the hormonal regulation of vitellogenin expression in daphnids indicated that Vtg2 mRNA levels are subject to suppression by ecdysteroids but are refractory to juvenoid hormones.

Discussion

Results from this study clearly demonstrate that product of the daphnid vitellogenin Vtg2 gene is susceptible to both increased and decreased accumulation in response to exposure to some environmental chemicals. However, this gene was not responsive to the estrogenic activity of chemicals. Increased Vtg2 mRNA accumulation in response to some estrogens (e.g. 4-nonylphenol) is due to some other properties associated with these compounds. For example, all of the compounds that appreciably increased Vtg2 mRNA levels reportedly cause oxidative stress (piperonyl butoxide (Muguruma et al., 2007), chlordane (Bondy et al., 2004), 4-nonylphenol (Dinan et al., 2001), cadmium (Sadik, 2008), chloroform (Burke et al., 2007)). Future studies should be directed towards identifying properties of chemicals that are responsible for modulating Vtg2 levels.

While not under the regulatory control of estrogens, Vtg2 mRNA levels were negatively regulated by ecdysteroids. Both 20-hydroxyecdysone and ponasterone A suppressed Vtg2 mRNA levels. Although, ponasterone A was appreciably more potent than was 20-hydroxyecdysone. Both 20-hydroxyecdysone and ponasterone A have high ecdysteroidal activity in crustaceans, with ponasterone A being recognized as the more potent hormone (Baldwin et al., 2001; Wang and LeBlanc, 2009). This responsiveness to ecdysteroids may explain some of the effects of xenobiotics on Vtg2 mRNA levels. Among the compounds that appreciably increased Vtg2 mRNA levels, 4-nonylphenol 0(Aydogan et al., 2008) and cadmium (Bodar et al., 1990) are reported to have anti-ecdysteroidal activity. These compounds may have increased Vtg2 mRNA levels by releasing the Vtg2 gene from the suppressive action of endogenous ecdysteroids. The most suppressive of the compounds evaluated, cyproterone acetate has been shown to bind the androgen receptor, the glucocorticoid receptor, the progesterone receptor, and the pregnane X receptor (Honer et al., 2003; Lehmann et al., 1998). Conceivably, cyproterone acetate could bind and activate the ecdysteroid receptor resulting in suppression of Vtg2.

The suppressive action of ecdysteroids explains, in part, the regulation of Vtg2 mRNA levels during the molt/reproductive cycle of daphnids. During the period of ovarian oocytes maturation, Vtg2 mRNA levels progressively increase. The regulatory factor responsible for this increase is presently unknown. Between 24 and 48 hours post-molt, Vtg2 mRNA levels decline. This decline corresponds to the endogenous increase in ecdysteroid levels that occur in preparation for the next exuviation (LeBlanc, 2007; Martin-Creuzburg et al., 2007). This decline in Vtg2 mRNA levels also temporally corresponds to the increase in nuclear receptor HR3 which is positively regulated by ecdysteroids (Hannas and LeBlanc, 2010). Thus, the pre-exuviation ecdysteroid surge that occurs in daphnids coordinates the regulation of a variety of genes involved in molting and reproduction.

The juvenoid hormone methyl farnesoate has been reported to stimulate ovarian maturation in the red swamp crayfish (*Procambarus clarkii*) (Laufer et al., 1998; Rodriguez et al., 2002) and it is attractive to speculate that methyl farnesoate, or a related juvenoid, is responsible for the increase in Vtg2 mRNA levels during oocyte maturation in daphnids. However, Tokishita et al. (Tokishita et al., 2006) reported that juvenoids suppress Vtg2 levels in *D.*

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magna; while, we were unable to detect any effect of several juvenoids on daphnid Vtg2 mRNA levels. This difference may be due to the age of the animals used in the respective studies. Tokishita et al. (2006) initiated their exposures with neonatal animals and measured Vtg2 levels through the first cycle of oocytes maturation. Thus, their animals were juveniles through most of the exposure period. Our exposures were initiated with molt- synchronized adult animals that had experienced a previous molt/reproductive cycle. Juvenoid hormones may differently impact Vtg2 gene expression among reproductive naïve animals as compared to animals that had previously undergone oocyte maturation. The role of juvenoids in the regulation of daphnid Vtg2 mRNA accumulation remains unresolved.

In conclusion, Vtg2 mRNA levels can be used in biomonitoring for exposure of daphnids to some environmental chemicals. This biomarker, however, does not have utility as an indicator of exposure to estrogenic chemicals. Chemical properties that are responsible for the elevation or suppression of Vtg2 mRNA levels are not completely resolved. However, Vtg2 levels are likely to be elevated in response to chemicals with antiecdysteroidal activity and suppressed in response to chemicals with ecdysteroidal activity. Temporal differences in Vtg2 mRNA levels over the molt/reproductive cycle require that molt synchronized animals be used in exposure assessments to maximize sensitivity of the assay. Future studies should address those chemical characteristics that are responsible for modulating Vtg2 mRNA levels and evaluating the relationship between altered Vtg2 mRNA levels and reproductive success.

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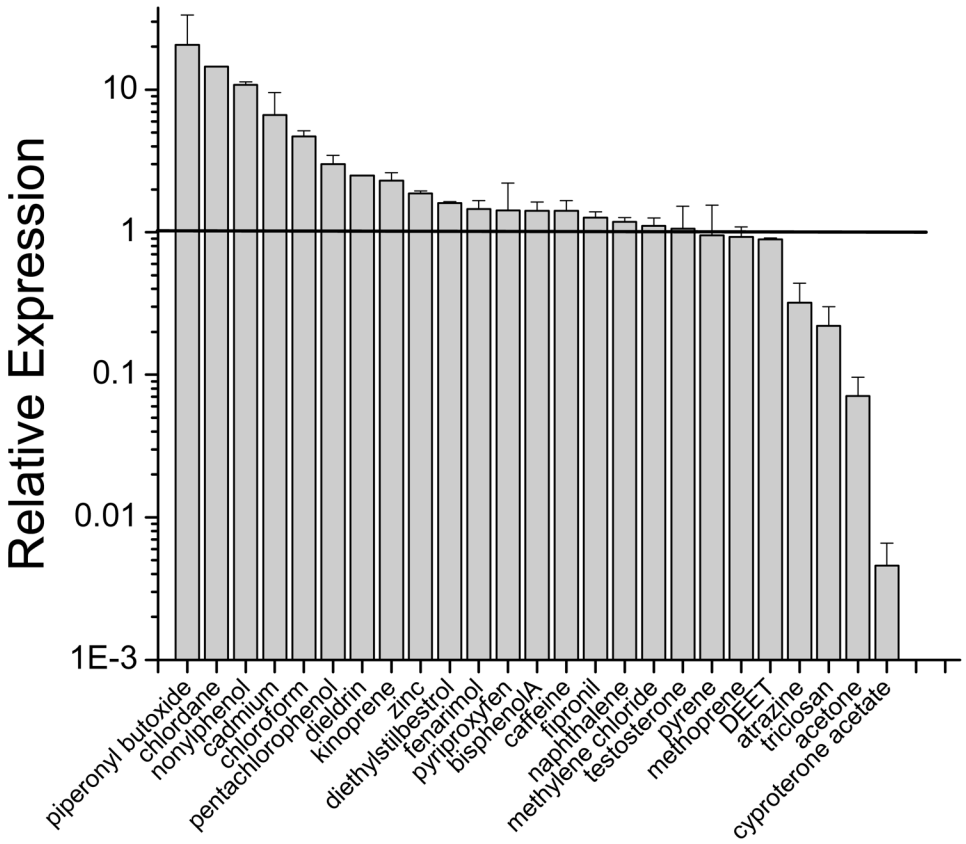

Figure 1.

Vitellogenin Vtg2 mRNA levels following 72 hrs exposure of daphnids to various chemicals. Exposure concentrations are reported in Table 2. Data are reported as the mean and SEM (n=2, with each experimental unit consisting of 5 individuals) expression relative to concurrently evaluated controls which were set at 1.0 (horizontal line).

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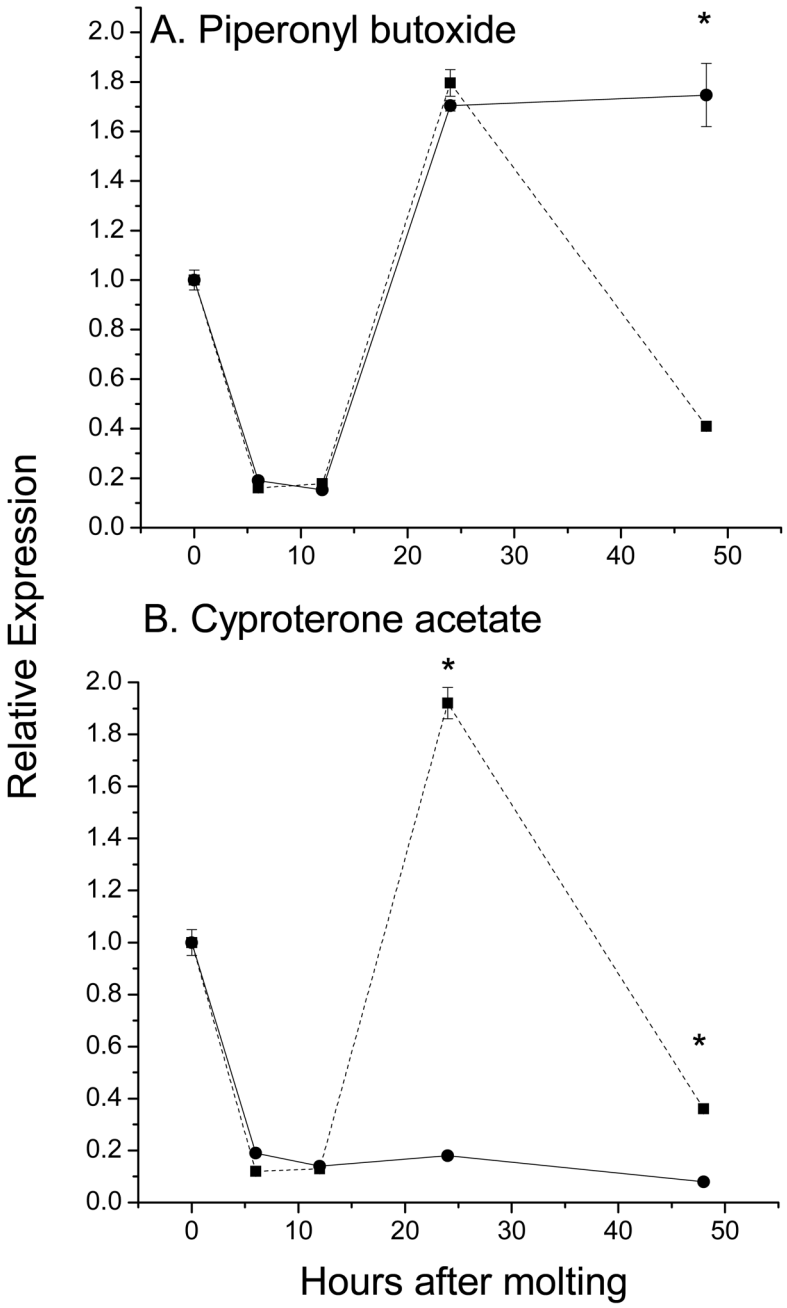

Figure 2.

Relative expression of vitellogenin Vtg2 mRNA during continuous exposure of molt-synchronized daphnids to piperonyl butoxide (300 μg/L, panel A) and cyproterone acetate (2100 μg/L, panel B). Error bars represent the SEM (n=3). An asterisk denotes a significant ($p<0.05$) difference between the treatment (solid line, circles) and control (dashed line, squares) (Student's t test).

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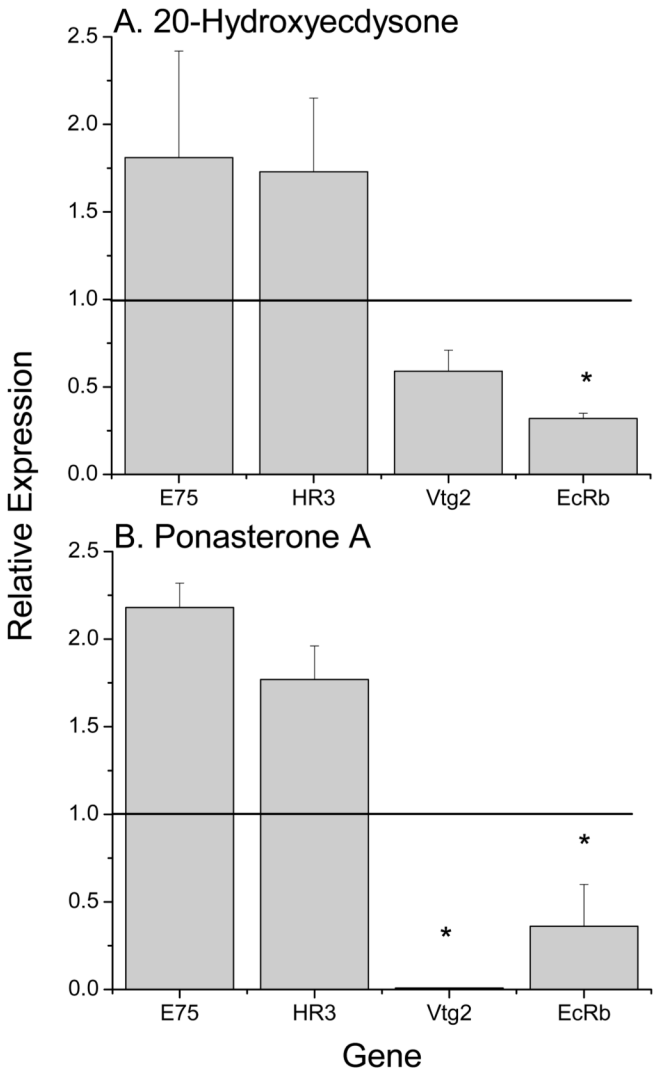

Figure 3.

Relative expression of selected mRNAs following 72 hrs exposure of daphnids to 20-hydroxyecdysone (481 μg/l, Panel A) or ponasterone A (232 μg/l, panel B). Data are reported as the mean and SEM (n=2, with each experimental unit consisting of 5 individuals) expression relative to concurrently evaluated controls which were set at 1.0 (horizontal line). An asterisk denotes a significant ($p<0.05$) difference between the treatment and control (Student's t test).

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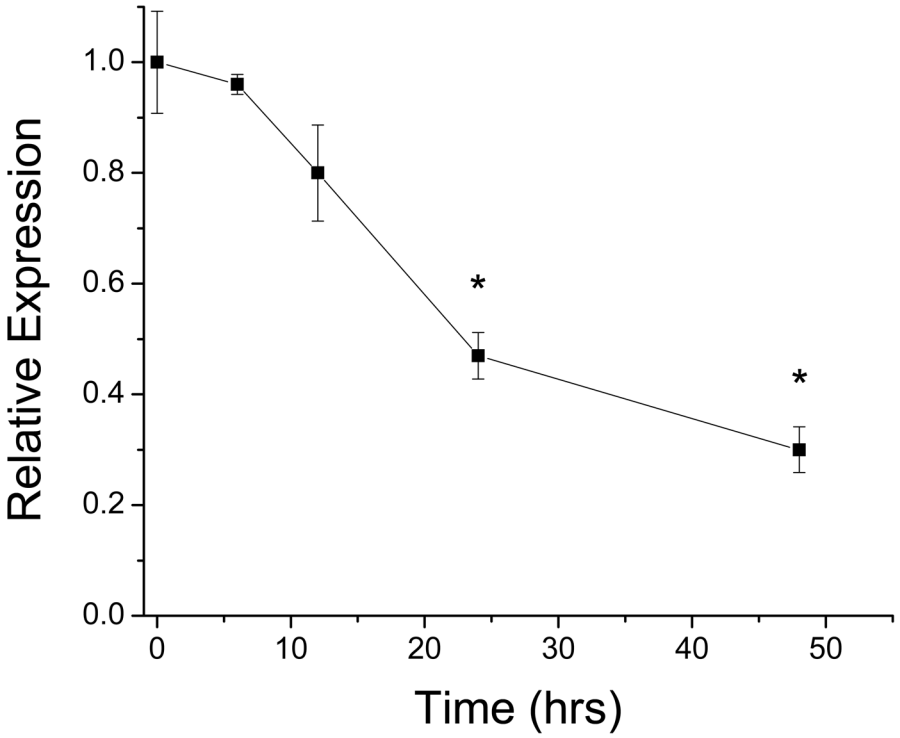

Figure 4.

Expression of vitellogenin Vtg2 mRNA levels during exposure to 481 μg/l 20-hydroxyecdysone. Values were normalized to concurrently evaluated control (untreated) animals. Error bars represent the SEM (n=3). An asterisk denotes a significant ($p<0.05$) reduction in Vtg2 level relative to levels at 0 hr (ANOVA, Tukey's Multiple Comparison).

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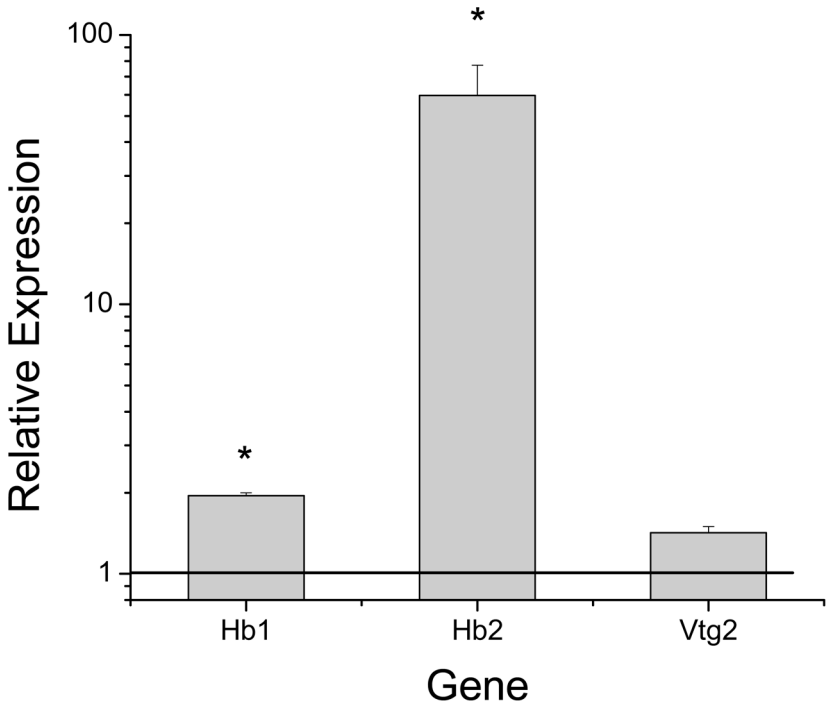

Figure 5.

Relative expression of selected mRNAs following 72 hrs exposure of daphnids to the juvenoid hormone fenoxycarb (1000 μg/l)). Data are presented as the mean ± SEM (n=2, with each experimental unit consisting of 5 individuals) expression relative to concurrently evaluated controls which were set at 1.0 (horizontal line). An asterisk denotes a significant ($p<0.05$) difference between the treatment and control (Student's t test).

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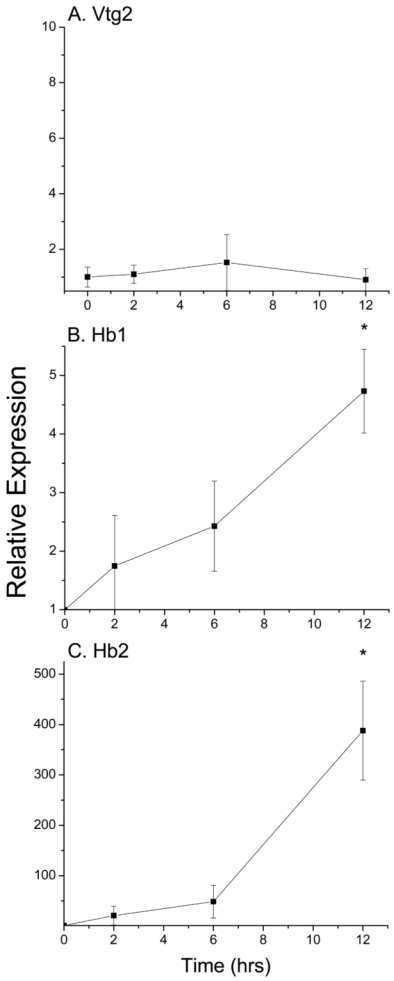

Figure 6.

Relative expression of selected mRNAs following 72 hrs exposure of daphnids to the juvenoid hormone methyl farnesoate (200 μg/l). Error bars represent the SEM (n=3–4, with each experimental unit consisting of 3 individuals). Data were normalized to concurrently maintained control (unexposed) daphnids. An asterisk denotes a significant ($p<0.05$) difference between the treatment and mRNA levels measured at 0 hr (ANOVA, Tukey's Multiple Comparison).

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Table 1

Chemicals and exposure concentrations used to assess the modulation of Vtg1 and Vtg2 mRNA levels in *D. magna.*

Chemical	Exposure concentration (μg/l)
Diethyl stilbestrol	500
4-Nonyl phenol	200
Piperonyl butoxide	300
Cyproterone acetate	2100
Kinoprene	1000
Methoprene	310
Pyriproxyfen	100
Fenoxycarb	1000
Bisphenol A	10000
Fenarimol	1300
Testosterone	3500
Chloroform	630
Zinc (as sulfate)	1000
Pentachlorophenol	39
Methylene chloride	195000
Naphthalene	2500
Acetone	3900
Caffeine	18200
N,N-diethyl-meta-toluamide (DEET)	10000
Atrazine	5400
Pyrene	0.4
Triclosan	40
Cadmium (as chloride)	20
Fipronil	19
20-hydroxyecdysone	481
Chlordane	10
Dieldrin	100
Ponasterone A	232
Methyl farnesoate	200

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Table 2

Oligonucleotide primer sequences used for gene-specified real time RT-PCR.

Gene	Forward	Reverse
Vtg2	CACTGCCTTCCCAAGAACAT	ATCAAGAGGACGGACGAAGA
HR3	AGTCATCACCTGCGAGGGC	GAACTTTGCGACCGCCG
E75	TCCGGAGAAGTATTCAACAAAAGA	TGCGAAGAATGGAGCACTGT
EcRb	AGTCCGTCAGACGAGCATTC	GGACGGTCCATTAATGTCAAG
Hb1	AAATTCAAACGTGGCACTCAAA	AAGTCCTCGTTGGGAGGGA
Hb2	CTGTTGGACGTTTTGGGTGC	TGAGTCCAGGTCGTCAGAGGA
Actin	TGGTCAGGTCATCACCATTG	CTCGTGGATACCGCAAGATT
GADPH	GGCAAGCTAGTTGTCAATGG	TATTCAGCTCCAGCAGTTCC