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Stress signaling: coregulation of hemoglobin and male sex determination through a terpenoid signaling pathway in a crustacean

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Summary

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 non-male-producing 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, the 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 be likely to 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, *Daphnia magna*.

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 in crustaceans as a major hormone 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 probably 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 such as a drop in food quantity or quality, a decrease in photoperiod, and crowding (Carvalho and Hughes, 1983; Klevien et al., 1992; Stross and Hill, 1968a,b) 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 coregulated 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 Straus 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, USA. 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 non-male producers (J. Dudycha, personal communication). All daphnids were cultured and experimentally maintained in deionized water reconstituted with 192 mg l^{-1} $CaSO_4{\cdot}H_2O$, 192 mg l^{-1} $NaHCO_3$, 120 mg l^{-1} $MgSO_4$, 8.0 mg l^{-1} KCl, 1.0 mg l^{-1} selenium and 1.0 mg l^{-1} vitamin B_{12}. *D. magna* and *D. pulex/pulicaria* cultures were maintained at a density of ~50 and ~200 brood daphnids l^{-1} 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 mass) 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 h:8 h L:D 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, USA), and the analogs pyriproxyfen (Chem Service, West Chester, PA, USA), 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, while 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 medium. For each experiment, 5–10 daphnids were exposed to the desired concentration of chemical and 5–10 daphnids were exposed to medium without chemical. Daphnids were provided food twice daily (1.4×10^7 cells of algae and 50 µl fish food suspension for *D. magna,* 5.6×10^6 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 h.

For male offspring production, daphnids were maintained under these conditions and were transferred to new medium 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 medium 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. (1995) with modifications. Individual adult daphnids were homogenized in 600 µl of distilled water by sonication for 5 s using a Vitra-Cell™ hand-held sonicator (Sonics & Materials Inc., Danbury, CT, USA). Particles were pelleted by centrifugation at 10 000 ***g***. A sample (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, USA; 1.0–60 µg hemoglobin in 400 µl) were used to generate standard curves. 24 µl of 0.10% KCN was added to each 400 µl sample. Samples were incubated for 5 min at room temperature, then absorbance (E) 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 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 (Table 1) 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, USA) 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 (10× magnification), then hemoglobin content of the individual daphnids was quantified by spectrophotometry. The relationship between the two measures of hemoglobin was determined (Fig. 1A,B). 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 per 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 be likely to 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 were assessed following native protein polyacrylamide gel electrophoresis. 17–20 adult *D. magna* were homogenized as described above, following exposure to either 3 nmol l^{-1} 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™ electrophoresis apparatus (Bio-Rad, Hercules, CA, USA) with a 5% running gel and a 4% stacking gel. Both gels were prepared at a pH 8.8. Sodium dodecyl sulfate was not added to any of the gels or buffers. Electrophoresis was performed for 30 min at 4°C and 200 V. 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. 30 control or hormone-exposed daphnids were crushed to a fine powder using a pestle and mortar containing liquid nitrogen. RNA was isolated from the powdered preparation using the SV Total RNA Isolation System (Promega, Madison, WI, USA). 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, USA) and the primer option with the lowest penalty (26.5) was selected. The hemoglobin primers were: forward 5′-TCCTCTGACGACCTGGACTCAT-3′, reverse 5′-CCATTA-GCCGAGGTTGAAATTG-3′. Constitutively expressed actin was used as a control for the reaction and to normalize hemoglobin results among samples (forward 5′-CCTGAGC-GCAAATACTCCGT-3′, and reverse 5′-CAGAGAGGCCA-AGATGGAGC-3′). All primers were acquired from Qiagen (Valencia, CA, USA) and were reconstituted in TE buffer (1 mol l^{-1} Tris, 0.5 mol l^{-1} EDTA, pH 8.0). The hemoglobin

Table 1. *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 9+ were placed on the color chart and final score (≥9) was based upon the color shade of the darkest region in the gonadal/visceral region.

hb2 gene product was selected for analyses because this gene is primarily responsible for the increase in hemoglobin mRNA in response to terpenoid signaling (Gorr et al., 2004).

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 ∞.

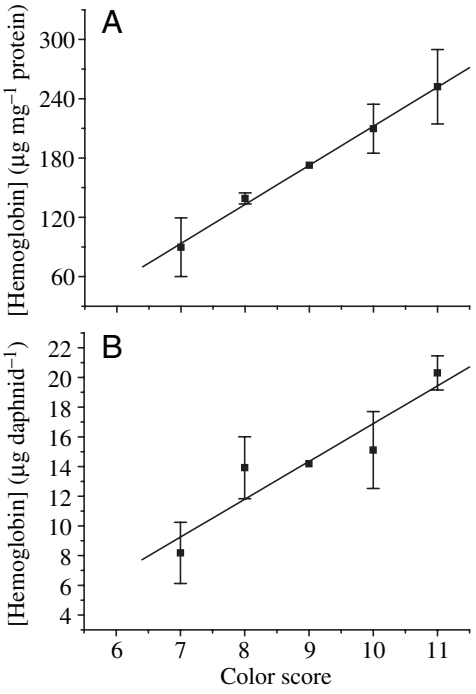

Fig. 1. Colorimetric scoring of hemoglobin content of daphnids. Criteria used to assign a color score to individual daphnids are listed in Table 1. Animals that were scored 9 were placed on a color grid of progressively darker shades of copper and a score was assigned based upon the best color match with the grid. Example relationships between color score assigned to daphnids and hemoglobin content normalized to the soluble protein content of the daphnids (A) and as total hemoglobin per daphnid (B). Values are means ± S.E.M. (*N*=2–4).

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, USA). 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 10× magnification (Olmstead and LeBlanc, 2000). Neonatal daphnids identified as male using this criterion

mature into completely differentiated males that exhibit normal male reproductive behaviors (G.A.L., 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 $P<0.05$ and all analyses were performed using JMP statistical software (SAS Institute, Cary, NC, USA).

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, 2003). Experiments were performed to determine whether hemoglobin is similarly regulated by terpenoid hormones. Hemoglobin levels were significantly elevated on exposure of daphnids to the crustacean terpenoid hormone methyl farnesoate (Fig. 2A) and several synthetic analogs of the hormone (Fig. 2B–D). Relative potency of the terpenoids to elevate hemoglobin levels and induce male offspring production were 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.

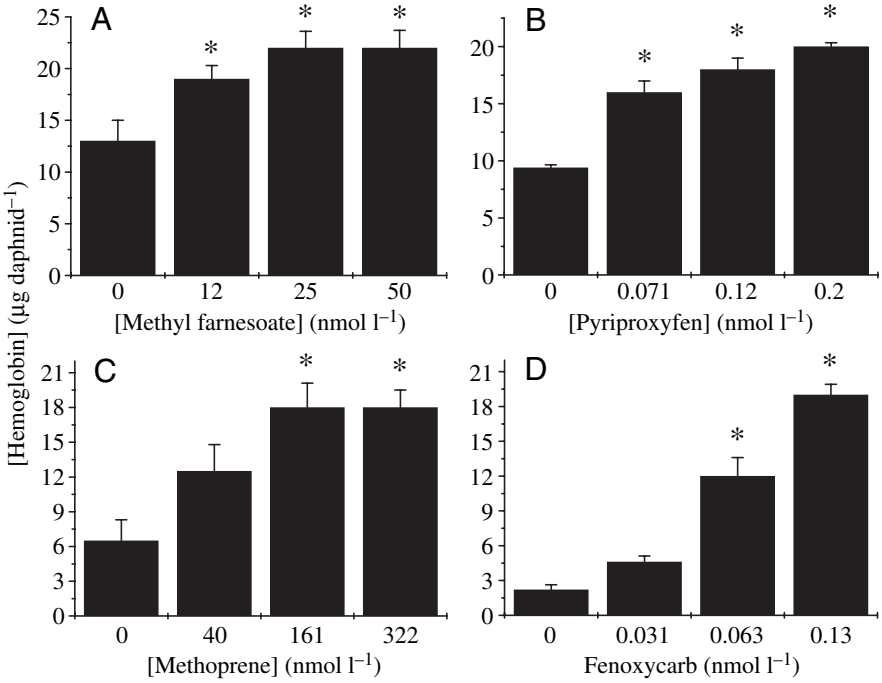

Fig. 2. Hemoglobin levels in juvenile daphnids (*D. magna*) following treatment with the crustacean terpenoid hormone methyl farnesoate (A) and three synthetic analogs (B–D). Hemoglobin levels were assessed colorimetrically after 48 h exposure to the terpenoids. Ten daphnids were exposed to each treatment. Values are means ± S.E.M. *Significant difference from the respective control (0 nmol l^{-1}; $P<0.05$, ANOVA, Dunnetts *t*-test).

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 h of exposure to pyriproxyfen and levels continued to increase linearly over at least the next 24 h (Fig. 5).

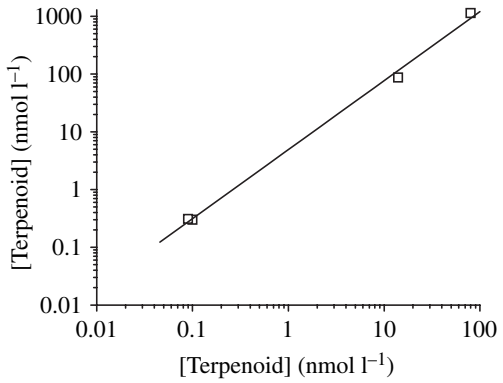

Fig. 3. Relationship between the concentrations of methyl farnesoate and analogs that elevated hemoglobin to 70% of the maximum level (*x* axis) and concentrations that caused a 50% incidence of male broods of offspring (*y* axis) in *D. magna*. Male offspring assessments were reported previously (Olmstead 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 $r^2=0.999$.

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 six 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*

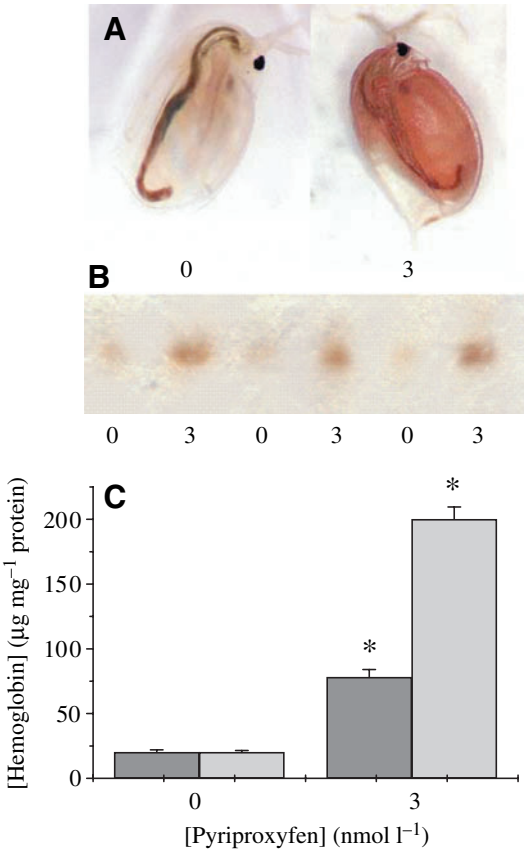

Fig. 4. Induction of hemoglobin in daphnids (*Daphnia magna*) exposed to 0 or 3 nmol l^{-1} pyriproxyfen. (A) Control (0) and exposed (3) daphnids. (B) Electrophoretically separated pigmented protein from individual control (0) and exposed (3) daphnids. (C) Hemoglobin protein levels (µg mg^{-1} protein) 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 are means ± S.E.M. (N=4 samples each prepared from 17–20 individuals). Hemoglobin mRNA levels are means ± S.E.M. (N=2 samples each prepared from 30 individuals). *Significant (P<0.05) difference between control and pyriproxyfen–treated daphnids (Student's t test).

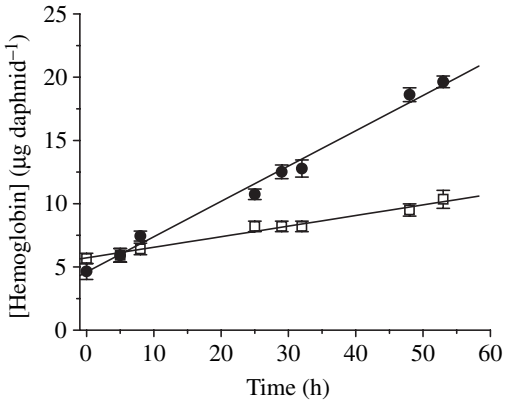

Fig. 5. Hemoglobin levels in juvenile daphnids (*D. magna*) during exposure to 0.93 nmol l^{-1} pyriproxyfen (circles) and concurrently maintained controls (squares). Values are means ± S.E.M., N=10 daphnids. Hemoglobin levels were measured colorimetrically.

(Table 2). All but one clone (MP5) also produced male offspring in response to methyl farnesoate (Table 2). 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 coresponsiveness 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., 2004). 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, 1968a). 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

Table 2. *Responsiveness of male producing (MP) and non-male producing (NP) clones of daphnids to methyl farnesoate*

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

Daphnids were exposed to either 0 or 220 nmol l^{-1} methyl farnesoate and evaluated for both male offspring and hemoglobin production as described in the Materials and methods. Values are means ± S.E.M. (N=5–10).

[a]Hemoglobin levels were estimated colorimetrically and reported values (μg/daphnid) were derived from a standard curve prepared using *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, personal communication).

*Significant increase as compared to the respective control (P<0.05).

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). The 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 be likely to 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 costimulate 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°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

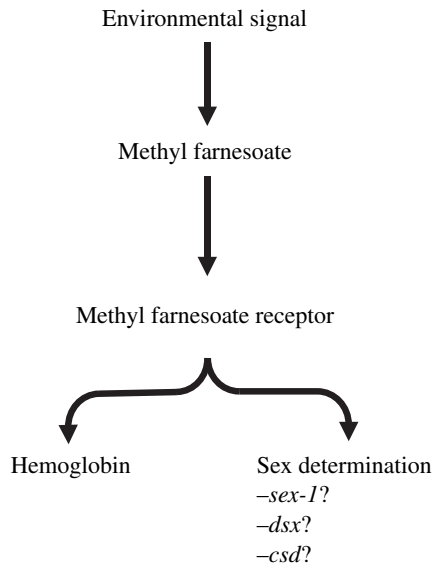

Fig. 6. Diagrammatic representation of the putative juvenoid signaling pathway.

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), its 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, two 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 these clones are 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 (G.A.L. and T.A.G., 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 coregulation 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, the 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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