RESEARCH ARTICLE

The role of nuclear receptor E75 in regulating the molt cycle of *Daphnia magna* and consequences of its disruption

Stephanie M. Street◉°, Stephanie A. Eytcheson◉°, Gerald A. LeBlanc◉*

Department of Biological Sciences, North Carolina State University, Raleigh, North Carolina, United States of America

◉ These authors contributed equally to this work.
* gerald_leblanc@ncsu.edu

Abstract

Biological rhythms regulate innumerable physiological processes, yet little is known of factors that regulate many of these rhythms. Disruption in the timing of these rhythms can have devastating impacts on population sustainability. We hypothesized that the timing of the molt infradian rhythm in the crustacean *Daphnia magna* is regulated by the joint action of the protein E75 and nitric oxide. Further, we hypothesized that disruption of the function of E75 would adversely impact several physiological processes related to growth and reproduction. Analysis of mRNA levels of several genes, involved in regulating the molt cycle in insects, revealed the sequential accumulation of *E75*, its dimer partner *HR3*, *FTZ-F1*, and *CYP18a1* during the molt cycle. Exposure to the nitric oxide donor sodium nitroprusside early in the molt cycle had no effect on *E75* or *HR3* mRNA levels, but delayed the peak accumulation of *FTZ-F1* and *CYP18a1* mRNA. The subsequent exuviation was also delayed consistent with the delay in peak accumulation of *FTZ-F1* and *CYP18a1*. These results supported our assertion that nitric oxide binds E75 rendering it incapable of binding HR3. Excess HR3 protein then enhanced the accumulation of the downstream products FTZ-F1 and CYP18a1. Similarly, suppression of *E75* mRNA levels, using siRNA, had no effect on mRNA levels of *HR3* but elevated mRNA levels of *FTZ-F1*. Consistent with these molecular responses, the suppression of E75 using siRNA increased the duration of the molt cycle and reduced the number of offspring produced. We conclude that the molt cycle of daphnids is regulated in a manner similar to insects and disruption of E75 results in a lengthening of the molt cycle and a reduction the release of viable offspring.

Introduction

Biological rhythms regulate the timing of many physiological processes, and often reflect organismal adaptation to rhythmic changes in the environment [1]. Infradian rhythms are chronobiological cycles that are greater than 24 hours in length. Infradian rhythms can span from a few days to over a century long. The twenty-eight-day menstrual cycle in humans,

yearly migratory and breeding patterns in animals, as well as thirteen or seventeen-year cicada emergence events are all examples of infradian rhythms [2–4].

Little is known about the molecular events involved in most infradian rhythms. The involvement of environmental cues in the entrainment of such rhythms are difficult to discern, as there does not seem to be one central regulatory clock for these rhythms, unlike with circadian rhythms [5]. Twenty-four hour circadian rhythms are typically entrained by light:dark cycles [6]. Many environmental cues seem to dictate the timing of infradian rhythms including, but not limited to, temperature, photoperiod, and food availability [7]. The range of potential entraining cues and the long timeframe of many of these rhythms makes their study challenging, but their ubiquitous nature underscores their importance to life and the regulation of biological processes.

Daphnids are crustaceans that are important constituents of freshwater zooplankton communities. Daphnids are highly responsive to environmental cues to regulate various aspects of their physiology [8]. The molt cycle in crustaceans consists of an environmentally entrained infradian rhythm [9]. This rhythm is closely aligned with physiological processes critical to population sustainability such as growth, maturation, and reproduction [10–12]. Adult *Daphnia magna* shed their exoskeleton (molt) every 3–4 days at 20˚C and a 16:8 hour light:dark cycle. Molting and reproduction are synchronized endocrine-regulated processes in this species, with brood release occurring just prior to molting. Molting also allows for growth to occur. Accordingly, elucidating the molecular events that dictate the molt rhythm will enhance our understanding of the susceptibility of the rhythm to environmental influences.

Previous studies conducted with various arthropod species provide a plausible hypothesis for the involvement of a gene expression cascade and nitric oxide in the regulation of the molt rhythm in daphnids. According to our model (Fig 1), the molt cycle in daphnids is initiated by a pulse of 20-hydroxyecdsone [13,14]. 20-Hydroxyecdysone induces the expression of two nuclear receptors E75 and HR3 [15]. The two nuclear receptors, E75 and HR3, can spontaneously dimerize, with E75 negatively regulating HR3's action as a transcription factor for downstream genes [16]. E75 is a thiolate hemeprotein and has been shown to be responsive to nitric oxide [17,18]. We propose that at the appropriate time in the cycle, nitric oxide synthase produces nitric oxide which binds to E75, releasing HR3 from E75 [18–20]. Free HR3 then regulates genes involved in orchestrating the synthesis of the next pulse of 20-hydroxyecdysone which denotes the end of one cycle and the beginning of the next. Nitric oxide, we propose, regulates the duration of each cycle within the molt rhythm through its manipulation of E75's function to ensure that the intermolt duration is timed appropriately to ensure proper development of embryos in the brood chamber. We tested aspects of this hypothesis in the present study by evaluating the sequence of expression of putative genes involved in regulating the molt rhythm and the ability of the nitric oxide donor sodium nitroprusside to alter this rhythm. We also investigated whether the targeted suppression of E75 using RNAi would elicit molecular and physiological consequences consistent the action of the nitric oxide donor.

Materials and methods

Daphnid cultures

D. magna used in these experiments were derived from cultures maintained at North Carolina State University. Cultures were housed in incubators at 20˚C and 16:8 hour light:dark cycle as described previously [21]. These conditions favor parthenogenetic reproduction over sexual reproduction by this species. Daphnids were housed in 1 L beakers and fed $1.4x10^7$ cells of green algae (*Raphidocelis subcapitata*) and ~4 mg dry weight of Tetrafin ® (Tetra, Spectrum Brands, Blacksburg, VA) fish food suspension twice daily. Fish food suspension was prepared
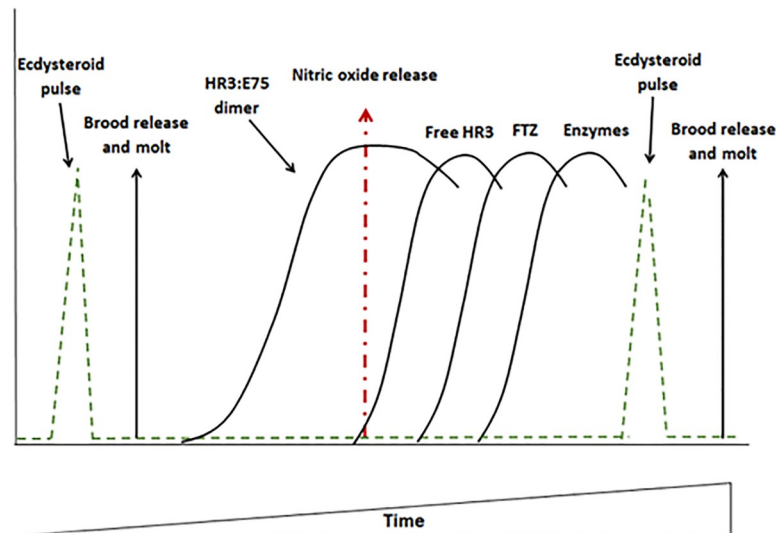
Fig 1. Proposed model for the regulation of the molt cycle duration by the sequential expression of nuclear receptors, nitric oxide, and enzymes involved in 20-hydroxyecdysone synthesis and inactivation in _Daphnia magna_. The pulse of 20-hydroxyecdysone at the initiation of the molt cycle induces transcription of the _E75_ and _HR3_ genes. Resulting HR3 and E75 proteins dimerize and progression of the cycle ceases. Nitric oxide is produced in response to environmental cues (nutrition, temperature, etc.) that influence the required time for embryo development during the molt cycle. The nitric oxide binds to E75 causing the release of HR3 protein and progression of the cycle continues. Free HR3 protein then initiates the transcription of the _FTZ-F1_ gene and resulting FTZ-F1 protein initiates the transcription of enzymes responsible for the next 20-hydroxyecdysone pulse designating the end of the molt cycle and the initiation of the next cycle within the rhythm.

as described previously [22]. Daphnid experiments and cultures used daphnid media composed of deionized water reconstituted with 192 mg/L $CaSO_4 \cdot H2O$, 192 mg/L $NaHCO_3$, 120 mg/L $MgSO_4$, 8.0 mg/L KCl, 1.0 µg/L selenium, and 1.0 µg/L vitamin B_{12}.

Gene expression during the molt cycle

Daphnids (<24 hours old) were reared in 1L beakers for 9 days and fed as described for cultures. On day 9, individual daphnids were placed into individual 50 mL beakers with 40 mL daphnid media and fed $1.4x10^6$ cells of green algae (_R. subcapitata_) and ~0.4 mg dry weight of fish food suspension daily. Beginning on day 10, beaker contents were evaluated every two hours for the presence of a molted exoskeleton. The time at which the exoskeleton was observed was designated as time zero for that individual and the daphnid was randomly assigned to a time point within the molt cycle at which it would be collected. Collection times occurred at 0, 12, 36, 48, 60, 72, 78, and 84 hours post-molt. Five daphnids from the same treatment and collected at the same time point were stored together in 100 µL RNAlater (Invitrogen, Carlsbad, CA) in a 1.7 mL tube, representing one sample. Three replicate samples were collected per time point for a total of 15 daphnids per time point. Samples were held for 24 hours at 4˚C and then stored at -80˚C until used for RNA extraction. The duration of the molt cycle was also determined in a group of daphnids not sampled for mRNA analyses by assessing the numbers of hours between one molt and the next.

Sodium nitroprusside treatment

Experiments were performed to disrupt the timing and duration of the putative pulse of nitric responsible for the dissociation of E75 and HR3. Daphnids were exposed to 12 µM of the nitric

oxide donor, sodium nitroprusside, for 8 hrs beginning at 36 hrs post molt. This dosing regimen approximated the maximum exposure that had no discernible effect on survival or overall behavior of the organisms. According to the proposed model, the pulse of nitric oxide would normally occur after the induction of HR3. Thus, the sodium nitroprusside treatment would cause a premature and prolonged pulse of nitric oxide within the organisms resulting in the excess accumulation of free HR3. Animals were collected and analyzed for gene expression and duration of the molt cycle as described above.

mRNA preparation and analyses

Frozen daphnids were homogenized with a bullet blender (Next Advance, Inc, Troy, NY) then RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI). RNA concentration was measured by absorbance at 260 nm and purity was confirmed by the 260/280 nm absorbance ratio (~2.0). cDNA was prepared using ImProm-II ™ Reverse Transcription System (Promega) with oligo (dT) primers.

Targeted mRNAs were measured using quantitative RT-PCR. Primer preparation for *D. magna Actin*, *GapDH*, *E75*, *HR3*, and *FTZ-F1* have been described previously [15,23]. Primers for *D. magna Neverland 2*, *CYP314a1*, and *CYP18a1* were derived from the literature [24] and primers *CYP307a1*, *CYP306a1*, *CYP302a1*, and *CYP315a1* were designed based upon sequences available in wFleaBase (http://wfleabase.org/) (Table 1). The sequences were amplified with an iCycler Thermal Cycler (Bio-Rad, Hercules, CA) using 2X PCR Mastermix (Promega) at 95°C for 1 min, followed by 35 cycles with each cycle consisting of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C. The amplified DNA fragments were cloned into the pCR pGEM-T Easy vector (Promega) or 4-TOPO vector (Invitrogen, Carlsbad, CA) following the manufacture's protocol. Plasmid DNA was sequenced by Eton Bioscience Inc. (San Diego, CA) to confirm that the primers were indeed targeting the gene of interest.

PCR was performed, following the manufacturers protocol, using the ABI PRISM® 700 Sequence Detection System with iTaq Universal Sybr Green Supermix (BioRad, Hercules, CA)

Table 1. Oligonucleotide primers used to amplify indicated gene products by quantitative real-time PCR.

Gene	Forward	T_m	Reverse	T_m	Amplicon (bp)	GenBank Accession or wFleabase scaffold
β-actin	5′ TGGTCAGGTCATCACCATTG	54.8	5′ CTCGTGGATACCGCAAGATT	54.8	97	AJ292554
GapDH	5′ GGCAAGCTAGTTGTCAATGG	54.1	5′ TATTCAGCTCGAGCAGTTCC	54.5	89	AJ292555
E75	5′ TCCGGAGAAGTATTCAACAAAAGA	54.1	5′ TGCGAAGAATGGAGCACTGT	57.0	72	EF369510.1
HR3	5′ AGTCATCACCTGCGAGGGC	60.0	5′ GAACTTTGCGACCGCCG	57.5	51	FJ755466
FTZ-F1[a]	5′ ATCGTGCAAGGGATTCTTCA	54.2	5′ ATCAGCGACGCAAGAATAGG	55.0	63	LC105701
CYP18a1	5′ TACCCGATCGTCGGTTACCT	57.2	5′ GAGCGCCGTCAGCTCTTC	58.8	63	AB839173
Neverland 2	5′ CGTCGGTGACTGCATCGA	57.5	5′ TGCCGTCGTTCCCATTG	55.8	59	AB839172
CYP307a1 (*spook*)	5′ CAGGGCTATGCTGTCGATTT	55.1	5′ CTCGTCGATGATCGTCTTGA	54.1	120	Scaffold02011
CYP306a1 (*phantom*)	5′ CGGACTGGAACGGAGAAAATGGTCCGGTT	65.2	5′ AATCTCGATCCGACCTTGTGGAGCGATCCG	65.7	152	Scaffold03376
CYP302a1 (*disembodied*)	5′ AATTCAGCTGGTGAGGCAGTTCCGTATCGA	64.2	5′ TGTTCGCTCGATGAAATTGAATTTCAATGG	58.4	155	Scaffold01863
CYP315a1 (*shadow*)	5′ CAACCTCGCCACTGATTCTT	55.2	5′ CCAACAGGGAAGCATGAAAT	53.3	121	Scaffold00687
CYP314a1 (*shade*)	5′ GACTGCTGAAGGCGTTGACA	57.9	5′ CGGCTGCCACTAGGTCGATA	58.9	62	AB257771.1

[a]The primers amplified both splice variants of *FTZ-F1*.

https://doi.org/10.1371/journal.pone.0221642.t001

in 96-well plates (Olympus Plastics, Genesee Scientific, SanDiego, CA) sealed with Thermal-Seal (Excel Scientific, Inc., Victorville, CA). mRNA levels were calculated from the raw data using GenEx software (Applied Biosystems) where the geometric mean of the Actin and GAPDH gene products were used for normalization [25]. The melting temperature of PCR products were 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. Amplification efficiencies were >85%.

dsRNA preparation

Oligonucleotide primers were designed to produce dsRNA targeting *D. magna* E75. Primers (forward: 5': TACTAATCTAGAGCGATGTCGCTATCCTCGGG, reverse: 5': TTAGTAGG TACCTTTGGTGGTTATAGTTCCAG) were synthesized (Integrated DNA Technologies, Inc. (Coralville, IA)) flanked by the XbaI and KpnI restriction enzyme sites. Each primer was amplified with the restriction enzyme sites according to the Phusion Hot Start II High-Fidelity PCR Master Mix protocol (ThermoFisher Scientific, Waltham, MA). The amplified target sequence (GCGATGTCGCTATCCTCGGGGGTCGTCAGTTCATTGACGTCGACCCATTCAACG CTGGCCCGCTCTCTGATGGAAGGCCCTAAAATGGTTAGCACTGAACAGCAGCGCCGCGCCG ATCTCATCGTCGCCAACATAATGAAGGGCAATGTTTCGGCATCGTCACCCACTCCTTCCTC ATCATCGTCCGGCCAAAATTATTCAATGTCTCCGTCACCTCATTCCAGCAACAACGGACAAC AGCAGAGGACCGTTCTGACGTCTGGTCCACTCTATGTCGGCTCACCCGCGCCATCTGGCAGT AGCAGCTGGAACTATAACCACCAAA) was double digested and ligated with the L4440 plasmid vector, a gift from Dr. Andrew Fire (Addgene plasmid #1654). The L4440 construct was then transformed into GC5 cells and plated on LB plates containing ampicillin (100 μg/mL, Sigma-Aldrich Corp., St. Louis, MO) and tetracycline (12.5 μg/mL, Sigma-Aldrich). Individual colonies were selected, and PCR was performed to identify inserts where ligation into the vector was successful. Successfully ligated inserts were sequenced (Eton Bioscience, San Diego, CA), and constructs were transformed into competent HT115 (DE3) cells obtained from the *Caenorhabditis* Genetics Center (funded by NIH Office of Research Infrastructure Programs (P40 Od010440)). Following transformation of HT115 cells containing the construct, stocks were prepared by growing the bacterial cells to OD_{595} = 0.040 (2.84×10^8 cells/mL) and freezing with 25% glycerol at -80°C.

The transformed cells were grown overnight in LB medium containing ampicillin and tetracycline for use in E75 RNAi experiments. Isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich) was added at a concentration of 2.0 mM to induce the T7 RNA polymerase and subsequent production of dsRNA of the target sequence. The concentration of transformed bacterial cells was determined by measuring the culture's optical density at 595 nm [26]. Cells were concentrated by centrifuge at room temperature for 10 minutes at 3000 rpm, LB medium was decanted, and cells were resuspended in daphnid medium. The concentration of cells was determined according to a standard curve, and the volume required to feed 7.2×10^7 cells/100 mL of medium was calculated.

Suppression of E75 via RNAi

Daphnids (< 24 hours old) were used to initiate experiments aimed at the suppression of E75. Individual daphnids were isolated in 40 mL of media and fed 2.88×10^7 cells of *E. coli* containing either the empty L4440 vector (no dsRNA insert) or containing the L4440 vector with the insert targeting *E75*. Daphnids were also provided 3.5×10^5 cells of green algae (*R. subcapitata*) as a nutrition source. Daphnids were evaluated every two hours for the presence of a molted exoskeleton beginning fourteen days after initiation of feeding the daphnids *E. coli*. The first

observed molt was designated time zero. Daphnids were then continually evaluated until their second molt in order to determine the length of the molt cycle, the number of offspring released, and the presence of developmental abnormalities among neonates.

Some daphnids were collected and stored in RNAlater after 14 days of feeding for mRNA preparation and analyses as described above. Three groups, with 3 daphnids per group, were sampled. Previous use of this method of mRNA suppression revealed that a minimum of 14 days of feeding *E. coli* containing dsRNA provided maximum suppression of targeted mRNA [26].

Statistical analyses

Statistical comparisons were made using Student's t tests upon establishing normality of the distributions (D'Agostino & Pearson and Shapiro-Wilk normality tests). Linear regressions were used to evaluate correlations between *FTZ-F1* and individual enzyme mRNA levels. All statistical analyses were performed using GraphPad Software (GraphPad Prism, La Jolla, CA) at an alpha level of 0.05.

Results

Gene expression cascade during the molt cycle

The temporal accumulation of mRNAs hypothesized to be involved in establishing the duration of the molt cycle was evaluated. *E75* mRNA levels attained maximum accumulation at 48 hours post-molt (Fig 2A). *HR3* mRNA levels peaked at 60 hours post-molt (Fig 2B). Maximum *FTZ-F1* mRNA levels were attained at 72 hours post-molt (Fig 2C). These results support our hypothesis that the mRNA accumulation of these nuclear receptors occurs sequentially over the molt cycle.

Next, we evaluated the mRNA accumulation profiles of the genes that contribute to the synthesis and inactivation of 20-hydroxyecdysone that ends one cycle of the molt rhythm and initiates the next. We hypothesized that the ecdysteroid synthesizing and inactivating genes were regulated by FTZ-F1 and thus would be expressed following the elevation of *FTZ-F1* mRNA levels. *Neverland 2* (Fig 2D) and *CYP307a1* (Fig 2E), the first two gene products, involved in the synthesis cascade, attained maximum accumulation at 48 hours post-molt. All subsequence mRNAs coding for biosynthetic enzymes were constitutively expressed with little evidence of increased expression during the molt cycle (Fig 2F–2I). *CYP18a1*, which is responsible for the inactivation/elimination of 20-hydroxyecdysone, mRNA levels peaked at 72 hours post-molt.

In summary, mRNA levels of the enzymes involved in 20-hydroxyecdysone synthesis did not increase after the rise in *FTZ-F1* mRNA levels indicating that these enzymes are not regulated by FTZ-F1. Rather, enzymes responsible for the initial steps in 20-hydroxyecdysone system appear to be regulated by some factor early in the cascade. Enzymes operative in the subsequent steps of 20-hydroxyecdysone synthesis appear to be constitutively expressed throughout the molt cycle. *Cyp18a1*, which is responsible for the inactivation of 20-hydroxyecdysone, mRNA levels were level commensurate with the rise in *FTZ-F1* mRNA levels suggesting that these components of the cascade may be co-regulated.

Modulation of the signaling cascade by sodium nitroprusside

We hypothesized that nitric oxide, delivered by sodium nitroprusside, would cause the excess and prolonged release of HR3 from E75. This free HR3 then would elevate and prolong the expression of FTZ-F1 along with enzymes that are regulated by either HR3 or FTZ-F1.

Treatment with 12 μM sodium nitroprusside had no effect on the timing of the accumulation of *E75* and *HR3* mRNAs, although the magnitude of accumulation of both mRNAs was
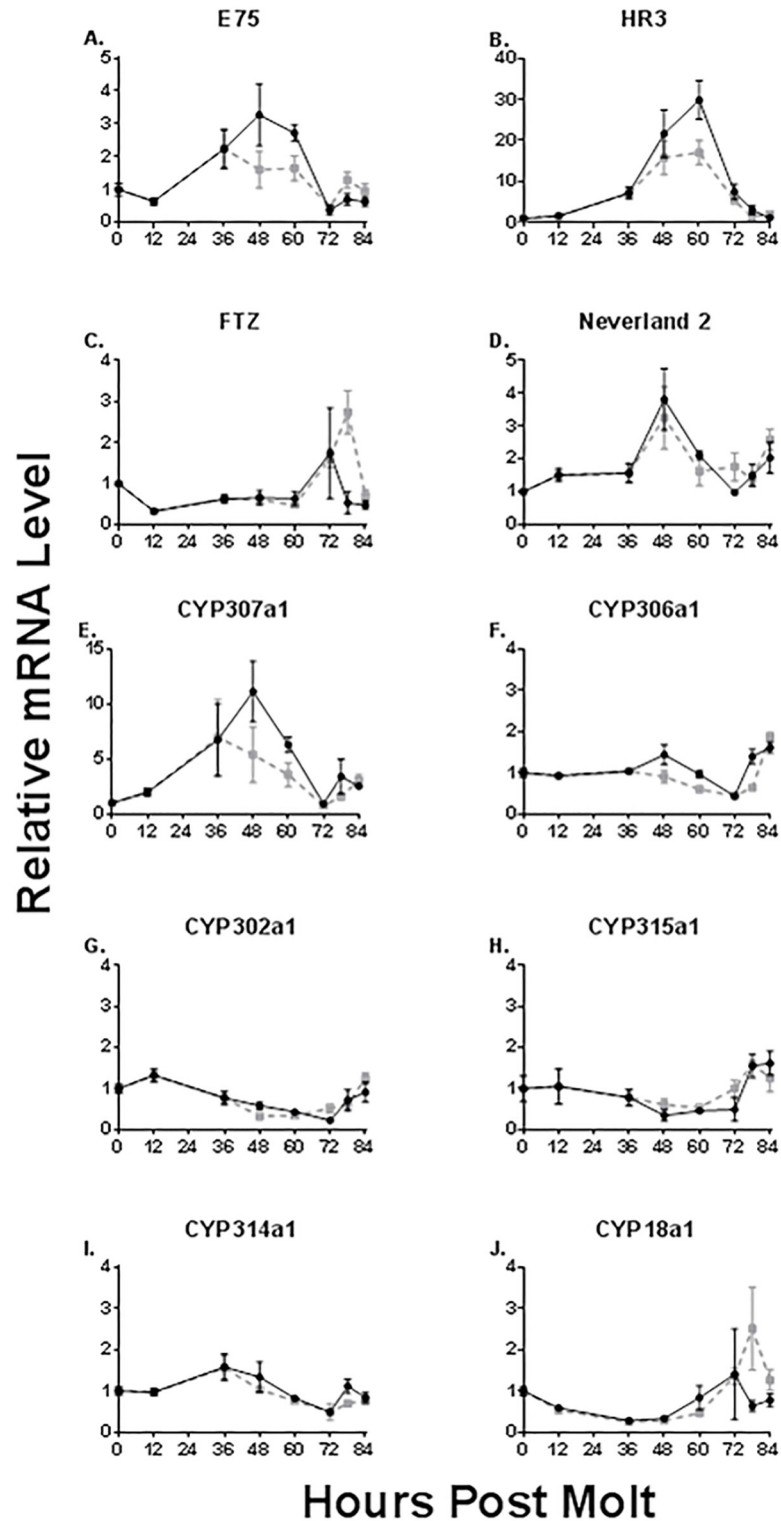
Fig 2. mRNA levels of relevant genes expressed during the molt cycle. Presented are temporal levels of mRNAs for *E75* (A), *HR3* (B), *FTZ-F1* (C), *Neverland 2* (D), *CYP307a1* (E), *CYP306a1* (F), *CYP302a1* (G), *CYP315a1* (H), *CYP314a1* (I) and, *CYP18a1* (J) during the molt cycle. Black solid lines represent control animals and gray dashed lines represent sodium nitroprusside-treated animals. Data are presented as mean and standard error (n = 3, with each sample consisting of mRNA from five daphnids). Raw values were normalized to their respective 0-hour value.

https://doi.org/10.1371/journal.pone.0221642.g002

reduced (Fig 2A and 2B). Sodium nitroprusside treatment extended the accumulation of *FTZ-F1* mRNA ultimately resulting in an increase in mRNA level, as compared to the maximum accumulation in untreated daphnids, and a delay in maximum accumulation by approximately 6 hours (Fig 2C). Sodium nitroprusside had little effect on the timing or magnitude of mRNA accumulation for the enzymes involved in 20-hydroxyecdysone synthesis (Fig 2D–2I). Similar to *FTZ-F1*, *CYP18a1* mRNA accumulated to greater levels with sodium nitroprusside treatment and the timing of peak accumulation was delayed by approximately 6 hours (Fig 2J).

The relationship between mRNA levels for FTZ-F1 and individual enzymes involved in the synthesis and inactivation of 20-hydroxyecdysone during the molt cycle were evaluated in an effort to discern which, if any, of the enzymes might be regulated or co-regulated with FTZ-F1 (Fig 3). Among the gene products evaluated, only *CYP18a1* mRNA levels significantly
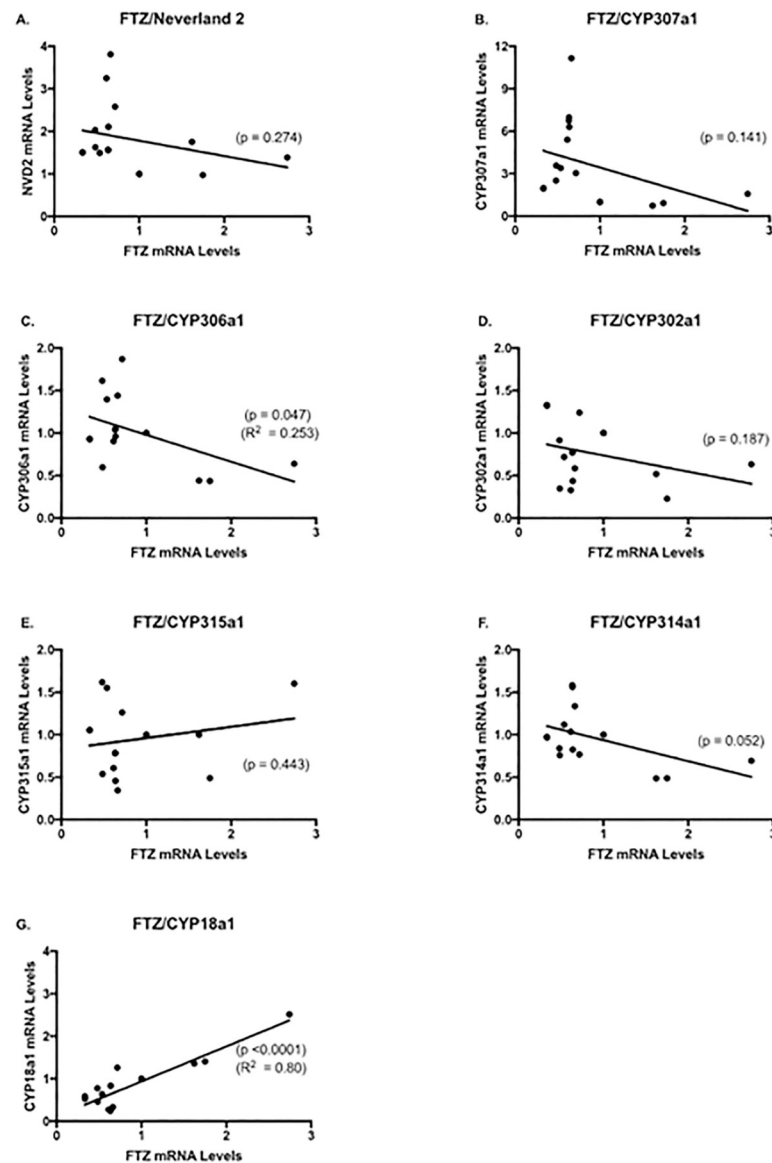

Fig 3. Correlation of mRNA levels for *FTZ-F1* and individual enzymes involved in the synthesis and inactivation of 20-hydroxyecdysone. Each data point represents relative mRNA levels from daphnids sampled at the same point during the molt cycle and from the same treatment (mean values, n = 3).

https://doi.org/10.1371/journal.pone.0221642.g003

(p<0.0001) positively correlated with *FTZ-F1* mRNA levels over the molt cycle (Fig 3G). These results support the assertion that only CYP18a1 is regulated by the E75/HR3/FTZ-F1 signaling pathway.

Impact of sodium nitroprusside on the timing of exuviation

Shedding of the exoskeleton is the ultimate event in the molt cycle. Delays in the pulse of mRNA accumulation of *FTZ-F1* and *CYP18a1* should similarly extend the time to exuviation if these molecular events are indeed components of the molecular clock that dictates the timing of the molt rhythm. Control daphnids molted approximately 75 hours after the previous molt (Fig 4). Molting among daphnids treated with sodium nitroprusside was delayed by ~6 hours. This delay is consistent with the delay in the peak accumulation of *FTZ-F1* and *CYP18a1* mRNA.

Suppression of E75 using RNAi

Results of experiments with sodium nitroprusside suggests that E75 is a critical determinant of the molt cycle duration. However, this conclusion is equivocal since sodium nitroprusside could be interacting with other molecular targets. Therefore, we sought to specifically inhibit E75 levels, and accordingly E75 activity, using RNAi. We hypothesized that the suppression of E75 would mimic the molecular and apical effects observed with sodium nitroprusside. Levels of *E75* mRNA were suppressed ~50% after 14 days of feeding *E. coli* containing E75 dsRNA (Fig 5A). Treatment with E75 dsRNA had no effect on *HR3* mRNA levels (Fig 5B). *FTZ-F1* mRNA levels increased after treatment with the E75 dsRNA (Fig 5C). This increase was consistent with the reduction of E75 protein levels causing an increase in free HR3 protein which elevated expression of FTZ-F1. *CYP18a1* mRNA levels were significantly reduced following treatment with E75 dsRNA (Fig 5D). This decrease was unexpected and may indicate that *CYP18a1* mRNA levels accumulate after FTZ-F1, where, at sampling, levels of *CYP18a1* mRNA had peaked among control daphnids but not yet in daphnids treated with E75 dsRNA.
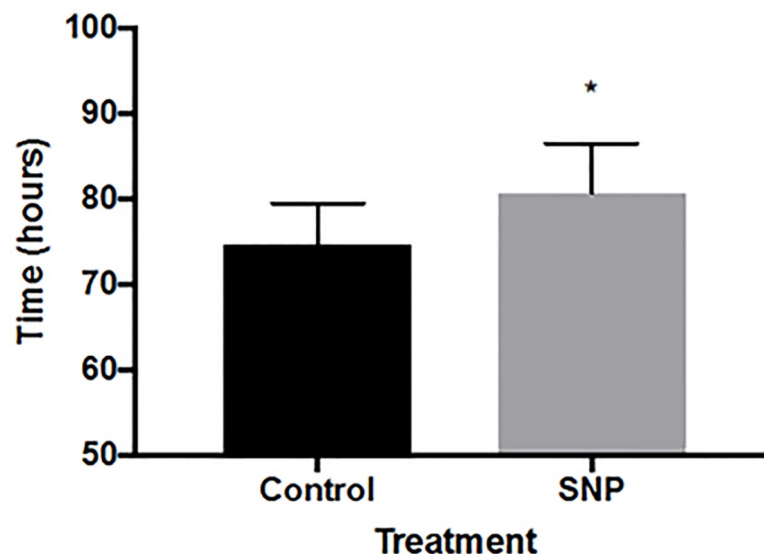

Fig 4. The influence of sodium nitroprusside on the duration of the molt cycle. The black bar represents control animals and the gray bar represents sodium nitroprusside (SNP)-treated animals. Data are presented as mean and standard error, n = 15 daphnids. An asterisk denotes a significant difference between control and sodium nitroprusside treated animals (p ≤ 0.05).
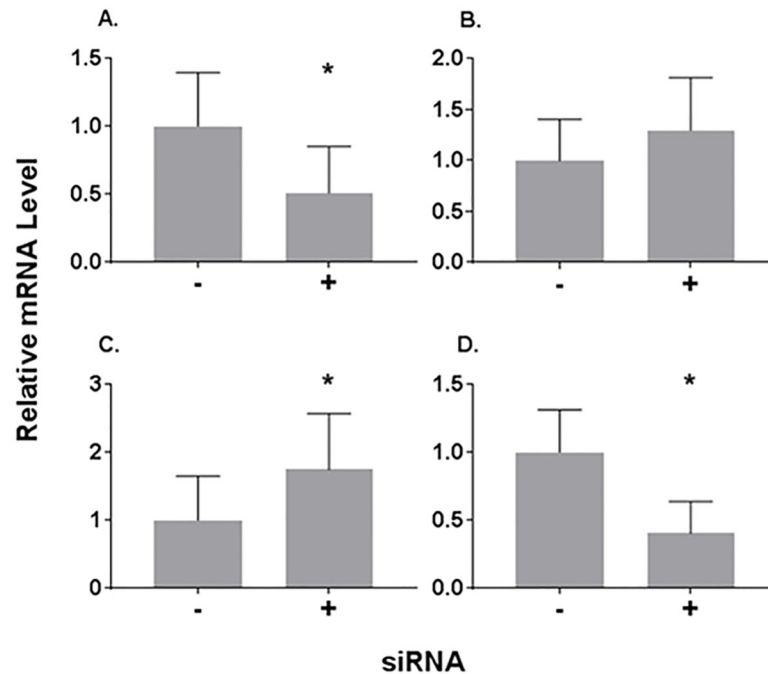
Fig 5. *E75*, *HR3*, *FTZ-F1*, *CYP18a1* mRNA levels with E75 dsRNA feeding. (A) *E75*, (B) *HR3*, (C) *FTZ-F1*, and (D) *CYP18a1*. Animals were sampled for mRNA analyses after 14 days of vector provision (empty vector for control, vector containing insert targeting *E75* for RNAi treatment). Sampling occurred at 36 hrs post molt. Data are presented as mean relative mRNA levels (n = 16). Error bars represent the standard errors. Significant difference (p<0.05) between treatment and control is indicated with an asterisk.

Apical effects of E75 suppression using RNAi

The targeted suppression of E75 significantly delayed molting (Fig 6A), further implicating E75 in the regulation of the daphnid molt cycle. E75 also significantly reduced the number of offspring produced by treated daphnids (Fig 6B). A low incidence of developmental abnormalities was observed in both control and E75-suppressed daphnids (Fig 6C) with no statistical significance associated with treatment.

Discussion

Two major objectives of this study were to investigate whether gene products shown to be important in the regulation of the molt cycle in insects might also be operative in daphnids, and to investigate whether the modulation of E75 activity might serve to dictate the duration of the molt cycle.

In insects, E75 is induced by the rise in 20-hydroxyecdysone levels that occur during the last half of the molt cycle [27,28,29]. Following the induction of E75 and as ecdysteroid levels peak, HR3 production commences [27,30]. Newly produced HR3 binds to E75 protein resident in the cell. This binding to E75 prevents HR3 from acting as a transcription factor [18,20].

We previously demonstrated that HR3 is induced by exogenous 20-hydroxyecdysone administration in daphnids [15]. Herein, we demonstrated that *E75* and *HR3* mRNA levels sequentially accumulate in daphnids during the molt cycle, consistent with the expression profile of insects.

In insects, FTZ-F1 is induced by HR3 and suppressed by 20-hydroxyecdysone, thus FTZ-F1 is maximally expressed at the 20-hydroxyecdysone nadir just prior to the pulse that
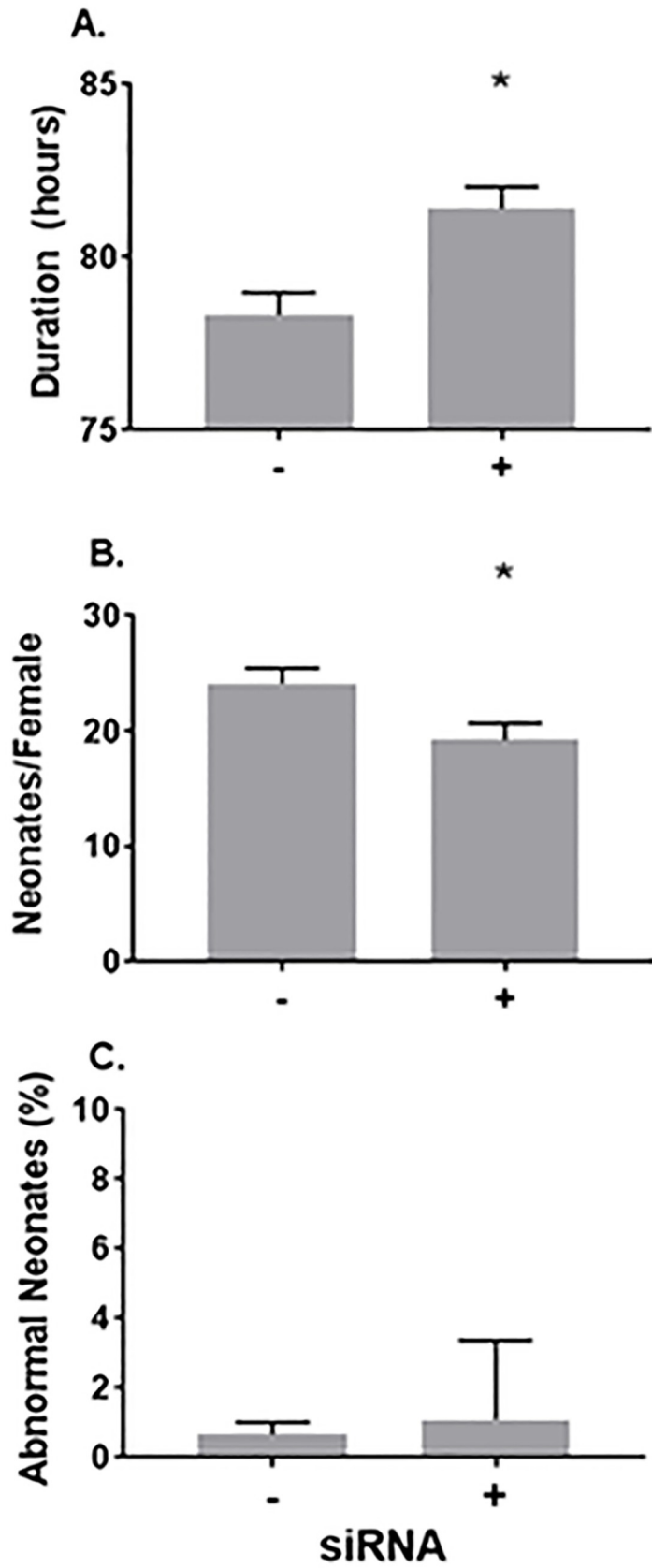
Fig 6. Impact of E75 suppression on (A) the duration of the molt cycle, (B) fecundity of maternal daphnids, and (C) incidence of developmental abnormalities among offspring. (A) Data are presented as the mean and standard error (n = 12 individual daphnids) duration of the molt cycle. (B) Data are presented as the mean and standard error (n = 19–20 individual daphnids) number of neonates per female in the first two broods. (C) Data are presented as the mean and standard error (n = 19–20 individual daphnids) percentage of abnormal neonates per maternal daphnid, determined from the same broods reported in figure B.

https://doi.org/10.1371/journal.pone.0221642.g006

regulates metamorphosis [29,30,31]. FTZ-F1 expression is considered necessary to orchestrate the varied activities related to tissue-specific metamorphosis and eclosion [32]. In daphnids, *FTZ-F1* mRNA levels are elevated following the peak in *HR3* expression suggesting positive regulation by free HR3 protein. Ecdysteroid levels were not measured in the present study. Nonetheless, results suggest that FTZ-F1 is regulated in daphnids in a manner similar to that in insects.

In insects, the functionality of HR3, as a transcription factor, is suppressed by its binding to E75 [18,33]. Nitric oxide can bind to the heme moiety of E75 causing the release of HR3 [18]. HR3 can then function to regulate the expression of downstream gene involved in ecdysis including FTZ-F1 [29,30]. Similar interactions have been demonstrated among nitric oxide and the human orthologs of E75 and HR3, rev-erb and ROR, respectively [20]. We previously demonstrated that daphnid E75 is capable of suppressing the ability of HR3 to function as a transcriptional activator of a reporter gene [16]. This suppression was not the result of competition between E75 and HR3 at the response element of the regulated gene suggesting that E75 protein binds and suppresses HR3 in daphnids as observed in insects and humans.

We posited that exposure of daphnids to the nitric oxide donor, sodium nitroprusside, would block E75's ability to bind HR3 causing a prolonged accumulation of free HR3 protein in cells. If FTZ-F1 is positively regulated by HR3, as reported in *Drosophila* [34], then we anticipated the over accumulation of *FTZ-F1* mRNA. Indeed, sodium nitroprusside caused the prolonged and increased accumulation of *FTZ-F1* resulting in a delay in its peak accumulation and a commensurate delay in molting. Similarly, provision of sodium nitroprusside to *Drosophila* delayed pupation [35]. While these results are inadequate to conclude that nitric oxide regulates the availability of free HR3 and that free HR3 regulates the expression of FTZ-F1 in daphnids, results are consistent with this hypothesis.

Maximum accumulation of *FTZ-F1* and *CYP18a1* mRNA routinely co-occurred in our experiments suggesting that these two components of the ecdysteroid signaling pathway are tightly co-regulated. In insects, *CYP18a1* is induced by the increased in 20-hydroxyecdysone levels [36]. This enzyme then functions in the inactivation of the hormone resulting the in 20-hydroxyecdysone pulse that is required for complete, successful ecdysis [37]. Daphnids similarly require the pulse of 20-hydroxyecdysone to successfully molt [38]. However, the molt cycle of daphnids is of short duration (~1–2 days in juveniles, ~3–4 days in adults) which may require novel regulation of factors involved in ecdysis. The timing of *CYP18a1* mRNA accumulation and subsequent ecdysis suggested that *CYP18a1* mRNA is expressed just prior to peak 20-hydroxyecdysone accumulation. This may be necessary to provide adequate CYP18a1 protein sufficiently early to complete the hormone pulse within the requisite time period. Thus, CYP18a1 expression may be regulated by HR3, FTZ, or some other late expression transcription factor within the ecdysteroid signaling pathway.

Importantly, enhanced accumulation of *FTZ-F1*, either through treatment with sodium nitroprusside or the suppression of E75 using RNAi, significantly lengthened the duration of the molt cycle. The molt cycle in daphnids is highly coordinated with reproduction as brood release occurs just prior to ecdysis and deposition of a new brood of embryos into the brood chamber occurs following ecdysis. Accordingly, the duration of the molt cycle must be

precisely timed for the embryos to fully develop. The time required for embryos to develop can be influenced by environmental factors such as temperature [39] and light [40], thus the duration of the molt cycle must be adjusted by these factors. We proposed that the molt cycle is in a state of stasis as long as HR3 is bound to E75 and that the production of nitric oxide frees HR3 allowing the progression of the cycle to completion. Under this scenario, nitric oxide might be produced in response to environmental cues to ensure that cycle is of the appropriate duration. A molt cycle timer that is related to environmental cues has been proposed for insects [41] and nitric oxide has been proposed as a regulator of the timing of development in *Drosophila* larvae via its interaction with E75 [33]. Nitric oxide has been shown to be produced by insects in response to environmental stimuli [42].

Humans have altered the global nitrogen cycle [43,44]. The production of nitrogen-based fertilizers have significantly increased the level of reactive nitrogen as ammonia, nitrate, and nitrite. These molecules can be converted to nitric oxide through various oxidation and reduction reactions [44,45]. The binding of nitric oxide to E75 can disrupt the molt cycle of arthropods resulting in various physiological perturbations, such as growth and reproductive disturbances, that would adversely impact population sustainability. Further, the human E75 ortholog, rev-erb, has been considered as a target of drugs with the goal of adjusting circadian rhythms such as the sleep cycle [46]. The presence of such compounds in the environment, from industrial or domestic sewage discharge, at sufficient concentrations could disrupt the molt cycle of arthropods in a manner similar to nitric oxide. Diligence is warranted to ensure that such disruptions do not become a reality.

Supporting information

S1 Dataset. Supporting dataset for Figs 2–6.
(PDF)

Acknowledgments

Author Contributions

Conceptualization: Gerald A. LeBlanc.

Data curation: Stephanie M. Street, Stephanie A. Eytcheson, Gerald A. LeBlanc.

Formal analysis: Stephanie M. Street, Stephanie A. Eytcheson.

Funding acquisition: Gerald A. LeBlanc.

Investigation: Stephanie M. Street, Stephanie A. Eytcheson.

Resources: Gerald A. LeBlanc.

Supervision: Gerald A. LeBlanc.

Validation: Gerald A. LeBlanc.

Writing – original draft: Stephanie M. Street, Stephanie A. Eytcheson.

Writing – review & editing: Gerald A. LeBlanc.

References

1. Reinberg A, Ashkenazi I. Concepts in human biological rhythms. Dialogues Clin Neurosci. Les Laboratoires Servier. 2003; 5:327–42. PMID: 22033796

2. American Academy of Pediatrics Committee on Adolescence, American College of Obstericians and Gynecologists Committee on Adolescent Heatlh Care. Menstruation in girls and adolescents: Using the menstrual cycle as a vital sign. Pediatrics. 2006; 118:2245–50. https://doi.org/10.1542/peds.2006-2481 PMID: 17079600

3. Dingle H, Alistair Drake V. What is migration? Bioscience. 2007; 57:113–21.

4. Lloyd M, Dybas HS. The periodical cicada problem. I. Population Ecology. Evolution. 1996; 20:133–49.

5. Eskin A. Identification and physiology of circadian pacemakers. Fed Proc. 1979; 38:2570–2. PMID: 499572

6. Takahashi JS, DeCoursey PJ, Bauman L, Menaker M. Spectral sensitivity of a novel photoreceptive system mediating entrainment of mammalian circadian rhythms. Nature. 1984; 308:186–8. https://doi.org/10.1038/308186a0 PMID: 6700721

7. Paul MJ, Zucker I, Schwartz WJ. Tracking the seasons: the internal calendars of vertebrates. Phil Trans R Soc B. 2008; https://doi.org/10.1098/rstb.2007.2143 PMID: 17686736

8. Colbourne JK, Pfrender ME, Gilbert D, Thomas WK, Tucker A, Oakley TH, et al. The ecoresponsive genome of *Daphnia pulex*. Science. 2011; 331(6017):555–61. https://doi.org/10.1126/science.1197761 PMID: 21292972

9. Aiken DE. Photoperiod, endocrinology and the crustacean molt cycle. Science. 1969; 164:149–55. https://doi.org/10.1126/science.164.3876.149 PMID: 5774187

10. Franke HD, Beermann J. The influence of intrinsic and extrinsic factors on developmental parameters and their relationships in the marine isopod *Idotea linearis* (Crustacea). Hydrobiologia. 2014; 732:197–212.

11. Supriya NT, Sudha K, Krishnakumar V, Anilkumar G. Molt and reproduction enhancement together with hemolymph ecdysteroid elevation under eyestalk ablation in the female fiddler crab, *Uca triangularis* (Brachyura: Decapoda). Chinese J Oceanol Limnol. 2017; 35:645–57.

12. Diez MJ, Lovrich GA. Moult cycle and growth of the crab *Halicarcinus planatus* (Brachyura, Hymenosomatidae) in the Beagle Channel, southern tip of South America. Helgol Mar Res. 2013; 67:555–66.

13. Mu X, LeBlanc GA. Synergistic interaction of endocrine-disrupting chemicals: Model development using an ecdysone receptor antagonist and a hormone synthesis inhibitor. Environ Toxicol Chem. 2004; 23:1085. PMID: 15095909

14. Martin-Creuzburg D, Westerlund SA, Hoffmann KH. Ecdysteroid levels in *Daphnia magna* during a molt cycle: Determination by radioimmunoassay (RIA) and liquid chromatography–mass spectrometry (LC–MS). Gen Comp Endocrinol. 2007; 151:66–71. https://doi.org/10.1016/j.ygcen.2006.11.015 PMID: 17222840

15. Hannas BR, LeBlanc GA. Expression and ecdysteroid responsiveness of the nuclear receptors HR3 and E75 in the crustacean *Daphnia magna*. Mol Cell Endocrinol. 2010; 315:208–18. https://doi.org/10.1016/j.mce.2009.07.013 PMID: 19631716

16. Hannas BR, Wang YH, Baldwin WS, Li Y, Wallace AD, LeBlanc GA. Interactions of the crustacean nuclear receptors HR3 and E75 in the regulation of gene transcription. Gen Comp Endocrinol. 2010; 167:268–78. https://doi.org/10.1016/j.ygcen.2010.03.025 PMID: 20346363

17. De Rosny E, De Groot A, Jullian-Binard C, Gaillard J, Borel F, Pebay-Peyroula E, et al. *Drosophila* nuclear receptor E75 is a thiolate hemoprotein. Biochemistry. 2006; 45:9727–34. https://doi.org/10.1021/bi060537a PMID: 16893174

18. Reinking J, Lam MMS, Pardee K, Sampson HM, Liu S, Yang P, et al. The *Drosophila* nuclear receptor E75 contains heme and is gas responsive. Cell. 2005; 122:195–207. https://doi.org/10.1016/j.cell.2005.07.005 PMID: 16051145

19. Forman BM, Chen J, Blumberg B, Kliewer SA, Henshaw R, Ong ES, et al. Cross-talk among RORα1 and the Rev-erb family of orphan nuclear receptors. Mol Endocrinol. 1994; 8:1253–61. https://doi.org/10.1210/mend.8.9.7838158 PMID: 7838158

20. Pardee KI, Xu X, Reinking J, Schuetz A, Dong A, Liu S, et al. The structural basis of gas-responsive transcription by the human nuclear hormone receptor Rev-erbβ. PLoS Biology. 2009; 7:384–98.

21. Rider C V, Gorr TA, Olmstead AW, Wasilak BA, LeBlanc GA. Stress signaling: coregulation of hemoglobin and male sex determination through a terpenoid signaling pathway in a crustacean. J Exp Biol. 2005; 208:15–23. https://doi.org/10.1242/jeb.01343 PMID: 15601873

22. Parks LG, LeBlanc GA. Inhibition of steroid hormone biotransformation/elimination as a biomarker of pentachlorphenol chronic toxicity. Aquat Toxicol. 1996; 34:291–303.

23. Thomson SA, Baldwin WS, Wang YH, Kwon G, LeBlanc GA. Annotation, phylogenetics, and expression of the nuclear receptors in *Daphnia pulex*. BMC Genomics. 2009; 10:500. https://doi.org/10.1186/1471-2164-10-500 PMID: 19863811

24. Sumiya E, Ogino Y, Toyota K, Miyakawa H, Miyagawa S, Iguchi T. *Neverland* regulates embryonic moltings through the regulation of ecdysteroid synthesis in the water flea *Daphnia magna*, and may thus act as a target for chemical disruption of molting. J Appl Toxicol. 2016; https://doi.org/10.1002/jat.3306 PMID: 26898244

25. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002; https://doi.org/10.1186/gb-2002-3-7-research0034 PMID: 12184808

26. Eytcheson SA, LeBlanc GA. Hemoglobin levels modulate nitrite toxicity to *Daphnia magna*. Sci Rep. 2018; 8:7172. https://doi.org/10.1038/s41598-018-24087-7 PMID: 29739949

27. Thummel CS. From embryogenesis to metamorphosis: the regulation and function of *Drosophila* nuclear receptor superfamily members. Cell. 1995; 83:871–7. https://doi.org/10.1016/0092-8674(95)90203-1 PMID: 8521511

28. Thummel CS. Puffs and gene regulation: molecular insights into the *Drosophila* ecdysone regulatory hierarchy. BioEssays. 1990; 12:561–568. https://doi.org/10.1002/bies.950121202 PMID: 2127884

29. Riddiford LM, Hiruma K, Zhou X, Nelson CA. Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster*. Insect Biochem. Mol. Biol. 2003: 33:1327–1338. PMID: 14599504

30. Huet G, Ruiz C, Richards G. Sequential gene activation by ecdysone in *Drosophila melanogaster*. The hierarchical eqivalence of early and early-late gene. Development 1995; 121;1195–1204. PMID: 7743931

31. Song Y, Villeneuve DL, Toyota K, Iguchi T, Tollefsen KE. Ecdysone receptor agonism leading to lethal molting disruption in arthropods: review and deverse outcome pathway development. Environ. Sci. Tech. 2017; 51:4142–57.

32. Cho KH, Daubnerova L, Park Y, Zitnan D, Adams ME. Secretory competence in a gateway endocrine cell confered by the nuclear receptor beta FTZ-F1 enables stage-specific ecdysone responses hroughut development in *Drosophila*. Dev. Biol 2014: 385:253–262. https://doi.org/10.1016/j.ydbio.2013.11.003 PMID: 24247008

33. Cáceres L, Necakov AS, Schwartz C, Kimber S, Roberts IJH, Krause HM. Nitric oxide coordinates metabolism, growth, and development via the nuclear receptor E75. Genes Dev. 2011; 25:1476–85. https://doi.org/10.1101/gad.2064111 PMID: 21715559

34. Parvy J, Blais C, Bernard F, Warren J, Petryk A, Gilbert L, et al. A role for βFTZ-F1 in regulating ecdysteroid titers during post-embryonic development in *Drosophila melanogaster*. Dev Biol. 2005; 282:84–94. https://doi.org/10.1016/j.ydbio.2005.02.028 PMID: 15936331

35. Lozinsky O V., Lushchak O V., Storey JM, Storey KB, Lushchak VI. Sodium nitroprusside toxicity in *Drosophila melanogaster*: Delayed pupation, reduced adult emergence, and induced oxidative/nitrosative stress in eclosed flies. Arch Insect Biochem Physiol. 2012; 80:166–85. https://doi.org/10.1002/arch.21033 PMID: 22692768

36. Williams DR, Fisher MJ, Rees HH. Characterization of ecdysteroid 26-hydroxylase: an enzyme involved in molting hormone inactivation. Arch Biochem Biophys. 2000; 376:389–398. https://doi.org/10.1006/abbi.2000.1731 PMID: 10775427

37. Rewitz KF, Yamanaka N, O'Connor BO. Steroid hormone inactivation is required during the juenile-adult transition in *Drosophila*. Dev. Cell. 2010; 19:895–902. https://doi.org/10.1016/j.devcel.2010.10.021 PMID: 21145504

38. Sumiya E, Ogino Y, Miyakawa H, Hiruta C., Toyota K, Miyagawa S, Iguchi T. Roles of ecdysteroids for progression of reproductive cycle in the fresh water crustacean *Daphnia magna*. Frontiers Zool. 2014; 11:60–72.

39. Lennon JT, Smith VH Williams K.. Influence of temperature on exotic *Daphnia lumholtzi* and implication for invasion success. J. Plank. Res. 2001; 23:425–434.

40. Buikema AJ, Jr.. Some effects of light on the growth, molting, reproduction, and survival of the Cladoceran, *Daphnia pulex*. Hydrobiologia. 1973, 41:391–418.

41. Suzuki R, Koyama T, Hiruma K, Riddiford LM, Truman JW. A molt timer is involved in the metamorphic molt in *Manduca sexta* larvae. Proc. Nat. Acad. Sci. 2013; 110:12518–12525. https://doi.org/10.1073/pnas.1311405110 PMID: 23852731

42. Cayre M, Malaterre J, Scotto-Lomassese S, Holstein GR,Martinelli GP, Forni C, Nicolas S., Aouane A, Strambi D., Strambi A. A role for nitric oxide in sensory-induced neurogenesis in an adult insect brain.

Eur. J. Neurosci. 2005; 21:2893–2902. https://doi.org/10.1111/j.1460-9568.2005.04153.x PMID: 15978001

43. Galloway JN, Cowling EB Reactive nitrogen and the world: 200 years of change. Ambio 2002; 31: 64–71. https://doi.org/10.1579/0044-7447-31.2.64 PMID: 12078011

44. Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, et al. Human alteration of the global nitrogen cycle: Sources and consequences. Ecol Appl 1997; 7: 737–750.

45. Hannas BR, Das PC, Li H, LeBlanc GA. Intracellular conversion of environmental nitrate and nitrite to nitric oxide with resulting developmental toxicity to the crustacean *Daphnia magna*. PLoS One. 2010; https://doi.org/10.1371/journal.pone.0012453 PMID: 20805993

46. Grant D, Yin L, Collins JL, Parks DJ, Organd-Miller LA, Wisely GB, et al. GSK4112, a small molecule chemical probe for the cell biology of the nuclear heme receptor Rev-erb alpha. ACS Chem. Biol. 2010; 5: 925–932. https://doi.org/10.1021/cb100141y PMID: 20677822