ABSTRACT

MEDLOCK KAKALEY, ELIZABETH KATHARINE. Characterization of Early Events in the Activation of the Methyl Farnesoate Receptor. (Under the direction of Gerald A. LeBlanc).

The purpose of this work was to characterize the early activation events of a crustacean methyl farnesoate hormone receptor. Methyl farnesoate orchestrates aspects of reproduction and development in crustaceans including, male sex determination in *Daphnia* sp. Further, several environmental chemicals have been shown to disrupt this endocrine signaling pathway. The elucidation of early activation events in this signaling pathway would aid in the development of endocrine-disrupting chemical detection methods.

We hypothesized that 1) methyl farnesoate activates a methyl farnesoate receptor complex (MfR), composed of the methoprene-tolerant transcription factor (Met) and steroid receptor coactivator (SRC). Further, we hypothesized 2) receptor activation is initiated with the dissociation of Met multimers and subsequent heterodimerization of Met and SRC subunits. We proposed 3) one or more of these activities associated with receptor activation would be suitable for use in the high-throughput screening of chemicals for interaction with this pathway. Finally, we hypothesized that 4) the activated methyl farnesoate receptor regulates downstream expression of reproductive and developmental genes by binding short consensus sequences in the gene promoter regions.

To address the first hypothesis, several candidate receptor complexes were evaluated, but only the Met and SRC heterodimer was activated by methyl farnesoate in a luciferase reporter gene assay. To further elucidate the signaling pathway, we utilized various methods to measure specific intra-molecular interactions between the methyl farnesoate receptor
subunits, and interactions between the MfR and DNA. Results demonstrated that methyl farnesoate mediated dissociation of daphnid Met multimers, and stimulated Met and SRC.

Our next aim was to identify the optimal method with which to measure MfR activation. We sought to determine if our newly constructed MfR assembly bioluminescence resonance energy transfer (BRET) assay was comparable to the “gold standard” of in vitro toxicity testing, the luciferase reporter gene assay. We concluded that the BRET assay was equally as sensitive but considerably more rapid than the luciferase reporter gene assay in detecting receptor activation.

Finally, we sought to identify the DNA sequence that serves as a docking site for the MfR. Candidate DNA sequences were determined based upon similarity to response elements from orthologous receptors in insects. Several candidates were identified upstream of genes known to be regulated by MfR agonists. However, we were unable to confirm binding of the receptor to any of these sequences using electrophoretic mobility shift assays.

The work described herein details the activation and disruption of the methyl farnesoate signaling pathway in daphnids. Further, we defined a novel approach to high-throughput chemical toxicity screening that is as sensitive, but more rapid, than current approaches.
Characterization of Early Events in the Activation of the Methyl Farnesoate Receptor

by

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DEDICATION

To my mother and father, who instilled in me the faith and perseverance necessary to finish this grave task.
BIOGRAPHY

On a record hot day in mid-July, Elizabeth Katharine was born unto Steve and Patricia Medlock. She spent her childhood in the small town of Guilford, Indiana, where the rolling foothills of the Ohio River Valley are covered in dizzying rows of corn and Angus cattle pastures. Elizabeth’s family soon grew to include her two younger brothers, Steven and Michael.

Upon graduation from East Central High School, St. Leon, Indiana, Elizabeth moved across the state to Greencastle; there she studied biochemistry as a DePauw University Science Research Fellow. While her collegiate career commenced with aspirations of pharmaceutical research, an internship with the United States Environmental Protection Agency diverted Elizabeth’s purpose. In the picturesque lakeside city of Duluth, Minnesota, an ecotoxicological study examining impacts of pharmaceuticals on a freshwater fish and the nearby Lake Superior inspired a renewed perspective. So, in the concluding years of her undergraduate studies, Elizabeth matured as a scientist, initiating a year-long independent study on seasonally-changing communities in local lakes, and opting for ecologically-based elective courses. In 2011, Elizabeth transplanted herself and newfound ambitions to Raleigh, North Carolina and soon became a doctoral candidate and research assistant in North Carolina State University’s environmental toxicology program.

Over the next couple years Elizabeth became acquainted with the greater Raleigh area; running greenways, volunteering for a non-profit and becoming a member of Cary Christian Church. There she happily served the pre-school ministry and food pantry. One bright and Sunday morning, while breakfasting at a church pitch-in, a strapping young man
named Daniel complimented Elizabeth’s savory side dish. The two were married on a crisp October afternoon two years later.

Elizabeth and Daniel currently reside in Cary, North Carolina, where they continue to run and serve together. Elizabeth looks forward to honing her skills as a toxicologist, and aims to employ them in ecological and/or agricultural applications.
ACKNOWLEDGMENTS

I would like to begin by thanking my committee chair and academic advisor, Dr. Gerald LeBlanc, not only for his guidance over the past several years, but for molding me into a confident speaker and conscious writer.

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INTRODUCTION

(A modified version of this chapter entitled, ““Males on demand: the environmental-neuro-endocrine control of male sex determination,” was published in FEBSJ 2015; 282, 4080-4093)

Daphnids, often referred to as the water fleas, are small freshwater crustaceans whose populations are abundant and widespread. The more than one hundred known species of these suspension filter-feeders inhabit most all types of freshwater excepting some extreme habitats, e.g., hot springs (1). Some daphnid species, such as *Daphnia pulex* and *Daphnia magna*, have amassed popularity as model laboratory species in disciplines from ecology to genetics. Due to their short reproductive cycle, rapid population growth, and other life history traits, daphnid species have been used in diverse applications including environmental risk assessment (2), population modeling (3), developmental gene mutagenesis studies (4) and chemical toxicity screening (5).

Daphnid life history involves both sexual and asexual reproduction as a means of maximizing population sustainability; this phenomenon is termed cyclic parthenogenesis. For example, when environmental conditions are well suited for rapid and expansive population growth (*i.e.* abundant resources, favorable temperatures) they reproduce asexually. However, asexually reproducing organisms have reduced capacity to purge the genome of deleterious mutations because genetic recombination does not occur (6). As a result, deleterious mutations can accumulate and ultimately result in population demise. And so, despite high costs of sexual reproduction (7), populations undergo intermittent cycles of sexual
reproduction to reap the benefits of this strategy i.e., producing offspring purged of deleterious mutations. Also, sexual reproduction is linked to the production of diapause or resting eggs which serves as a dispersal and survival strategy when under unhospitable conditions.

Asexually expanding populations consist completely of clonal females which, at maturity, release large broods of offspring every three to four days. The first step in the switch from the asexual to sexual phase of reproduction is the production of male offspring, or male sex determination (MSD) (8). Males can be distinguished from females by their smaller size, larger first antennae (antennule), a modified post-abdomen, and first thoracic legs which are armed with hooks used in reproductive clasping (1).

Upon maturation, males mate with females that have produced meiotic haploid eggs in contrast to the diploid eggs produced during the asexual phase (Fig. 1). The resulting fertilized eggs are encased in a protective capsule, called an ephippium, and expelled into the environment at the next molt (Fig. 1) (9). These fertilized eggs are in a resting state (diapause at blastulation). Ephippia are rather hydrophobic and will either deposit in sediments, adhere to substrates or disperse, via air currents or attachment to fur and feathers, to new locations. The resting eggs can remain in a diapause state for decades and hatch when conditions are again suitable for asexual reproduction (10).

A critical component of cyclical parthenogenesis is the phenomenon of environmental sex determination. Environmental sex determination contrasts with genetic sex determination in that the environment, and not genetic factors, determines whether a progeny will be male or female (11). The environmental and physiological processes that are responsible for environmental sex determination have arguably been best described in daphnids. Daphnid
species typically possess 10–12 chromosome pairs with no sex chromosomes (12-14).

Females appear to be the default sex and male sex is determined by environmental factors. Following are descriptions of known linkages in a cascade of events initiated by environmental cues and terminating with male sex determination of progeny. Evidence for the identity of yet unidentified linkages in the cascade are provided.

*Environmental cues initiate male sex determination*

As the initiating event in the sexual reproductive cycle, male sex determination must be exquisitely attuned to environmental cues that signal approaching environmental conditions that will be inhospitable to the asexually reproducing population. These environmental factors, that initiate the male sex-determining cascade, may reflect the change of season, exhaustion of resources or loss of habitat.

*Change of season*

Species inhabiting regions that are subject to significantly cold seasons should be attuned to the onset of the cold season, for the production of resting eggs that can survive the cold temperatures. The most plausible environmental cues of approaching winter are temperature and photoperiod. Photoperiod has been shown to be a male sex-determining factor in some species/strains of daphnids, although temperature may be a contributing factor.

Stross (15) observed that *Daphnia pulex* derived from a population from New York, USA produced a significant number of male offspring (36%) at a cool temperature of 13 °C with an 11-h photoperiod. Both temperature and photoperiod appeared to be required for
male sex determination because an increase in male sex determination was not observed at 13 °C under a shorter photoperiod or at an 11-h photoperiod at the warmer temperature of 19 °C. With this strain, the combination of temperature and photoperiod might ensure the production of males prior to the full onset of winter and not during transient periods of cold that are not associated with an approaching winter.

Kleiven et al. (16) and Hobaek et al. (17) evaluated male sex determination in *D. magna* under various conditions at three photoperiods: 8, 16 and 24 h of light. The proportion of male offspring in *D. magna* populations produced under various conditions was consistently higher with short photoperiod (8-h of light) compared with the longer photoperiods (16 and 24-h of light). Similarly, the WTN6 strain of *D. pulex* produced primarily female (> 95%) offspring under a long day length (14-h light) but produced large numbers of male offspring under a shorter day length (10-h light) (18). Two strains of *D. magna* derived from a Canadian tundra pond were evaluated for potential male sex determination under three photoperiod regimens (8, 16 and 24 h light) (19). Male sex determination was consistently higher at the 16-h photoperiod than the 8 or 24-h photoperiod. Interestingly, the 8-h photoperiod stimulated females to produce haploid eggs suggesting that, for this strain, the decreasing photoperiod coordinates both the introduction of males into the population, and the subsequent production of eggs that must be fertilized and are destined for diapause. Two strains derived from an English temperate pond that was permissive of year round parthenogenetic reproduction were not responsive to photoperiod and produced few males and no haploid eggs under any of the light scenarios (19). Taken together, these results demonstrate that photoperiod alone or in conjunction with temperature can serve as a cue for male sex determination in some species and strains of daphnids. These
cues are likely calibrated to geographic differences in the seasonal timing at which parthenogenetic reproduction will no longer be favorable.

Exhaustion of food resources

Clearly, abundant resources are favorable to rapid population expansion, but as a population grows exponentially the food resources dwindle to depletion. Some studies have implicated a decline in food availability as an environmental signal to trigger male sex determination among daphnid offspring. In a study evaluating the combined action of photoperiod, food deprivation, and crowding on D. magna resting egg and male offspring production, a dependence on all three stimuli for the production of diapause eggs and male sex determination was demonstrated (16). The experimental design was deficient in individual factor assessment and could not detect if food reduction functions independently in stimulating male sex determination. However, in a similar set of experiments by Hobaek and Larsson (20) examining the role of various environmental stimuli on male sex determination, food restriction alone did not stimulate male sex determination. Olmstead and LeBlanc (21) also were able to show that food deprivation alone did not stimulate male sex determination, but in combination with crowding did stimulate this phenomenon.

Population density

Several early studies involving cladocerans suggested that crowding of adult females promoted male sex determination among their offspring (22-25). Since then this stimulus has received considerable attention. Stross and Hill (26) noted that a short-day photoperiod in conjunction with a population density-dependent factor, presumably an eliminated product of
metabolism, triggered male sex determination in *D. pulex*. However, Kleiven et al. (26) reared daphnids in media derived from high population density cultures to discern the contributions of short-day photoperiod, food restriction and soluble metabolites produced by a high-density population on environmental sex determination. The investigators were unable to detect a significant contribution of metabolites from high-density cultures on male sex determination. Similarly, Barker and Hebert (27) were unable to discern a role for population density on sex ratios among *D. magna* from high and low-density naturally occurring populations. Hobaek and Larsson (20) undertook a definitive laboratory evaluation of environmental signals that are responsible for male sex determination in *D. magna*. They found that media derived from high-density daphnid cultures stimulated male sex determination independent of photoperiod or food restriction. The recognition of metabolites as a signal for impending threats to sustainability may be relevant to species/strains derived from temporary habitats.

Olmstead and LeBlanc (21) investigated the individual and interactive effects of crowding and food restriction on male sex determination in *D. magna* by using physically crowded organisms, instead of media containing the probable metabolite. They observed that neither crowding nor food restriction independently stimulated male sex determination. However, in combination, these factors did stimulate the production of male offspring. The involvement of a volatile or otherwise unstable metabolite (26) may account for the discrepant observations.

Collectively, these studies suggest that environmental signals responsible for initiating male sex determination vary in type and number, as well as among species and even among strains within a species. This diversity is expected because organisms in
different habitats, latitudes and geographies are likely subject to different threats to population sustainability and accordingly require different environmental signals that foretell of the relevant threat.

*Hormonal cues transduce the environmental signal*

The mechanism by which environmental signals are received by daphnids and converted to an internal physiologic signal is not known but likely involves environmental signal reception by sensory neurons. Such sensory neurons have been identified in the daphnid peripheral nervous system and have the ability to suppress methyl farnesoate, a relevant reproductive and developmental hormone in decapod crustaceans (discussed in more detail below) (28, 29).

Daphnids also are known to possess neurosecretory cells that produce allatostatin and allatotropin peptides (30, 31). Allatostatins and allatotropins inhibit or stimulate juvenile hormone (the insect methyl farnesoate ortholog) synthesis, respectively, in insects (32) and likely inhibit or stimulate methyl farnesoate synthesis in crustaceans (33). Thus, neurosensory cells likely possess the dual role of detecting environmental signals that dictate sexual or asexual reproduction in daphnids and responding to these signals by releasing methyl farnesoate stimulatory or inhibitory neurohormones.

Using a strain of *D. pulex* that produces female offspring under long day length and male offspring under short day length, Toyota *et al.* (34) noted significant activation of expression among genes involved in ionotropic glutamate receptor signaling in response to short day length. Further, exposure of daphnids to agonists or antagonists of the ionotropic glutamate receptor subtype, the N-methyl-D-aspartic acid (NMDA) receptor increased or
decreased, respectively, male sex determination among offspring. Because exposure to methyl farnesoate had no effect on expression of these genes, the authors concluded that NMDA likely is a photoperiod-responsive regulator of sex determination early in the sex determining neuroendocrine cascade. It is enticing to speculate that the NMDA receptor serves as a sensor of environmental signals that dictates sex.

The NMDA receptor in vertebrates requires three simultaneous factors to open calcium channels in neuronal cells; glutamate binding, glycine binding and a strong depolarization (35). As such the receptor serves as a coincidence detector (36). Considering, male sex determination in daphnids typically requires two or three stimuli (16, 21), the NMDA receptor becomes additionally attractive as the point of coincident activation by several environmental stimuli.

*Methyl farnesoate endocrine signaling*

The initial indication for the hormonal control of male sex determination arose from studies with the insecticidal juvenile hormone mimic, methoprene. Continuous methoprene exposure of *D. magna* at concentrations as high as 160 nM resulted in no significant effect on male sex determination under conditions that favored the production of female (21). However, a methoprene concentration-dependent increase in male sex determination did occur under conditions of crowding and food restriction that were permissive of male sex determination. Later experiments revealed that methoprene alone could stimulate male sex determination when present at sufficiently high exposure concentrations (37). The investigators speculated that crowding and food restriction resulted in the production of low
levels of a male sex-determining factor with which methoprene acted additively to stimulate male sex determination.

The discovery of a compound with juvenile hormone-like activity, that could stimulate male sex determination, led to the search for an endogenous juvenoid hormone responsible for this phenomenon. The insect hormone, juvenile hormone III, was found to stimulate male sex determination \((38)\) albeit at high exposure concentrations \((\text{EC50} \sim 4 \text{ µM})\) \((39)\). Analysis of crustacean \((Libinia emarginata)\) hemolymph for the presence of juvenile hormone III revealed only trace amounts compared to the unexpoxidated form of the hormone, methyl farnesoate \((40)\). Methyl farnesoate is produced in, and secreted by, the mandibular organ of decapod crustaceans \((41, 42)\) and has been detected in branchiopod crustaceans such as \(Artemia\) \((43)\) and \(Daphnia\) (LeBlanc, unpublished). Two groups have reported methyl farnesoate as a male sex determinant in daphnids at physiologically relevant exposure concentrations \((39, 44)\). Oocytes were found to be susceptible to the sex-determining influence of methyl farnesoate, specifically during the late stages of maturation in the ovaries \((44)\).

A major contributing enzyme in methyl farnesoate synthesis in decapod and branchiopod crustaceans is farnesoic acid O-methyl transferase (FAMT, also known as JHAMT) \((18, 45, 46)\). The enzyme is negatively regulated in decapods by crustacean hyperglycemic hormone-like neuropeptides \((41)\). As in insects \((47)\), FAMT may also be negatively or positively regulated by allatostatin and allatotropin, respectively. Thus, FAMT serves as the regulatory linkage between neuropeptide secretion and methyl farnesoate production in decapod crustaceans. The \(famt\) gene has been identified in \(D. pulex\) \((48)\) and likely serves a similar role as the site of regulation of methyl farnesoate production in
response to environmental signals in branchiopod crustaceans. Indeed, famt gene expression is elevated in concert with the photoperiod-induced period of susceptibility of *D. pulex* embryos to male sex determination (18).

**Search for the methyl farnesoate receptor (MfR)**

The identification of methyl farnesoate as a male sex determinant in daphnids, and the newly sequenced genome of *D. pulex* allowed LeBlanc *et al.* to attempt to identify the protein receptor that mediates the action of this hormone. Using the newly sequenced genome of *D. pulex* as a roadmap, 25 nuclear receptors were identified in this species (49). The retinoid X receptor ortholog (Dappu-RXR, NR2B) was viewed as a viable candidate for the MfR. This possible role of the Dappu-RXR was based upon the demonstration that: (a) the methyl farnesoate analog, methoprene, activated human RXRα in a gene transcription reporter assay (50); (b) methyl farnesoate bound the *Drosophila* RXR ortholog (Ultraspiracle) with high affinity (51); and (c) methyl farnesoate influences ecdysteroid signaling in daphnids where the ecdysteroid receptor forms a heterodimer with RXR (52). However, methyl farnesoate did not activate the *D. magna* RXR in a gene transcription reporter assay (53). Methyl farnesoate did synergize with 20-hydroxyecdysone to enhance transcription of a reporter assay containing both *D. magna* RXR and ecdysteroid receptor (53). This synergistic activity with 20-hydroxyecdysone also was observed with the methyl farnesoate analog pyriproxyfen (53) and the potent daphnid RXR agonist tributyltin (54). Corroborating evidence that RXR does not serve as the MfR is that trybutyltin is a potent agonist to daphnid RXR but is not a male sex determinant in this species (54). These results demonstrated that methyl farnesoate does indeed bind to the daphnid RXR and can modulate
the activity associated with the RXR partner receptor/ligand, but independently, is not the MfR. Several members of the nuclear receptor family from \textit{D. pulex} were cloned and evaluated in gene transcription reporter assays for responsiveness to methyl farnesoate ((55), LeBlanc, unpublished). No evidence was found that a member of this protein family functioned as the MfR.

Mounting evidence suggesting the juvenile hormone receptor in insects is composed of two basic helix–loop–helix-Per-Arnt-Sim (bHLH-PAS) proteins, Methoprene-tolerant (Met) and Steroid Receptor Coactivator (SRC) (aka FISC, Taiman) (56, 57) prompted the evaluation of these receptors as mediators of methyl farnesoate signaling in crustaceans. Met, cloned from \textit{D. pulex}, when expressed with the mosquito SRC ortholog (FISC) was activated by methyl farnesoate and its functional analog pyriproxyfen (55). Similar results were obtained using Met and SRC cloned from both \textit{D. magna} and \textit{D. pulex} (58). Additionally, these investigators demonstrated that a single amino acid substitution in the ligand-binding domain of the Met protein could explain the divergence in the ligand affinity of Met between crustaceans (methyl farnesoate) and insects (juvenile hormone III).

Insect Met is known to dimerize with other proteins (e.g., Cycle) (59) to form active transcription factors. However, various synthetic juvenile hormone analogs (pyriproxyfen, methoprene, hydroprone, fenoxycarb, epofenonane) and other compounds have been evaluated for their ability to activate the MfR in gene transcription reporter assays (55, 58). The potency of these compounds as an activator of Met:SRC correlates well with their potency to stimulate male sex determination \textit{in vivo}. This evidence supports Met:SRC as being the MfR complex responsible for male sex determination.
**Additional physiological consequences of methyl farnesoate signaling**

Methyl farnesoate signaling has been shown to regulate various physiological processes in daphnids that appear to be distinct from its role in male sex determination.

**Hemoglobin induction**

The inductive effect of methyl farnesoate and analogs on hemoglobin was first reported in *D. magna* where chemical exposure was noted to result in an increase in red coloration of the organisms (60). More detailed analyses revealed that the coloration was due to increased hemoglobin levels associated with induction of the *hb2* gene (60). The increase in hemoglobin levels was also noted in *D. pulex* and *D. pulicaria* following methyl farnesoate treatment (60). Exposure of juvenile *D. pulex* to the MfR agonist fenoxycarb upregulated 15 members of the hemoglobin gene battery (61). The ecophysiological significance of hemoglobin induction via the methyl farnesoate signaling pathway is not known. However, increased capacity to survive a hypoxic environment may represent a component of a generalized stress response to environmental adversity that is regulated by this signaling pathway (60).

**Vitellogenin regulation**

Methyl farnesoate and its analogs have been reported to suppress the expression of the vitellogenin *vtg1* and *vtg2* genes in *D. magna* (62). Vitellogenin is yolk protein that is produced by maternal organisms and packaged into eggs. Similar observations were made with the methyl farnesoate analog fenoxycarb (63). However, Hannas et al. (64) were unable to detect any effect of methyl farnesoate and the analog fenoxycarb on *vtg2* mRNA levels.
Toyota et al. (61) evaluated the effects of several methyl farnesoate analogs on the vitellogenin gene battery of *D. magna*. Consistent with the results of Hannas et al. (64), vtg2 mRNA levels were neither elevated nor reduced in response to these compounds. The suppression of mRNA levels of some members of the vitellogenin gene battery have been reported (61); however, the vitellogenin forms were not identified by specific gene name. These discrepant results may reflect the complexity of the vitellogenin gene battery in daphnids with most forms being unaffected from exposure to methyl farnesoate and analogs and a few forms being suppressed.

*Regulation of cuticle proteins*

Cuticle proteins possess mechanical characteristics that contribute to strength, flexibility and other characteristics of arthropod exoskeletons (65). The methyl farnesoate analog fenoxycarb has been shown to suppress mRNA levels of *D. magna* cuticle 12 protein (63). Subsequent studies revealed complex interactions among methyl farnesoate agonists and members of the cuticle protein battery in *D. magna*, with mRNA levels being elevated, suppressed or not affected in relation to agonist, agonist concentration and mRNA species evaluated (61). Currently, the significance of methyl farnesoate regulation of cuticle proteins is not known however, this regulatory function may more closely align with methyl farnesoate’s putative role in molting rather than in sex determination (52).

Microarray analyses of mRNA transcripts following exposure of *D. pulex* to methyl farnesoate revealed changes in transcript levels for over 50 distinct genes (66). The
alterations in expression of the various genes hint at a methyl farnesoate-regulated shift in energy production and usage and a coordinated redirection of receptor-mediated signaling processes.

Cyclomorphosis

Many species of daphnids exhibit a form of polyphenism known as cyclomorphosis (8). Cyclomorphosis is characterized by various changes in body structure such as the development of neck teeth, elongation of the head capsule and alterations in the length of the tail spine. These changes occur in response to predation threat, and the resulting phenotypic changes increases the organism’s resistance to predation (67, 68). Exposure of D. galeata to methyl farnesoate, or its analog fenoxycarb, stimulated cyclomorphosis characterized by an elongation of the head capsule and shortening of the tail spine (69). Methyl farnesoate may indeed be the morphogen responsible for cyclomorphosis in daphnids, because predation threat also reportedly induced the Met gene in D. pulex (70) and stimulated male sex determination in D. magna (71). The regulation of cuticle protein expression by methyl farnesoate may contribute to these morphogenic changes.

Gynandromorphism

Olmstead and LeBlanc (72) noted a low (< 0.01%), but detectable, incidence of bilateral gynandromorphic individuals among cultured D. magna. Investigation into the potential role of methyl farnesoate in this phenomenon revealed that an increased incidence of bilateral gynandromorphs among offspring could be generated when maternal organisms were exposed to 48–50 µg/L methyl farnesoate, the concentration range that also generated a
50: 50 sex ratio among offspring. The authors surmised that this marginal male sex-determining concentration of methyl farnesoate occasionally programmed one of the cells in a two-celled embryo that was present during the window of susceptibility to methyl farnesoate. Upon further cell divisions, one half of the individual originated from the male-programmed cell and the other half originated from the default female cell. This hypothesis was supported by the observation that when cultured at an elevated temperature the proportion of bilaterally gynandromorphic individuals significantly increased, presumably because the elevated temperature advanced the rate of development of the embryo resulting in more two-celled embryos being present during the window of susceptibility to methyl farnesoate (72).

Resurrection of the male phenotype

Branchiopods that inhabit stable environments often lose their ability to produce males and populations persist through asexual reproduction. Parthenogenetic production of females is apparently favored because the generation of more offspring outweighs the advantages of genetic variability in these stable environments. Kim et al. (73) proposed that exogenous administration of methyl farnesoate to cladoceran species for which males have never been described, might resurrect the male phenotype. Indeed, administration of methyl farnesoate to three non-male-producing lacustrine species resulted in the production of male offspring. However, the male phenotype cannot be rescued in all non-male-producing clones of daphnids (60). Taken together, results from these studies indicate that the male phenotype is lost in some water flea species/clones due to loss of the ability to produce methyl farnesoate, reduced sensitivity of the Mfr to the hormone, or loss of some component of the
sex determining pathway downstream of methyl farnesoate signaling. In all cases, these modifications to the hormone-signaling pathway relegate the species/clone to asexual reproduction or the recruitment of mates from sympatric species.

What follows MfR activation?

Sex determination in insects involves a cascade of events leading to the differential expression of genes in either the male or female phenotype. In Drosophila, and apparently other insects (74), the cascade is initiated by the differential expression of the Sex Lethal gene (Sxl). In Drosophila, the functional sex lethal protein is produced in female embryos but not in male embryos (75). The SXL protein is an RNA splicing factor that processes the RNA of the Transformer gene resulting in active TRA protein in females, but not males (76). TRA protein is also an RNA splicing factor that splices RNA produced by the Doublesex (Dsx) gene resulting in the production of a functional transcription factor in females (77). The DSX protein binds to regulatory elements on responsive genes that are transcribed to produce the female phenotype. The unprocessed DSX protein in males functions as a transcription repressor of the responsive genes in females resulting in the male phenotype.

Gene sequence analysis has revealed significant evolutionary relatedness between insects and branchiopod crustaceans (78), and this conservation appears to carry into the sex-determining cascade. A putative sxl gene has been identified in the D. pulex genome (79), and the annotated putative SXL protein has characteristics associated with RNA binding activity. The tra gene has been cloned in both D. magna (80) and D. pulex (81) and is expressed in both males and females embryos of D. magna with no apparent differences in mRNA splicing between sexes (80). These observations have led to the suggestion that, in
contrast to insects, *tra* is not involved in daphnid sex determination (80). Using *D. pulex*, *tra* was shown to be expressed at slightly higher levels in adult males than in adult females (81). However, sex differences in *tra* expression during the critical period of embryonic sex determination were not evaluated.

The *dsx* gene has been cloned from *D. magna, D. pulex, D. galeata, Ceriodaphnia dubia* and *Moina macrocopa* (82, 83). Two *dsx* paralogs were identified in *D. magna, D. pulex, D. galeata* and *C. dubia*, but only one form was found in *M. macrocopa* (82, 83). In *D. magna*, expression of *dsx1* during the period of embryo susceptibility to male sex determination results in the male phenotype (82). The *dsx2* gene also is expressed in embryos destined to be males during the period of susceptibility to sex determination; however, its role is not currently known. Inhibition of *dsx1* in male embryos results in the female phenotype, whereas ectopic expression of *dsx1* in female embryos resulted in a partial male phenotype (82).

DSX is clearly a sex determinant in daphnids. However, its role in daphnids differs from that in insects. In most insects, a functional DSX splice variant directs the female phenotype, whereas a nonfunctional DSX splice variant inhibits female sex differentiation resulting in the male phenotype. In *D. magna*, sex-specific splice variants of DSX are not produced, DSX directs male, not female, differentiation, and the action of DSX is dictated by DSX expression levels during the period of sex determination. Accordingly, TRA might not function as a RNA splicing factor in daphnids, but rather may function in the regulation of DSX protein levels.
Endocrine disruption by environmental chemicals

Recent advances in insect endocrinology and widespread applications of hormone analogs known as “third generation pesticides,” threaten crustaceans as the recipients of chemical exposure, and subsequent reproductive and developmental endocrine disruption. One such family of pesticides, the insect growth regulators (IGRs), include the juvenile hormone analogs described above. IGRs target the juvenoid signaling pathways of insects by binding the Methoprene-tolerant transcription factor, an ortholog to the daphnid protein of the same name (56, 84, 85). Further, these compounds have been shown to activate the methyl farnesoate signaling pathway, similar to the hormone ligand itself, but in some cases with higher potency (55).

Since their first official IGR registration in 1975, both the variety of pest control uses and the geographical range of use have increased. The entire chemical class includes an overwhelming 4000+ compounds (86, 87), with uses including large turfed area coverage, mosquito (West Nile virus vector) population control, household uses, insect control on cattle, and leafy vegetable, fruit, and nut crop applications (87-89). As such, the U.S. Environmental Protections Agency estimated environmental concentrations of pyriproxyfen, in surface waters adjacent to agricultural applications, exceeding concentrations necessary for male production in D. magna (55, 90). Further, detectable levels of juvenile hormone agonist IGRs have been reported as high as 250 ng/g organic carbon of bed sediment (91) and 0.1-0.6 µg/L in surface waters in the northwestern United States (92).

Due to the specific mode of action, juvenile hormone agonist, of these IGRs adverse effects in exposed non-target arthropods during widespread use and application are possible. Such pesticide contamination, either through direct application to aquatic environments, or
indirectly from agricultural runoff, can cause injury to both commercial and ecologically important aquatic invertebrate species (21, 93-95).

Given the greater environmental implications of potential reproductive and developmental endocrine disruption in crustacean species, and the prevalence of relevant endocrine disrupting chemicals, the goals within this study were to elucidate some of the underlying molecular signaling processes between environmental cues and phenotypic outcomes. More specifically, we aimed to 1) identify the daphnid methyl farnesoate receptor, 2) elucidate the molecular interactions of the MfR subunits; including receptor sub-unit gene expression, ligand-mediated protein-protein and sequence-specific protein-DNA interactions, and 3) develop and utilize the most sensitive and efficient MfR molecular interaction assay for a novel approach to high-throughput chemical toxicity screening. We then 4) validated the novel approach using the positively screened chemicals and assessing reproductive and developmental endpoints in *in vivo* toxicity assays.

**Research Outline**

We hypothesized that the daphnid methyl farnesoate hormone (MF) activates the methyl farnesoate hormone receptor complex (MfR), composed of the methoprene-tolerant transcription factor (Met) and steroid receptor coactivator (SRC). MF initiates the dissociation of Met multimers and subsequent heterodimerization of Met and SRC subunits. Further, the activated MfR regulates downstream expression of reproductive and developmental genes by binding short consensus sequences in the gene promoter regions, therefore making receptor activation a suitable endpoint for a high-throughput chemical toxicity screening approach.
Chapter 1 addressed the first sub-hypothesis: the Methoprene-tolerant transcription factor (Met) and the steroid receptor coactivator (SRC) compose the daphnid MfR. Although several candidates were tested, the Met: SRC dimer was the only transcription factor combination activated by methyl farnesoate in a luciferase reporter gene assay. Further, in vivo experiments suggested epigenetic modifications may be involved in this environmental-endocrine signaling pathway due to transgenerational effects (to an unexposed F2 generation), like reduced overall offspring numbers.

In chapter 2, the intricate hormone-mediated molecular interactions of the previously identified MfR were elucidated. Using a relatively new tool, termed Bioluminescence Resonance Energy Transfer (BRET), several fusion MfR subunit proteins were constructed to monitor these molecular interactions; including the dissociation of the Met homomultimer, Met: SRC receptor assembly and gene transcriptional activation. Results suggest MF initiates the dissociation of multimeric Met proteins and the commensurate assembly of the Met and SRC MfR subunits. This activated receptor initiated downstream gene transcription.

Chapter 3 addressed the sub-hypothesis: MfR activation is a suitable endpoint for a high-throughput chemical toxicity screening approach. The highly sensitive MfR dimerization BRET assay, created within the studies of chapter 3, was used to screen twenty-nine environmental chemicals. Many chemical classes were represented in this low-throughput screen, but positive reads for reproductive and/or developmental toxicity only resulted from insect growth regulating (IGR) insecticides. The screening assay was validated by assessing relative phenotypic endpoints e.g., male sex determination and total offspring production, after in vivo chemical exposures to positively screened IGRs.
The final chapter details our search for the methyl farnesoate receptor response element (MfRE). Mounting evidence suggests orthologous receptors, in phylogenetically similar species, bind a short 9-12 nucleotide sequence upstream of relevant developmental genes (56, 59, 96). Chapter 4 details multiple genome-wide searches for the MfRE. The mathematical parameters and consensus sequences for each search are outlined, and the resulting promoter region sequences are listed. Although binding assays were unsuccessful, a complete list of trial sequences are provided.

Concluding Remarks

Overall, results of this research have built upon the foundation of knowledge concerning the daphnid methyl farnesoate receptor signaling pathway. The receptor and process of ligand-mediated activation have been identified, and as a consequence, we constructed a novel and accurate approach to screening chemicals for potential endocrine disruption. However, after a vast search for downstream gene transactivation sites for genes producing relevant phenotypes, the MfRE remains unknown. The work described herein advanced our understanding of reproduction and development in an ecologically important species, as well as afforded a useful approach pertinent to environmental chemical risk assessment.
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Figure 1. Reproductive cycle of daphnids Red lines depict the asexual parthenogenetic reproductive phase. Blue lines depict the sexual reproductive phase.
CHAPTER ONE: A TRANSGENERATIONAL ENDOCRINE SIGNALING PATHWAY IN CRUSTACEA

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Abstract

Background: Environmental signals to maternal organisms can result in developmental alterations in progeny. One such example is environmental sex determination in Branchiopod crustaceans. We previously demonstrated that the hormone methyl farnesoate could orchestrate environmental sex determination in the early embryo to the male phenotype. Presently, we identify a transcription factor that is activated by methyl farnesoate and explore the extent and significance of this transgenerational signaling pathway.

Methodology/Principal Findings: Several candidate transcription factors were cloned from the water flea Daphnia pulex and evaluated for activation by methyl farnesoate. One of the factors evaluated, the complex of two bHLH-PAS proteins, dappuMet and SRC, activated a reporter gene in response to methyl farnesoate. Several juvenoid compounds were definitively evaluated for their ability to activate this receptor complex (methyl farnesoate receptor, MfR) in vitro and stimulate male sex determination in vivo. Potency to activate the MfR correlated to potency to stimulate male sex determination of offspring (pyriproxyfen>methyl farnesoate>methoprene, kinoprene). Daphnids were exposed to concentrations of pyriproxyfen and physiologic responses determined over multiple generations. Survival, growth, and sex of maternal organisms were not affected by pyriproxyfen exposure. Sex ratio among offspring (generation 2) were increasingly skewed in favor of males with increasing pyriproxyfen concentration; while, the number of offspring per brood was progressively reduced. Female generation 2 daphnids were reared to reproductive maturity in the absence of pyriproxyfen. Sex ratios of offspring (generation 3)
were not affected in this pyriproxyfen lineage, however, the number of offspring per brood, again, was significantly reduced.

**Conclusions**: Results reveal likely components to a hormone/receptor signaling pathway in a crustacean that orchestrates transgenerational modifications to important population metrics (sex ratios, fecundity of females). A model is provided that describes how these signaling processes can facilitate population sustainability under normal conditions or threaten sustainability when perturbed by environmental chemicals.

Keywords: methyl farnesoate, methoprene-tolerant gene, FISC, SRC, p160, environmental sex determination, daphnia, pyriproxyfen
**Introduction**

Hormones in the fetal environment regulate a variety of processes that orchestrate physiologic function in the resulting offspring. For example, intrauterine fetal position of mice, with respect to the sex of its adjacent litter mates and thus the hormonal environment of the fetus, influences later events such as the timing of puberty and sexual behavior (1). Perturbations in the prenatal hormonal milieu can result in inter-individual variability in the expression of these programmed traits as well as disease (2). Indeed, administration of hormones or hormone mimics to maternal rodents has resulted in the production of offspring with increased susceptibility to prostate cancer (3), mammary tumors (4), obesity (5), and glucose intolerance (5).

Changes in fetal programming due to alterations in the hormonal environment of the developing fetus, be it from maternal influences, in utero sibling influences, or maternal exposure to environmental chemicals and drugs, are generally considered to be caused by disruptions or alterations in hormonal regulation of epigenetic programming events. Various components of the epigenetic machinery are under the control of hormones and fetal exposure to hormones or their mimics have been shown to alter epigenetic modifications of several genes (6). However, a precise understanding of the linkage between endocrinology and fetal programming is lacking.

Environmental sex determination provides a plausible phenomenon that could serve well to define the mechanistic linkages between endocrinology and fetal programming. Environmental sex determination is the ubiquitous process among metazoans whereby sex is determined, not by sex chromosomes allocated to the fetus by its parents, but by
environmental influences on the maternal organism or fetus. Environmental factors responsible for sex determination of offspring include temperature (7), nutrition (8), photoperiod (9), and population density (10). Environmental sex determination serves to provide population sex ratios that will maximize sustainability of the population under incipient environmental conditions (11). Generally, the environmental cue is considered to stimulate the release of a chemical signaling molecule (i.e., hormone) that orchestrates the sex programming of the neonate (12). Despite the ubiquity with which environmental sex determination occurs, the process itself remains poorly understood.

Branchiopod crustaceans, such as Daphnia sp., are cyclic parthenogens that are subject to environmental sex determination (13). Under suitable environmental conditions, daphnid populations consist largely of females that reproduce asexually. This clonal reproduction provides for the rapid expansion of the population. However, in response to specific environmental cues, that typically represent a limiting factor to unregulated population growth, the daphnids will produce male offspring. Male sex determination is under endocrine control. The sequiterpenoid hormone methyl farnesoate programs oocytes in late stages of maturation to develop into male offspring (14, 15). The males mate with sexually receptive females producing embryos that are more genetically diverse and less likely to carry gene mutations (16). These embryos are typically in a diapause state and can develop once in a different time or space that is more conducive to parthenogenetic population expansion.

Daphnids can serve as an ideal model for the evaluation of transgenerational signaling owing to: a) environmental sex determination in this taxa is highly suitable to mechanistically evaluate transgenerational signaling; b) populations can be readily reared and
offspring sex can be controlled in the laboratory (14, 17); and c) the genome of a member of this taxa (*Daphnia pulex*) has been fully sequenced (18). In the present study, we sought to identify the endocrine-related transcription factors that translate environmental signals received by the mother to sex determination of her offspring. Three transcription factors were characterized and evaluated for involvement in environmental sex determination. DappuPNR and dappuDSF are member of the NR2E group of nuclear receptors (19). Members of this group of nuclear receptors are important in various aspects of neural development including sexual orientation sex-specific and reproductive behavior (20, 21). Thus, members of this group of transcription factors in daphnids were considered as candidates for contributing to environmental sex determination. The methoprene-tolerant (Met) protein is a member of the bHLH-PAS family of transcription factors and is a component of the juvenoid hormone signaling pathway in insects (22). Consequently, we considered this protein to be a candidate for mediating the action of methyl farnesoate, the unepoxidated form of juvenile hormone III, in crustaceans. We also explored the significance of this transgenerational signaling pathway with respect to population sustainability parameters.

Results

*Transcription factor cloning*

The transcription factors dappuPNR, dappuDSF, and dappuMet were cloned from *D. pulex* using the deduced gene sequences derived from the published sequenced genome of...
the organism (wFleaBase.org) (18, 19). Nucleotide sequences of the cloned genes (cDNAs) are presented in the Supporting Information (Figs. S1, S2, and S3). Deduced amino acid sequences for the gene products are provided in Figs 1, 2, and 3. The dappuPNR gene product was 548 amino acids in length and contained DNA-binding and ligand-binding sites characteristic of most other members of the nuclear receptor family. Its DNA-binding site was 89% identical and its ligand-binding site was 61% identical to those of PNR from *Drosophila melanogaster*. The dappuDSF gene product was 613 amino acids in length and also contained DNA-binding and ligand-binding sites. Its DNA-binding site was 90% identical and its ligand-binding site was 66% identical to those of DSF of *D. melanogaster*.

The Met cDNA was cloned from both *D. pulex* (dappuMet; Fig. S3) and *D. magna* (dapmagMet; Fig. S4) since *D. magna* was used for subsequent whole animal experiments and Met proved to be most relevant to these experiments. The sequenced dappuMet cDNA was highly similar to the sequence derived from wFleaBase. Overall, the two sequences were 97% identical with 100%, 91%, and 98% identity within the bHLH, PAS-A, and PAS-B domains, respectively. The major difference between the two sequences was an additional stretch of 10 nucleotides in the sequenced cDNA just 3’ of the bHLH domain which may have been lost in the wFleaBase sequence due to an error in intron/exon designations. The sequenced dappuMet and dapmagMet cDNAs were also highly similar with 100%, 98%, and 88% identity in the bHLH, PAS-A, and PAS-B domains, respectively (Fig. 3). The bHLH domain is typically involved in protein dimerization and, in some cases, DNA binding (23). The PAS domains are typically involved in dimerization to partner transcription factors or in binding, as a co-activator, to transcription factors, depending upon the specific function of
the bHLH-PAS protein (23). No evidence of dappuMet paralogs was discerned during the cloning of the dappuMet cDNA.

The sequenced dappuMet was 64%, 36%, and 26% identical to the bHLH, PAS-A, and PAS-B domains of the *Drosophila melanogaster* Met, respectively (Fig. 3). In contrast, these domains were 62%, 24%, and 21% similar to the respective domains of the *D. melanogaster* Gce, a paralog of Met (Fig. 3). Taken together, the evidence supports the identification of the sequenced cDNAs from *D. pulex* and *D. magna* as being Met and not a Met paralog. Results also support the use of *D. magna* as a surrogate to *D. pulex* in subsequent whole animal experimentation.

**Activation of the transcription factors by methyl farnesoate**

Constructs of the transcription factors containing the Gal4 DNA binding domain were used in transcription reporter assays where luciferase was the reporter gene which contained GAL4 binding sites upstream of the transcription start site. In the initial screen, none of the transcription factors stimulated luciferase expression either alone or in the presence of 10 μM methyl farnesoate (Fig. 4). SRC is a bHLH-PAS protein that is known to associate with a number of nuclear receptor family of proteins, as well as, bHLH-PAS transcription factors (24). We therefore, co-transfected insect SRC (previously identified as mosquito-FISC (25)) into the transfection reporter assays and evaluated methyl farnesoate responsiveness. SRC had no effect in reporter assays involving dappuPNR and dappuDSF (Fig. 4). However, dappuMet did activate gene transcription in response to methyl farnesoate when SRC was added to the assay (Fig. 4). Concentration-response analyses revealed that methyl farnesoate activated the dappuMet –SRC complex, hereafter referred to as the methyl farnesoate
receptor (MfR), with maximum activation of ~9-fold with a potency (EC_{50}) of 16 \mu M (Fig. 5A).

Three compounds that function as juvenile hormone mimics in insects were selected to determine whether these compounds also activated the MfR. Of the three compounds selected, only pyriproxyfen activated the MfR (Fig. 5B). Maximum activation of the complex was ~2/3 of that observed with methyl farnesoate though this compound appeared more potent with an estimated EC_{50} of 4.8 \mu M (Fig. 5B). Neither methoprene nor kinoprene activated the MfR at concentrations as high as 120 \mu M (Figs. 5C, D).

**Male sex determination**

We have shown that methyl farnesoate is a male sex determinant in daphnids (14). Experiments next were performed to determine whether the relative potency of methyl farnesoate and the juvenile hormone mimics correlated to the relative potency of these compounds to activate the MfR. Both methyl farnesoate and pyriproxyfen stimulate male sex determination among offspring of exposed maternal organisms (Fig. 6 A,B) with pyriproxyfen being more potent. EC_{50} values for male offspring production were 34 nM and 0.22 nM for methyl farnesoate and pyriproxyfen, respectively. Neither, methoprene nor kinoprene stimulated male offspring production at the maternal exposure concentrations tested which were limited by toxicity (methoprene) or solubility (kinoprene) (Fig. 6 C,D). The potency ranking of the four compounds were comparable with respect to the activation of the MfR and male sex determination. Although, the magnitude of difference between methyl farnesoate and pyriproxyfen was much greater for male sex determination as compared to activation of the MfR.
**Transgenerational impacts on life history parameters**

Having demonstrated that pyriproxyfen was most potent in activating the MfR we next evaluated whether elevated levels of the MfR ligand in the maternal organisms (generation 1) elicited responses specifically in offspring (generation 2) or next generation offspring (generation 3). Continuous exposure of first generation organisms to concentration of pyriproxyfen ranging from 0.084 to 0.62 nM had no discernible effect on longevity (Fig. 7A), growth (Fig. 7B) or molt frequency (Fig. 7C). All individuals exposed to pyriproxyfen, as well as controls, matured as reproductively competent females. However, male:female sex ratios of offspring (generation 2) increased with increasing concentration of pyriproxyfen and ranged from all female offspring at the exposure concentration of 0.084 nM pyriproxyfen to all male offspring at 0.56 nM pyriproxyfen (Fig. 7D). The magnitude of this effect was comparable to that observed in previous experiments (Fig. 6B) indicating that the effect of pyriproyfen was not cumulative over the duration of exposure but rather reflected the magnitude of exposure as it occurred during a selected window of susceptibility of the prenatal second generation organisms. Further, the number of second generation individuals within a brood decreased with increasing concentration of pyroproxyfen (Fig. 7E) suggesting that pyriproxyfen decreased the number of oocytes recruited for maturation or increased the number of oocytes/embryos lost during the maturation process. Thus, pyriproxyfen had no discernible effect on parental organisms while modifying the development of neonates.

One female second generation neonate derived from each of ten first generation organisms exposed to 0.22 nM pyriproxyfen was isolated and reared to maturity in the absence of pyriproxyfen. These second generation female neonates all were derived from broods that contained both male and female offspring. Thus, even female offspring were
likely exposed to a near sex-determining concentration of pyriproxyfen during prenatal development. Ten control neonates were similarly isolated and reared. There were no significant differences in survival and growth between the second generation pyriproxyfen-exposed lineage and the control daphnids (Fig. 8A, B). Furthermore, all offspring produced (third generation daphnids) in this experiment were female (Fig. 8C). However, consistent with reduced brood sizes observed among pyriproxyfen-exposed daphnids in the previous generation, broods of third generation organisms produced by the pyriproxyfen-exposed lineage were significantly smaller than broods produced by control daphnids (Fig. 8D).

**Discussion**

It has been recognized for decades that the hormone methyl farnesoate plays many important roles in crustacean development and reproduction (26). Yet the receptor protein that mediates the activity of methyl farnesoate has remained an enigma. The close structural and function identity of methyl farnesoate to the insect hormone JHIII has led to speculation that these two hormones may function through some signaling pathway common to insects and crustaceans (27). Ultraspiracle, the retinoid X receptor ortholog in *D. melanogaster*, was hypothesized to be the functional target of JHIII binding in this insect species (28). However, we found no evidence to suggest that daphnid RXR is activated by methyl farnesoate (29, 30). Although, methyl farnesoate did appear to bind to daphnid RXR resulting in synergistic activation of the daphnid ecdysteroid receptor complex (EcR:RXR) by 20-hydroxyecdysone (29). Recently, we identified the nuclear receptors PNR and DSF within the *D. pulex* genome (19) and presently, we cloned the respective cDNAs. Both
nuclear receptors were viewed as candidate methyl farnesoate receptors as members of this nuclear receptor group (NR2E) contribute to sexually dimorphic development in insects (31). Neither receptor was activated by methyl farnesoate in the reporter gene assay.

We also cloned the methoprene tolerant (Met) gene ortholog from *D. pulex* and *D. magna*. This bHLH-PAS protein was recently shown to be a strong candidate as a JHIII-dependent transcription factor in mosquito (25). Daphnid Met alone was unable to activate the reporter gene in the presence of methyl farnesoate. However, when co-transfected with SRC derived from mosquito (25), a functional methyl farnesoate-dependent activator of gene transcription was created. We refer to this receptor complex (Met-SRC) as the methyl farnesoate receptor (MfR). Efforts to clone and express the daphnid SRC are underway, but has proven challenging due to the large size of the gene (>7600 bp). Presently, it is not known whether SRC functions as a partner transcription factor to Met (i.e., contributes to DNA binding) or functions as a non-DNA binding coactivator. It is highly improbable that SRC was independently responsible for reporter gene activation since it did not possess the GAL4 DNA-binding domain. Furthermore, the presence of SRC in experiments involving PNR or DSF did not result in reporter gene activation. Previously, we demonstrated that methyl farnesoate is a male sex determinant in daphnids (*D. magna*) (14). Subsequently, we and others have shown that methyl farnesoate functions as a sex determinant in other Cladoceran species and some insecticidal juvenile hormone mimics are capable of mimicking this action of methyl farnesoate (15, 17, 32, 33). Having now identified a candidate MfR in daphnids, we evaluated whether the potency of putative ligands of the MfR correlated to their ability to stimulate male sex determination. The insecticide pyriproxyfen was a potent activator of the MfR and was extremely potent at stimulating male sex determination in vivo.
Pyriproxyfen was approximately 3-times as potent as methyl farnesoate in activating the MfR in the mid-range of the concentration-response curve. However, the insecticide was approximately 150-times more potent in stimulating male sex determination. This increased potency in vivo may be due to differences in in vivo-relevant pharmacokinetic parameters such as uptake, distribution, metabolism, and elimination between the two ligands.

The JHIII mimics, methoprene and kinoprene, were unable to activate the MfR and also were inactive as male sex determinants in vivo. Methoprene was previously shown to have weak activity as a male sex determinant (34). This subtle difference in response between studies may reflect strain differences in the MfR or differences in the manufacturers lots of methoprene used. Regardless, potent activators of the MfR (methyl farnesoate and pyriproxyfen) were shown to be potent male sex determinants in vivo; while, JHIII mimics that were inactive with the MfR also were unable to stimulate the production of male offspring in vivo. These observations support the hypothesis that MfR activation by methyl farnesoate is responsible for male sex determination in daphnids. Additional studies of MfR-ligand, MfR-protein, and MfR-DNA interactions are warranted to definitively establish this putative mechanistic linkage between MfR activation by methyl farnesoate and male-sex determination.

Experiments on the physiologic responses of daphnids to the potent MfR ligand pyriproxyfen demonstrated the profound multigenerational consequences of activation of this hormonal pathway. Though pyriproxyfen produced no discernible effects on the endpoints measured among parental (generation 1) organisms, these organisms produced progressively more male offspring (generation 2) with increasing exposure concentration of the hormone mimic. Further, female offspring (generation 2) derived from a pyriproxyfen-exposed
lineage but whose only potential for exposure to pyriproxyfen was early in development produced fewer offspring (generation 3) than organisms derived from an unexposed lineage. These effects provide novel insight into the manner in which methyl farnesoate may regulate daphnid populations through multiple generations (Fig. 9). Under conditions of food abundance, daphnids reproduce asexually with maternal organisms producing large broods of all-female offspring. These offspring mature and continue the asexual reproductive cycle resulting in rapid population growth (Fig. 9, Phase 1). Ultimately, food resources are depleted and population density is very high (Fig. 9, Phase 2). These duel conditions cause an elevation in methyl farnesoate in maternal organisms resulting in activation of the MfR and the production of male offspring and a reduction in the rate of offspring production (Fig. 9, Phase 3). Population density declines, the population now has viable males, and through presently unidentified stimuli, females produce haploid eggs and become sexually receptive. The population density continues to decline due to the transgenerational suppression of fecundity by the original activation of the methyl farnesoate signaling pathway and fertilized diapause embryos (resting eggs) are introduced into the population (Fig. 9, Phase 4). The reduced density of feeding organisms allows for recovery of food resources, diapause eggs hatch, and the asexual population growth cycle is restored (Fig. 9, Phase 5). A significant data gap in this hypothesis is the present lack of demonstration that methyl farnesoate levels are elevated in daphnids in response to food restriction and high population density (which are known to stimulate the production of male offspring in D. magna (10)).

Recently generated information on the molecular contributors to the sex determining pathway of Cladocera provides for assembly of a credible chain of events that link the initiating event (environmental signals) to the apical event (male sex determination) (Fig.
We had previously demonstrated that low food resources coupled with high population density are the initiating environmental signals for male sex determination in *D. magna* (10). We also were the first to demonstrate that the crustacean hormone methyl farnesoate programs maturing oocytes to develop into males (14). Presently, we show that the Met:SRC complex (MfR) provides a functional target for mediating the activity of methyl farnesoate. The *transformer* gene (Tra) has been identified as the initial determinant of sex differentiation in several insect species (35). The Tra gene has been identified in *D. magna* but its functionality in the sex-determining pathway is yet to be determined (36). We propose that methyl farnesoate-activated MfR orchestrates a sex-specific modification to Tra that dictates downstream events leading to male or female differentiation. Essentially, we propose that the default sex in daphnids is female, but activated MfR triggers a “sex switch” that initiates a trajectory for Tra towards male sex differentiation. In insects, the *doublesex* gene (Dsx) is the target of Tra (37). Dsx protein then orchestrates male or female sex differentiation (38). In *D. magna*, Dsx expression during early embryogenesis also is responsible for male sex differentiation (39). Thus, the sex switch may involve the induction of doublesex expression by Tra. A major gap in this proposed pathway is the lack of functional characterization of Tra in Cladocerans.

Results of the present study not only help to elucidate the molecular signaling pathway that links environmental stimuli to sex differentiation, but provide insight into how environmental chemicals can disrupt such signaling pathways resulting in profound transgenerational consequences. Here, we demonstrate that exposure of maternal daphnids to extremely low (parts per trillion) concentrations of an insecticide could dramatically alter sex ratios in the subsequent generation and compromise fecundity of reproductively competent
females for at least two generations. Short term reductions in population size of this important food source for juvenile fishes would likely occur under this scenario. Although, long-term consequences are questionable due to the ability of daphnid populations to rapidly recover (40). None the less, the scenario described herein provides a model that depicts why concern exists for the presence of endocrine disrupting chemicals in the environment. 1) The model chemical targeted a specific receptor with high potency resulting in the capacity to elicit toxicity at very low exposure levels. 2) Processes that are critical to population sustainability were disrupted as a consequence of the initial chemical:target interaction. 3) Adverse consequences of the initial exposure event persisted into subsequent unexposed generations. The identification of such pathways and the characterization of their susceptibility to disruption by environmental chemicals can significantly refine the hazard risk characterization process.

Materials and methods

Daphnids

Transcription factors were cloned from tissues of D. pulex (clone NP6 (15)) since we had previously identified and annotated several transcription factors from this species (19). Life cycle experiments were performed with D. magna (clone NCSU1 (15)) due to the greater fecundity associated with this species. Animals were cultured and used in experiments under rearing conditions described previously (30). Cultured daphnids were raised in media reconstituted from deionized water (41). D. pulex were maintained at a density of 20 daphnids in 40 ml of media and were fed once daily with 1.4 X 10^7 cells of
algae (*Pseudokirchneriella subcapitata*) and 0.4 mg (dry weight) Tetrafin™ fish food suspension prepared as described previously (42). *D. magna* were reared at a density of 40 daphnids in 1 liter of media and were fed twice daily with 1.4x10⁶ cells of *P. subcapitata* and 4 mg dry weight of fish food suspension. Media was changed 3 times per week. Cultured daphnids were kept in incubators maintained at 20°C with a 16/8 hour light/dark cycle.

**Transcription factor cloning**

The SV Total RNA Isolation System (Promega) was used to isolate RNA from female *D. pulex*. Oligonucleotide primers were designed to cover the open reading frame of dappuMet, dappuPNR and dappuDSF based on wFleaBase: the Daphnia Genome Database (http://wfleabase.org/). Primer sequences used to amplify the respective cDNAs are provided in Table 1. Amplification of the dappuMet sequence was performed with an iCycler Thermal Cycler (Bio-Rad, Hercules, CA) using 0.25 U Phusion Hot Start DNA Polymerase (New England Biolabs, Ipswich, MA), 5 µl of 5× Phusion GC Buffer, 0.75 µl DMSO, 200 µM dNTP, 0.5 µM primers, 100 ng template cDNA for a total amount of 25 µl. PCR conditions consisted of hot start at 98°C for 30 sec, followed by 40 cycles with each cycle consisting of 10 sec at 98°C, 30 sec at 58°C, and 45 sec at 72°C. Amplification of dappuPNR and dappuDSF were similarly performed but with 2X PCR Mastermix (Promega) at 94°C for 2 min, followed by 40 cycles with each cycle consisting of 30 sec at 94°C, 30 sec at 54.5°C, and 2 min at 72°C. The amplified DNA fragments were cloned into the pCR 4-TOPO vector (Invitrogen, Carlsbad, CA) following the manufacture’s protocol. Plasmid DNA was sequenced by Eurofins MWG Operon (Huntsville, AL). The Met gene from *D. magna* also was cloned (dapmagMet) using procedures as described for dappuMet.
Luciferase reporter gene assays

Chimeric constructs consisting of the transcription factor and a Gal4 DNA binding domain were prepared for use in luciferase-based transcription reporter assays. DNA encoding the 489 nucleotides of the Gal4 DNA binding domain within the pBIND vector (Promega) was amplified using the oligonucleotide primers described in Table 1. The amplified DNA fragments were digested with SpeI and BstBI and cloned into the PMTB vector (Invitrogen). This construct was designated the PMT-Gal4 vector. DNA encompassing the DEF domain of dappuPNR and dappuDSF and the PAS domains of dappuMet were amplified using oligonucleotide primers depicted in Table 1. Amplified sequences are underlined in the transcription factor nucleotide sequences provided in the Supplementary Information (Figs. S1, S2, and S3). The PCR products were digested with the appropriate enzymes (dappuMet: EcoRI and MluI; dappuDSF and dappuPNR: EcoRI and BstBI) and cloned into the PMT-Gal4 vector. Vector containing the SRC gene (pAC 5.1/V5-His A-FISC), isolated from mosquito (Aedes aegypti), was a generous gift from Dr Jinsong Zhu, Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA. The reporter gene vector used in the assay (pGL5-Luc, Promega) contained the luciferase gene with five upstream GAL4 binding sites. The pPAC-β-gal vector, containing the β-galactosidase gene, served as a control for transfection efficiency and was a kind contribution from Dr. Robert Tjian (University of California, Berkeley).

Reporter gene assays were performed in Drosophila Schneider (S2) cells (Invitrogen). Drosophila S2 cells were grown in Schneider’s medium (Gibco, Carlsbad, CA, USA), containing 10% heat inactivated fetal bovine serum (Gibco), 50 units/ml penicillin G (Fisher Scientific, Pittsburgh, PA), 50 µg/ml streptomycin sulfate (Fisher Scientific) and
incubated at 23°C under ambient air atmosphere. Cells were seeded at a density of 3×10^6 in a 35 mm plate and transfected 16-23 hours after plating when the cells were at 50-70% confluence. Transfections were performed by calcium phosphate DNA precipitation with the relevant plasmids. Following transfection, cells were washed and transcription induced with the addition of CuSO_4 at a final concentration of 500 μM for 24 hours. Transfected cells were treated with the chemicals for 24 hours with Ex-cellTM 420 insect serum-free medium with L-glutamine (SAFC Biosciences, Sigma, St. Louis, MO) and harvested for luciferase and β-galactosidase determinations. Luciferase activities were measured using the luciferase Assay System (Promega), and normalized to β-galactosidase activities which were measured by the β-galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega), according to the manufacturer’s recommendation. Each experiment was repeated at least three times.

Compounds evaluated in the transcription reporter assays were: methyl farnesoate (95%, Echelon Biosciences Inc., Salt Lake City, Utah), pyriproxyfen (99.5%, Chem Service, West Chester, PA), methoprene (99%, Chem Service) and kinoprene (96%, Chem Service). Chemicals were dissolved in DMSO for reporter assays at a final assay concentration of 0.050%, v/v.

Male sex determination

The potency of several juvenoid analogs in stimulating male sex determination of daphnids was determined generally as described previously (34). Compounds used were the same as used in the transcription reporter assays. All test compounds were dissolved in ethanol with a final ethanol concentration in treatments and controls of 0.050%, v/v. Female daphnids, carrying embryos in their brood chambers, were selected from the cultures and
placed individually in 50-ml beakers containing 40 ml media and the desired concentration of juvenoid analog. Test solutions were changed daily and daphnids were observed daily for the release of broods of offspring. Food was provided to each beaker as $7 \times 10^6$ cells of algae ($P. subcapitata$) and 0.20 mg (dry wt) of fish food homogenate (42) daily. Treatments were replicated 10-times (ie., one animal per beaker, 10 beakers per treatment). Assays were terminated when all maternal daphnids in the experiment had released their second brood of offspring.

The number of offspring present in the second brood released by each maternal daphnid was quantified and sex of individual daphnids within that brood was determined. Sex of individual offspring was established microscopically with males being discerned from females by the longer first antennae (14). Daphnids typically produce only female offspring under these culture and assay conditions in the absence of juvenoid compound.

**Life cycle assessment**

Daphnids ($D. magna$) were exposed to concentrations of pyriproxyfen over their life cycle to test the hypothesis that maternal exposure to this methyl farnesoate mimic causes transgenerational effects. Individual female daphnids were exposed to a series of tightly spaced dilutions of pyriproxyfen for 21 days during which time effects on parental survival, growth, and molt cycle duration was evaluated. In addition, effects of pyriproxyfen on brood size and sex ratio of offspring was determined. Results were compared to those derived from 10 control organisms that were exposed only to the solvent used to deliver pyriproxyfen (ethanol, 0.020%, v/v). Animals were exposed individually in 50 ml beakers containing 40 ml of media. Solutions were exchanged every 2-3 days. Test beakers were provided
3.5X10^6 cells of algae (*P. subcapitata*) and 0.10 mg (dry wt) of fish food homogenate (42) twice daily for daphnids <7 days old and twice these amounts, for animals >7 days old. Experiments were maintained in incubators at 20°C and a light:dark photoperiod of 16:8 hr. This experimental design has been described in detail previously (43).

Experimental animals were examined daily for survival, ecdysis, and offspring production. Exuvia and offspring were removed from the beakers when observed and sex of individual offspring was determined microscopically based upon the length of the first antennae (14). At 21 days exposure, length of individual parental organisms was determined as the distance from the top of the helmet to the base of the shell spine.

One female offspring derived from a mixed (males and females) brood from each of 10 maternal daphnids exposed to 0.22 nM pyriproxyfen were raised to reproductive maturity in the absence of pyriproxyfen. Ten offspring from unexposed daphnids were similarly isolated and raised to reproductive maturity. Survival and length of these organisms, size of their first brood of offspring and sex of individuals within the first brood produced by these organisms were determined as additional indicators of transgenerational effects of pyriproxyfen.

*Statistics and modeling*

Significant differences between treatment and controls were evaluated using Student’s t test at p=0.05. All concentration-response curves were generated using the logistic equation. Statistics and curve generation were performed using Origin software (OriginLab Corp., Northampton, MA). The amino acid sequences were deduced from the
nucleotide sequences using ExPASy software (http://www.expasy.org/). Amino acid sequence alignments were performed using ClustalW (http://www.genome.jp/tools/clustalw/).

Acknowledgements

The authors acknowledge the assistance of David Anick and Hong Li in the performance of some experiments.
References


Figures

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Figure 1. Amino acid sequence of D. pulex PNR deduced from the nucleotide sequence of dappuPNR (Fig. S1) and aligned to PNR from D. melanogaster. The D. melanogaster sequence was deduced from the nucleotide sequence provided in GeneBank (accession # NP_611032.2). The DNA-binding domain (DBD) and the ligand-binding domain (LBD) are indicated. Common amino acids between the two sequences are shaded.
**Figure 2.** Amino acid sequence of *D. pulex* DSF deduced from the nucleotide sequence of dappuDSF (Fig. S2) and aligned to DSF from *D. melanogaster*. The *D. melanogaster* sequence was deduced from the nucleotide sequence at Gene Bank (accession number AAD05225.1). The DNA-binding domain (DBD) and the ligand-binding domain (LBD) are indicated. Common amino acids between the two sequences are shaded.
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<th>Length</th>
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<td>D. pulex</td>
<td>EVAIKVLEBLJSRSGDTGHIQHGNHNNNTSK-</td>
<td>613</td>
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<tr>
<td>D. melanogaster</td>
<td>---------------------------------- ---------------------------------</td>
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<tr>
<td>D. pulex</td>
<td>NSWAPLRELMETMEPAQVDK-----------------------------------------------------</td>
<td>693</td>
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**Figure 3.** Aligned amino acid sequences of *D. magna* and *D. pulex* Met deduced from the nucleotide sequences of dapmagMet and dappuMet (Figs. S3 and S4, respectively) and aligned to Met and Gce from *D. melanogaster*. The *D. melanogaster* sequences were deduced from the nucleotide sequence at GeneBank (accession numbers NM_078571 and NP_001259566.1). The bHLH and PAS domains (A and B) are indicated. Identical amino acids are indicated by the same shading.
D. pulex-Met  -TDQHYFPGEKLKVNTGSSP-  687
D. magna-Met  TADQHYTHSLKVNAGTNSA-  695
D. melan-Met  ----------------------
D. melan-Gce  DSERNQEQPFPPLQEDDQD--  959
**Figure 4.** Activation of a GAL4-driven luciferase reporter gene by dappuPNR-GAL4, dappuDSF-GAL4, and dappuMet-GAL4 in the presence and absence of SRC (1 μg plasmid DNA transfected) and methyl farnesoate (MF, 10 μM). An asterisk denotes a significant difference (p<0.05) from the respective assay performed in the absence of MF. All data are represented by the mean and standard deviation of three replicate assays.
Figure 5. Activation of a GAL4-driven luciferase reporter gene by the dappuMfR (Met-GAL4:SRC) by different concentrations of putative ligands. Data represents the mean (data point) and standard deviation (error bars) of three replicate assays.
Figure 6. Percentage maternal daphnids (*D. magna*; *n*=10) that produced male-containing broods following exposure to putative MfR ligands. Red dots denote the percentage male-containing broods among 10 daphnids that were not exposed to ligands (negative control).
**Figure 7.** Physiological responses of daphnids (*D. magna*) exposed to concentrations of the MfR ligand pyriproxyfen through their life cycle. Each black data point represents the response of a single daphnid. Red dots depict the performance (mean ± standard deviation) of ten unexposed daphnids.
Figure 8. Physiologic performance of daphnids (*D. magna*), produced by maternal organisms that were exposed to either 0.00 or 0.22 nM pyriproxyfen. These offspring were reared in the absence of pyriproxyfen. Data represent the mean and standard deviation (where appropriate) of ten individuals. An asterisk denotes a significant (p<0.05) difference between the treatments.
**Figure 9.** Proposed transgenerational population consequences of activation of the MfR resulting from depleted food resources and high population density.
Figure 10. Proposed mechanistic linkage whereby environmental signals receive by material organisms results is sex determination of next generation individuals.
**Table 1.** Oligonucleotide primers used in the PCR amplification of various transcription factors. Bold denotes added restriction sites and italics denote spacer nucleotides added to facilitate proper cutting of the sequence. Some primers used in the reporter assay constructs were situated upstream or downstream of the sequence targeted for amplification.

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<tr>
<td></td>
<td></td>
<td>reverse: 5’-TACTATCGGCATTACGAGATCTACCTTCCAG-3’</td>
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Figure S1. Open reading frame nucleotide sequence of the of the dappuPNR cDNA.

Underlined sequence denotes the portion that was used in the transcription reporter assays.
Figure S2. Open reading frame nucleotide sequence of the dappuDSF cDNA.

Underlined sequence denotes that which was used in transcription reporter assays.
Figure S3. Open reading frame nucleotide sequence of the of the dappuMet cDNA.

Underlined sequence denotes that which was used in transcription reporter assays.
Figure S4. Open reading frame nucleotide sequence of the dapmagMet cDNA.
CHAPTER TWO: AGONIST-MEDIATED ASSEMBLY OF THE CRUSTACEAN METHYL FARNESOATE RECEPTOR (MfR)

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Abstract

The methyl farnesoate receptor (MfR), composed of two βHLH-PAS proteins, methoprene-tolerant transcription factor (Met) and steroid receptor coactivator (SRC), orchestrates certain aspects of reproduction and development, such as male sex determination in branchiopod crustaceans. Although phenotypic endpoints regulated by the receptor have been well-documented, the molecular interactions involved in receptor activation remain elusive. We hypothesized that maximum MfR subunit expression would coincide with the previously described timing of methyl farnesoate-mediated male sex determination in daphnids. Further, we hypothesized that methyl farnesoate activates MfR by orchestrating the assembly of its subunits, Met and SRC. We measured expression levels of the MfR subunit mRNAs over two successive reproductive (molt) cycles. Met was expressed rhythmically during the reproductive cycle, with peak expression mid-cycle (36 hrs post-molt). In contrast, SRC mRNA levels did not significantly change over the reproductive cycle. Using bioluminescence resonance energy transfer (BRET) technology, we showed that Met proteins self-associate in the absence of methyl farnesoate. Met multimers dissociate in the presence of methyl farnesoate with subsequent dimerization to SRC to form the active transcription factor. Results demonstrated that Met expression increases just prior to the period of susceptibility to male sex determination and exists in self-assembled configuration. The production of methyl farnesoate, in response to environmental cues to produce male offspring, would result in the liberation of Met subunits, which dimerize with SRC to transcriptionally initiate the male sex determination process.
Introduction

Methyl farnesoate is an acyclic sesquiterpenoid hormone and the unoxidated form of the orthologous juvenile hormone III (JHIII) in insects. In some branchiopod crustaceans, methyl farnesoate is synthesized by juvenile hormone acid o-methyltransferase (JHAMT) (1) and involved in various aspects of reproduction and development in crustaceans (2-4). For example, some branchiopod crustaceans produce asexual broods entirely of female offspring under favorable environmental conditions however the production of methyl farnesoate in response to environmental cues stimulates male sex determination (MSD) (2, 5, 6). Further, some environmental chemicals, known as insect growth regulating insecticides (IGRs), initiate the same male phenotypes in several crustacean species (7, 8).

While environmental cues, such as changes in photoperiod (9), temperature (10), or the simultaneous reduction in food availability and high population density (11, 12) act as environmental cues for male sex determination, exogenous exposure to methyl farnesoate has been shown to stimulate male sex determination in many crustacean species (2, 7, 13, 14). The highest susceptibility to exogenous methyl farnesoate (2) and methyl farnesoate agonist (15) exposure is during oocyte maturation. Exposure during a 12-hour developmental window to a range of concentrations of methyl farnesoate, similar to those measured in hemolymph of other crustaceans (3), resulted in a concentration-dependent response in male sex determination (2).

Previously, we determined that methyl farnesoate activated the daphnid methoprene-tolerant transcription factor (Met) in the presence of the steroid receptor coactivator (SRC) ortholog derived from Aedes aegypti (16), FISC (βFtzF1 interacting steroid receptor
coactivator) (17, 18). Subsequent experiments performed with Met and SRC cloned from *D. magna* and *D. pulex* corroborated our previous results and confirmed the protein complex as a methyl farnesoate responsive receptor (MfR) (19). Further, several IGRs activated the daphnid MfR in gene transcription reporter assays (16, 19), with similar relative potency as observed with the stimulation of male sex determination *in vivo*. These experiments support Met and SRC as the receptor protein complex mediating male sex determination.

Although the environmental signals that stimulate male sex determination have been elucidated and the MfR receptor subunits identified, the intra-molecular interactions of daphnid MfR activation remain elusive. However, some of the interactions of the orthologous juvenile hormone receptor (Met :FISC/Taimen), in insects, have been characterized. *Drosophila melanogaster* Met proteins form spontaneous homomultimers (18, 20), but dissociate in the presence of juvenile hormone (20), and juvenile hormone analogs/IGRs, pyriproxyfen and methoprene (18). JHIII binds with high affinity to the PAS ligand-binding domain of Met in some species (18) and activates the functional JH receptor to initiate gene transcription of some developmental genes (17, 21).

Herein we hypothesized that daphnid *Met* and *SRC* mRNA levels increase just prior or during the period of susceptibility to male sex determination in daphnids. Further, we hypothesized that methyl farnesoate orchestrates the assembly of the Met: SRC transcriptionally activated receptor dimer, the prequel to male sex determination.
Materials and Methods

Methyl farnesoate (Echelon Biosciences Inc., Salt Lake City, Utah), used in bioluminescence resonance energy transfer dimerization/dissociation experiments and luciferase reporter gene transcription experiments, was dissolved in DMSO for delivery to the assay solutions. Final DMSO concentration was 0.001% v/v in BRET assays and 0.0005% v/v in luciferase reporter gene assays.

Cloning of SRC

SRC was cloned from tissues of Daphina pulex (clone Busey16, provided by Dr. Jeffery Dudycha, University of South Carolina, USA). Animals were cultured under parthenogenetic rearing conditions described previously (22), where cultured daphnids were kept in incubators set at 20°C and a 16:8 hour light/dark cycle. The daphnids were maintained at a density of 20 daphnids in 40 ml of media and were fed once daily with 1.4 x 10⁸ cells of algae (Pseudokirchneriella subcapitata) and 0.4 mg (dry weight) Tetrafin™ fish food suspension prepared as described previously (23).

The Aedes aegypti FISC (AaFISC) nucleotide sequence was used to search for the daphnid SRC in the wFleaBase: the Daphnia Genome Database (http://w fleabase.org). Total RNA from adult female D. pulex was isolated using the SV Total RNA Isolation System (Promega). RNA integrity was verified by agarose gel electrophoresis (2.0%), and purity by the 260/280 nm ratio. Forward (5’-GGGATTCTAAAACAAAATTGGTACC-3’) and reverse (5’-GAGTCAAGGTCTTGGTTGGATTC-3’) oligonucleotide primers were designed to amplify the entire daphnid SRC open reading frame. Amplification of dappuSRC was
performed with an iCycler Thermal Cycler (Bio-Rad, Hercules, CA) using 0.25 U Phusion Hot Start DNA Polymerase (New England Biolabs, Ipswich, MA), 5 µl of 5x Phusion GC Buffer, 0.75 µl DMSO, 200 µM dNTP, 0.4 µM primers, 50 ng template cDNA in 25 µl total. PCR conditions consisted of an initial denaturation at 98°C for 30 sec, followed by 40 cycles of 10 sec at 98°C, 30 sec at 58°C, and 4 min at 72°C, followed 5 min at 72°C for final extension. Amplified DNA fragments were cloned into pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. Plasmid DNA was sequenced by Life Technologies-ThermoFisher Scientific.

The European Bioinformatics Institute’s web-based sequence alignment tool, Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to align the deduced dappuSRC amino acid sequence to AaFISC, as well as the recently cloned *D. magna* SRC (19).

**MfR Subunit Expression**

Three hundred molt-synchronized adult female *D. magna* were reared as described by Hannas *et al.* 2010 (24) for use in MfR subunit gene expression analysis. Daphnids were kept individually in 40mL daphnid media and sampled in triplicate where each replicate contained 5-10 daphnids. Beginning at 0 hrs post-molt, daphnids were sampled every 12 hrs for 144 hrs (two reproductive/molt cycles). Replicates were kept in RNAlater® at 4°C for 24 hrs, then stored at -80°C. Whole animal tissue was homogenized using Next Advance Bullet Blender®, and RNA isolation and reverse transcription was completed as previously described (24).

Oligonucleotide primers were designed to measure relative amounts of each MfR subunit (*Met* and *SRC*) mRNA over the course of the daphnid molt cycle. Using forward (5’-
CGTGACAAGCTCAATGCCTA-3’) and reverse (5’-GGCTTCATTCGAAGATCCAC-3’) primers, a 149 base pair sequence was amplified from the conserved *dapmagMet* bHLH DNA-binding domain (19). Another forward (5’- TGTCGAGATCAACAAGTGTC-3’) and reverse (5’- CGCCAGCTCTTCAATGTAAAC -3’) primer set, based on the *D. pulex* *SRC* sequence, amplified a 74 base pair sequence derived from the conserved *D. magna SRC* bHLH DNA-binding domain (19). Amplicon sequences were confirmed by cloning and sequencing (Eton Bioscience, Inc.).

*Met* and *SRC* mRNA levels were quantified using 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) and amplification mixtures consisting of 12.5 uL 2x SYBER green (ThermoFischer Scientific), 300 nM primers, 500 ng DNA in a total volume of 25 uL. Reaction mixtures were heated to 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 sec then 60 °C for 1 min. Mixtures were then heated to 90 °C for 15 sec, cooled to 60 °C for 1 min, reheated to 90 °C for 15 sec and re-cooled to 60 °C for 15 sec. A single melting peak was detected for each sample, indicating amplification occurred only for the target DNA sequence. The comparative C\(_T\) method (2\(^{-\Delta\Delta CT}\)) was used to assess relative levels of *Met* and *SRC* mRNA (normalized to levels of actin and gapdh within the same cDNA sample). Each relative *Met* and *SRC* expression time point was normalized to the 0 hr time point.

**Fusion Protein Construction**

The association of *Met* and *SRC* was assessed using BRET. Daphnid *SRC* was fused to the *Renilla* luciferase 2 protein (RLuc2), which served as the photon donor during BRET (substrate: coelenterazine 400A, emission: 410nm). Daphnid *SRC* gene was amplified (with a stop codon) from the TOPO cloning vector using primers harboring AgeI (forward) and
BstBI (reverse) restriction enzyme sites, and sub-cloned into the pMT-B vector (ThermoFischer Scientific). *Rluc2* (Dr. Sanjiv Gambhir, Stanford University, School of Medicine, Stanford, CA) was amplified from its original storage plasmid (pcDNA) using primers harboring XhoI (forward) and BstBI (reverse). The reverse BstBI primer also contained a short 24 nucleotide “linker” sequence (AGCGGAAGTGGTAGCGGAAGTGGC) to lengthen the distance between the two proteins and decrease probability of incorrect folding. The *Rluc2*-linker sequence was sub-cloned at the 5’-terminus of the pMT-SRC plasmid, to create pMT-*Rluc2*-linker-SRC (*Rluc2*-SRC).

The previously cloned *Met* (16) was fused to yellow fluorescent protein *mAmetrine* (*mAme*) to serve as the photon acceptor during BRET (excitation: 510nm, emission: 535nm). The *Met* gene was amplified (with a stop codon) using primers harboring NotHFI (forward containing linker sequence, (ATAGCGGAAGTGGTAGCGGAAGTGGT) and BstBI (reverse) restriction enzyme sites, and sub-cloned into the pMT-B vector. *mAme* was amplified from pBad cloning vector using primers harboring KpnI (forward) and ApaI (reverse) restriction enzyme sites, and sub-cloned at the 5’-terminus of the pMT-*Met*, to create pMT-*mAme*-linker-*Met* (*mAme*-Met).

*Met* was also fused to *Rluc2*, for use with Met fused to *mAme*, to assess spontaneous association and dissociation of Met multimers. The *Met* gene was amplified (with stop codon) using primers harboring NotHFI (forward containing linker sequence, AGCGGAAGTGGTAGCGGAAGTGG) and BstBI (reverse) restriction enzyme sites, and sub-cloned into the pMT-B vector. *Rluc2* was amplified from pcDNA storage vector with primers harboring KpnI (forward) and EcoRI (reverse) restriction enzyme sites and sub-cloned at the 5’-terminus of the pMT-*Met*, to create pMT-*Rluc2*-linker-*Met* (*Rluc2*-Met).
BRET Assay

BRET assays were performed in *Drosophila* Schneider (S2) cells (Invitrogen). Cells were grown in Schneider’s medium (Gibco, Carlsbad, CA, USA), containing 10% heat inactivated fetal bovine serum (Gibco), 50 mg/ml penicillin G (Fisher Scientific, Pittsburgh, PA), 50 mg/ml streptomycin sulfate (Fisher Scientific) and incubated at 23°C under ambient air atmosphere. Cells were seeded at a density of 3 x 10^6 in 35 mm x 6 well plates and transfected 24 hours after plating.

Transfections were performed by calcium phosphate DNA precipitation with the relevant plasmids. Total DNA transfected was constant across treatments, while the donor: acceptor ratio was held at an optimized ratio (producing highest energy transfer), 1: 6 *Rluc2-SRC/Met: mAme-Met*. Transcription of the transfected genes was induced with CuSO_4_ (500 µM). Twenty-four hours later, cells were treated with methyl farnesoate for 1-2 hours in phosphate-buffered saline. Coelenterazine 400A (5 µM) was then added and light emission was measured immediately at 410 ± 40nm and 535 ± 15nm (mAmetrine fluorescence emission) using a FluoroStar fluorimeter (BMG Labtech). The ratio of light emitted at 535nm/410nm (corrected for basal level donor emission of Rluc2 (25, 26)) is termed a BRET ratio. A BRET ratio of methyl farnesoate-treated cells significantly different from the BRET ratio of control-treated cells is indicative of a change in the amount of protein-protein binding.

Background energy transfer was evaluated for both mAme-Met : Rluc2-Met and mAme-Met : Rluc2-SRC BRET protein interactions; to ensure that energy transfer was due to MfR subunits binding and not random luminescent and fluorescent protein proximity. In these background experiments, treatment groups consisted of cells transfected with a donor
protein, Rluc2 (fused or not fused to a MfR subunit protein), and an acceptor protein, mAmetrine (fused or not fused to a MfR subunit protein) (4 transfection treatment groups total). Each treatment groups was assayed in the presence and absence of 30 (Met : Met ) or 100 µM (Met : SRC) methyl farnesoate.

**Luciferase Reporter Gene Assay**

Luciferase-based reporter gene transcription assays were conducted to assess the ability of the activated receptor dimer to initiate gene transcription. S2 cells were transfected with a plasmids containing Met fused to the Gal4 DNA binding domain (16), SRC, *Renilla luciferase* (pRL-CMV, internal transfection control, Promega) and the reporter gene vector (pGL5-Luc, Promega). Following transfection, transcription was induced with CuSO₄ (500 µM for 24 hours). Cells then were treated with methyl farnesoate in Ex-cellTM 420 insect serum-free medium with L-glutamine (SAFC Biosciences, Sigma, St. Louis, MO). Cells were harvested after 24 hours of incubation with methyl farnesoate. Firefly and *Renilla* luciferase activity were assessed using the Dual-Glo® luciferase system (Promega) and manufacturer’s protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity, and each methyl farnesoate treatment group was normalized to DMSO control treated cells.

**Statistical Analysis**

Significant differences between controls and hormone treated cells in the assessment of background BRET fluorescence were evaluated using Student’s t test (p < 0.01). All other statistical differences were detected using a one-way analysis of variance (ANOVA),
followed by a Tukey’s multiple comparison procedure ($\alpha \leq 0.05$), to evaluate significant differences between concentrations/time points. Using three replicated experiments ($n = 9$), a meta-analysis was completed for the mAme-Met : Rluc2-Met interaction. Statistics were performed using Origin software (OriginLab Corp., Northhampton, MA.)

Results

SRC Cloning

The sequence of the daphnid SRC cDNA was identified using the published *Daphina pulex* genome, and the complete open reading frame was successfully cloned from whole animal tissue. The *dappuSRC* nucleotide sequence of our cloned gene (cDNA), presented in the Supporting Information (Fig. S1), was 97.82% similar to the same gene previously reported by Miyakawa et al., 2012 (19). The deduced amino acid sequence, and an inter-species sequence comparison, for the proposed translated product is provided in Figure 1. *DappuSRC* has 28.50% sequence similarity to the *Aedes aegypti* FISC sequence, deduced from the nucleotide sequence originally used to identify the daphnid gene in the online database. At 2,357 amino acids *dappuSRC* is much longer than *AaFISC* and contains an extended C-terminal end. Not surprisingly, the gene is more comparable in length and sequence similarity, 79.71%, to *Daphnia magna* SRC. Like *dapmagSRC*, *dappuSRC* contains several “LXXLL” transcription factor binding domains in the C-terminal. The *dappuSRC* basic helix-loop-helix (bHLH) domain, has 100% sequence similarity to *D. magna* and 52% to *A. aegypti*. The *dappuSRC* Per-Arnt-Sim (PAS) domain, has 100% sequence similarity to *D. magna* and 37.5% to *A. aegypti*. 
**MfR Subunit Expression**

Analysis of *Met* mRNA levels revealed that the MfR subunit transcript levels oscillate over the course of each molt cycle, with base level expression at the beginning and end of each cycle, and peak expression at 36 hours post molt (Fig. 2A). *SRC* mRNA levels did not significantly vary over the course of two molt cycles, though expression levels maintained a similar trend of peaking 36 hours post molt (Fig. 2B).

**MfR Subunit Interaction**

Several BRET experiments were performed to evaluate the intra-receptor interactions among the MfR subunits (Fig. 3). We compared the BRET signal generated in the presence of both Met fusion proteins (Rluc2-Met and mAme-Met) to the signals generated by various combinations of fusion and free Rluc2 or mAme proteins (Fig. 4). Cells with Rluc2-Met fusion protein and free mAme, and cells with free Rluc2 and mAme-Met fusion protein produced significantly lower BRET signals than those cells transfected with two Met fusion proteins. Further, methyl farnesoate had a significant impact on the BRET signal only when both intact Met fusion proteins were present (Fig. 4). Expression of free Rluc2 and mAme also produced a weaker BRET signal that was non-responsive to methyl farnesoate. These results demonstrated that Met forms multimeric associations which allowed for energy transfer to occur between photo donor and acceptor proteins. Results also provided initial evidence that methyl farnesoate stimulates dissociation of these Met multimers.

Unlike the relatively high basal BRET signal generated with Rluc2-Met and Rluc2-SRC in the absence of methyl farnesoate, Rluc2-SRC and mAme-Met produced negligible energy transfer. The BRET signal generated from the two fusion proteins was
indistinguishable from signals generated from free Rluc2 and mAme, in the absence of receptor proteins (Fig. 5). Further, BRET signals, upon addition of methyl farnesoate, increased significantly only when both fusion proteins were present. These results indicate that negligible spontaneous dimerization of Met and SRC occurs, but methyl farnesoate stimulates association of the two subunits.

Concentration-response analysis revealed that the BRET ratio generated from cells with both Met fusion constructs decreased in response to increasing concentrations of methyl farnesoate, with a maximum decrease at 3 µM (Fig. 6A). The decrease in BRET signal, in response to methyl farnesoate, represented an approximately 50% dissociation of the Met proteins. Thus, Met proteins exist in cells as multimeric complexes and the Met agonist methyl farnesoate stimulates the partial dissociation of these complexes.

Concentration-response analysis with the mAme-Met and Rluc2-SRC constructs confirmed that methyl farnesoate stimulates Met and SRC dimerization, with significant association beginning at 10 µM (Fig. 6B). Therefore, methyl farnesoate stimulates both the dissociation of Met multimers and the formation of Met : SRC dimers (Fig. 7).

**Met: SRC Ligand-Mediated Transcriptional Activation**

Lastly, we evaluated the ability of the cloned Met and SRC proteins to initiate gene transcription in response to methyl farnesoate. For these experiments, cells contained the daphnid Met protein fused with the gal4 DNA binding domain, the daphnid SRC protein, and a gal4-luciferase reporter gene. The fusion of Met to gal4 was necessary as the endogenous MfR response element is not known. The relative amount of reporter gene transcribed
increased as concentration of methyl farnesoate increased, with significant transcriptional activation at concentrations \( \geq 10 \mu M \) (Fig. 6C).

**Discussion**

It has long been established that environmental conditions, such as photoperiod, population density and food availability alter reproductive patterns in crustacean species \( (9, 10, 12) \), and that methyl farnesoate is the mediating hormone of these reproductive and developmental changes in crustacean species \( (27) \). The recent identification of the MfR \( (16, 19) \) enabled further elucidation of how methyl farnesoate mediates reproduction and development.

We previously identified the methyl farnesoate receptor, using the SRC ortholog derived from *Aedes aegypti*, and determined that methyl farnesoate activated Met-mediated gene transcription in the presence of the insect SRC \( (16) \). However, to elucidate some of the finer molecular interactions between the two subunits, cloning SRC from *Daphina spp* was essential. For example, with only 37.5% sequence similarity in their PAS domains, the daphnid and mosquito-hybrid receptor may have interacted differently with the Met subunit. PAS domains play integral roles in heterodimerization with other PAS proteins \( (28) \), and upon mutation of these domains those protein-protein interactions are diminished \( (29) \).

The cloned daphnid SRC contained additional conserved binding domains near the C-terminus. Members of the steroid receptor coactivator family typically house 4-6 “LxxLL” binding motifs (where “L” is leucine and x represents any amino acid), responsible for binding ligand-bound receptors \( (30) \) and transcription coactivator recruitment \( (31) \), and at
least one glutamine (Q)-rich region, known for histone acetyltransferase chromatin remodeling (28). Seven LxxLL motifs and two regions of repeated glutamines are present in our cloned daphnid SRC (Fig. 1). In previous transcriptional activation studies we used the orthologous AaFISC which lacks the extended C-terminal and respective binding domains (Fig. 1). The presence of these binding domains are likely responsible for the drastic increase in methyl farnesoate-mediated transcriptional activation (18-fold) (Fig. 7C), compared to the lower level of activation in previous studies using the shorter AaFISC (9-fold) (16).

The relative expression of SRC does not significantly change over the course of the daphnid molt cycle (Fig. 2B), conceivably due to its probable promiscuity in other reproductive (32) and metabolic gene transcription (33). However daphnid Met oscillates over the course of each molt cycle, peaking in expression at 36 hours post molt, just prior to the window of oocyte susceptibility to methyl farnesoate (2) and an agricultural insecticide (15) (Fig. 2A). We postulate, that approximately 36 hours post-molt, Met mRNA levels peak and subsequently are transcribed and translated over the course of the following 30 hours post molt. At approximately 60 hours post molt, Met proteins interact with methyl farnesoate to stimulate the male offspring phenotype (2). Future studies should seek to determine whether Met levels are modulated in the maternal organism, in the oocytes, or both.

There was a significant decrease in BRET signal, indicative of protein-protein dissociation, in cells transfected with Rluc2-Met and mAme-Met in the presence of methyl farnesoate compared to cells not exposed to the hormone (Fig. 4). Upon concentration response analysis, we discovered the high basal BRET signal was not completely diminished even in the presence of relatively high concentrations of the hormone (Fig. 6A). For example, cells transfected with Rluc2-SRC and mAme (not fused to Met) represented random energy
transfer, or baseline energy transfer, indicating methyl farnesoate mediated the dissociation of approximately 50% of the multimeric Met proteins at even the highest exposure concentrations (Fig. 6A).

The incomplete dissociation of Met multimers may represent a smaller version (homotrimer or -dimer) of the Met multimer in the activated MfR complex. However, many bHLH-PAS proteins operate in heterodimeric protein complexes (34), although at least one group has reported homodimeric bHLH-PAS protein complex binding a respective response element (35). Further, we and others have shown steroid receptor coactivators are necessary for transcriptional activation, not enhancement, of hormone-mediated receptor proteins (16, 19, 36, 37). These studies support our model of MfR activation (Fig. 7), in which SRC binds Met to form a heterodimeric receptor. The inability to detect complete Met multimer dissociation may be attributed to the limitations of our BRET dissociation assay.

Unlike the diminishing methyl farnesoate-mediated signal from Met: Met multimers, the significant increase in BRET signal from cells transfected with both Met and SRC demonstrates protein binding in response to hormone ligand addition (Fig. 5). Concentration-response analysis confirmed a ligand-dependent assembly of the subunits (Fig. 6B). We postulate that subunit assembly does not necessitate DNA promoter binding, as both fusion MfR genes were transfected in excess of 2 x 10^6 times the number of genes, or potential transcription start sites (17,000), in the drosophila genome (flybase.org). Conceivably, the daphnid Met and SRC proteins likely do not bind orthologous Drosophila response elements due to low sequence similarity in DNA-binding domains. The bHLH domains of daphnid Met has 64% sequence similarity to the same domain in the orthologous dmMet (16), while
the daphnid SRC bHLH domain has only 45% to the bHLH domain in the orthologous
Drosophila protein (38).

As expected the activated MfR initiated gene transcription in a concentration-response
manner. However, our previous results demonstrated a maximum 9-fold increase in
transcription in the presence methyl farnesoate (16), while results depicted in Fig. 7C
indicate relatively larger 18-fold maximum. As previously described, this fold increase can
be contributed to the use of the daphnid SRC gene.

In conclusion, we have further elucidated some of the receptor-activating subunit
interactions in a daphnid environmental-endocrine signaling pathway. A representative
model for these interactions is depicted in Figure 7. Firstly, Met spontaneously forms a
multimer (Fig. 7A), which we have shown can be, at least partially, dissociated in the
presence of the hormone ligand (Fig. 7B). Upon dissociation, the free Met may bind with
SRC in the presence of methyl farnesoate (Fig. 7C), and the activated protein dimer can
initiate gene transcription in the presence of hormone (or chemical ligand) (Fig. 7D).

Understanding these molecular interactions involved in the activation of a reproductive
and developmental hormone receptor will support accurate chemical assessment for
population relevant toxicity. For example, although environmental chemicals have been
shown to initiate male sex determination (39) and gynandromorphism (23) in daphnid
offspring, we present novel points of possible endocrine disruption (Fig. 7A-C) in the MfR
signaling pathway, not evaluated in previous chemical toxicity assessments (9, 16, 40).

Acknowledgements
We would like to thank Stephanie Street for the use of daphnid cDNA samples utilized in the gene expression time course analysis.
References


9. K. Toyota, H. Miyakawa, K. Yamaguchi, S. Shigenobu, Y. Ogino, N. Tatarazako et al., NMDA receptor activation upstream of methyl farnesoate signaling for short day-induced male offspring production in the water flea, Daphnia pulex. *BMC Genom* 16, (2015).


Figure 1. Alignment of the deduced dappuSRC, AaFISC and dapmagSRC amino acid sequences The highlighted sequences indicate the conserved bHLH (DNA-binding) and PAS (ligand-binding) domains, as well as the C-terminal LXXLL domains in dappuSRC (transcription factor-binding).
<table>
<thead>
<tr>
<th>AaFISC</th>
<th>DapmagSRC</th>
<th>DappuSRC</th>
</tr>
</thead>
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<td>LSYELPQGNDGLHGRVWDSPNMGESPSTMPSTRTNTMEEAPRPADPQMSLLLKOLLSE-</td>
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</table>
Figure 2. MfR subunit gene expression Expression of *dapmagMet* and *dapmagSRC* over the course of two reproductive/molt cycles. Each time point is normalized to the 0 hour time point. Error bars represent standard error and *denotes significant difference in *dapmagMet* expression compared indicated time point (p<0.05).
Figure 3. **BRET method and fusion proteins** a) When two proteins, protein A (tagged with *Renilla* luciferase, Rluc2) and protein B (tagged with a yellow fluorescent protein, mAmetrine), are an endogenous dimer, energy is transferred. Upon ligand-mediated dissociation, energy is not transferred and the overall BRET ratio drops. b) When two proteins, protein A and protein B are part of a ligand-activated dimer, the absence of ligand will prevent energy transfer. Upon the addition of ligand, the protein dimer is activated, resulting in energy transfer and an increased BRET ratio.
Figure 4. Spontaneous multimerization and ligand-dependent dissociation of Met White bars indicate vehicle control treated cells, while grey bars indicate cells treated with 30µM methyl farnesoate. Error bars represent standard deviation and *denotes significant drop in Met: Met binding compared to DMSO control (p<0.05)
Figure 5. MfR ligand-dependent dimerization White bars indicate vehicle control treated cells, while grey bars indicate cells treated with 100µM methyl farnesoate. Error bars represent standard deviation and *denotes significant increase in Met-SRC dimerization compared to DMSO control (p<0.05).
Figure 6. Concentration responses for ligand-dependent MfR subunit interactions

White bars indicate vehicle control treated cells, while grey bars indicate cells treated with methyl farnesoate. Error bars represent standard deviation and *denotes significant increase/decrease compared to DMSO control (p<0.05). A) Met-Met spontaneous multimerization and ligand-mediated dissociation B) ligand-mediated Met: SRC dimerization C) ligand-mediated MfR
**A.**

BRET Ratio

Methyl Farnesoate (µM)

0.000
0.005
0.010
0.015
0.020
0.025
0.030

0 1 3 10 30 mAme

**B.**

BRET Ratio

Methyl Farnesoate (µM)

0.000
0.005
0.010
0.015
0.020
0.025
0.030

0 1 3 10 30 100

**C.**

Relative Luciferase Units

Methyl Farnesoate (µM)

0
5
10
15
20
25

0 1 3 10 30 100
Figure 7. MfR endocrine signaling pathway model A representation of the molecular interactions detected. a) Met spontaneously forms a multimer which can be b) be dissociated in the presence hormone ligand. Upon dissociation the free Met may bind with SRC in the presence of methyl farnesoate (and other chemical ligands) and d) the activated protein dimer can initiate gene transcription in the presence of hormone and chemical ligand.
Fig S1. The complete open reading frame of the steroid receptor coactivator gene cloned from *Daphnia pulex* (*dappuSRC*)
>Dp-SRC (Open Reading Frame)
ATGCCTGACGGATACCCGATTTCCGGATGATGCACAGAGTTTGGGTGCAATTCCATGCAGGAGTCCCTATCGTCCGAGCCGTGCTGGGCCAACATGAACACGCTCAGCAGCGGCGGAGGTGGTGGTGGTGGCAGCAGCAGCAACAGCAACAGCCCCGGTCTC
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CGTCTCGTCGAGGCCCAGCTCGCGTCAGAGCGCTTCATCCACACCGCGACCGCCCAGCGTTTCGTCAGCCTTCAGTCCGGCTCCCAATTCCGTTTTGGGCGTCGTGCATC
CCTCCCCCGTCTCAGTCCAATGTGCTCATCCGGCCAGGGAATGATGAG
CTCGGCCACAGCCACACCATTGTGCTCTGCTCGTCTCAGTCATCGGCAGCGCCACAGCACCC
AGTCCGGCAGCGATGACGAGCGCAGCCTTTAGCAACAATTTCCCTTCTAGCTCGCTCC
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GTCTGACGGAATGCAGCGCAGTGCTGCTGCAGTCCTGACGAGCCAGGGAAGG
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CCAGGAATACGATGGGAAGAAGCTCAGGGCAGGAGCTGATCTTCAATAGTGTCCTTC
TCAAGCGACTACTCTCGGAGTGTA
CHAPTER THREE: LIGAND-MEDIATED RECEPTOR ASSEMBLY AS AN ENDPOINT FOR HIGH-THROUGHPUT CHEMICAL TOXICITY SCREENING

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Abstract

Methyl farnesoate is a crustacean hormone that activates the methyl farnesoate receptor (MfR) and regulates various aspects of male sex determination and differentiation. Recently, we characterized early activation events in this endocrine signaling pathway, which afforded a novel method for detecting endocrine disruption. The novel method quantifies hormone receptor activation using assembly of the MfR subunits, the Methoprene-tolerant transcription factor (Met) and steroid receptor coactivator (SRC), and bioluminescence resonance energy transfer (BRET) technology. We hypothesized that the MfR assembly BRET assay would be more rapid and cost-effective in detecting xenobiotic receptor interactions than current methods without compromising sensitivity. We proposed that chemicals screening positive in the assay would accurately reflect phenotypic outcomes in daphnids that result from MfR signaling. BRET assays were performed, where daphnid Met was fused with the fluorophore, mAmetrine (Met-mAme), and daphnid SRC was fused with the photon generator, Rluc2 (SRC-Rluc2). Ligand-mediated receptor assembly was measured by the transfer of photons from SRC-Rluc2 to Met-mAme resulting in fluorescence emission from the mAmetrine. Six of the 29 chemicals evaluated screened positive for stimulating MfR subunit association (kinoprene > methoprene > pyriproxyfen > hydroprene > diofenoaln > fenoxycarb). Only those chemicals that screened positive in the BRET assay activated a luciferase reporter gene with similar lowest observable effect concentrations, supporting the accuracy and sensitivity of the MfR BRET assay. All chemicals that screened positive in the in vitro assays also stimulated MfR-regulated male sex determination among offspring of exposed maternal D. magna. The BRET assay chemical exposure duration is only 60 minutes.
and endpoint assessment is not dependent upon reporter gene transcription and translation.

Therefore, we concluded the novel assay is equally as sensitive, but significantly more rapid and cost effective, than the traditional luciferase reporter assay.
Introduction

Endocrine signaling pathways are crucial to survival and reproduction, however these pathways are often susceptible to disruption by environmental chemicals resulting in perturbations in normal physiology (1-4). Environmental exposure to endocrine disrupting chemicals has been associated with reproductive dysfunction (5), perturbations in reproductive development (6, 7), and population demise (8).

As a result, significant effort has gone into screening and testing method development for detecting endocrine-disrupting properties of chemicals, and hazard assessment associated with the use of these chemicals (9-13). Screening assays used to detect endocrine-disrupting activity of chemicals often consist of hormone-receptor binding assays or reporter gene transcription assays (9-11). Chemicals that screen positive in one or more of these assays then become a candidate for more definitive testing to assess whole organism consequences of this activity and the exposure concentrations at which effects occur (12). However, receptor-binding assays are relatively uninformative, because they provide no information on the consequence of binding (e.g., receptor activation, inhibition, or no consequence). Reporter gene transcription assays are more informative, however these assays rely upon reporter gene transcription and translation which can take days to provide a measurable gene product.

Most hormone receptors consist of homo- or hetero-dimers (14). The first step in activation of many of these receptors is ligand-stimulated dimerization (14-16). This receptor assembly endpoint may serve to be an early event in the adverse outcome pathway (17) for
many endocrine signaling pathways, and would conform to a high-throughput screening format.

Methyl farnesoate has long been recognized as a crustacean hormone involved in reproduction and development (18-20). For example, methyl farnesoate stimulates male sex determination, in branchiopod crustaceans (18, 19) and male sex differentiation in some decapod crustaceans (21). Recently, we and others identified the protein receptor complex that mediates the actions of methyl farnesoate, the methyl farnesoate receptor (MfR) (22, 23). Further, we demonstrated methyl farnesoate stimulates the multimer dissociation of the MfR subunit called Methoprene-tolerant transcription factor (Met), and stimulates the association of Met with the protein called steroid receptor coactivator (SRC) (24). These assembled proteins comprise the active MfR (22-24).

Bioluminescence resonance energy transfer (BRET) technology has gained prominence as a means of measuring protein-protein interactions in cells and in real time (25, 26). The method involves the construction of fusion proteins whereby one protein of interest is fused to a luciferase protein. The other protein of interest is fused to a fluorophore. When the proteins associate, photons from the donor can excite the fluorophore resulting in fluorescence emission. BRET has been extensively used in the study of G-protein coupled receptors (26-28), but more recently has been used in the study of homo- and heterodimerization of hormone nuclear receptors (15, 16).

Herein, we describe the construction and optimization of a novel approach to screening chemicals for hormone receptor activation using the MfR cloned from daphnids. Further, we screened several compounds for comparison of specificity and sensitivity to the more traditional luciferase reporter gene transcription assay. Finally, compounds that
screened positive in the BRET assay were evaluated in vivo to determine whether results from the cell-based assay accurately predicted phenotypic outcomes in the whole organism.

**Material and Methods**

Methyl farnesoate (Echelon Biosciences Inc., Salt Lake City, Utah), and all other chemicals (Sigma-Aldrich Corp., St. Louis, MO) screened in BRET and luciferase reporter gene assays, were dissolved in DMSO for delivery to assay solutions. Final DMSO concentrations were 0.001% v/v in the BRET assays and 0.0005% v/v in the reporter gene assays. Hydroprene and diofenolan were dissolved in ethanol for in vivo experiments, where the final concentration of ethanol was 0.0003% v/v.

**Fusion Protein Construction**

Four fusion proteins were constructed to identify those constructs that provided the optimum BRET signal. The daphnid met open reading frame, described previously (22), was fused to the mAmetrine open reading frame (mAme) (excitation: 510nm, emission: 535nm) (Addgene, Cambridge MA) at both the 5’ and 3’ ends of met. The daphnid SRC open reading frame, described previously (24), was fused to the Renilla luciferase 2 open reading frame (RLuc2) (substrate: coelenterazine 400A, emission: 410nm) (Dr. Sanjiv Gambhir, Stanford University, School of Medicine, Stanford, CA) at both the 5’ and 3’ ends of SRC.

The met gene was amplified using primers harboring NotHFI (forward containing linker sequence, ATAGCGGAAGTGGTAGCGGAAGTGGT) and ApaI (reverse) restriction enzyme sites, and sub-cloned into the pMT-B vector (ThermoFischer Scientific). mAme was
amplified from the pBad cloning vector using primers with KpnI (forward) and NotHFI (reverse) sites, and subcloned at the 5’-terminus of the pMT-Met, to create pMT-mAme-linker-Met (mAme-Met).

A similar procedure was used to construct the pMT-Met-linker-mAme (Met-mAme), with some exceptions. Primers harboring KpnI (forward) and NotHFI (reverse) sites were used to amplify met, while primers with NotHFI (forward containing linker sequence) and BstBI (reverse) sites amplified mAme.

SRC was amplified from the TOPO cloning vector using primers harboring BstB1 (forward) and AgeI (reverse) restriction enzyme sites, and was sub-cloned into the pMT-B vector. Rluc2 was amplified from the pcDNA storage vector using primers harboring XhoI (forward) and BstBI (reverse) sites. The reverse primer also contained a nucleotide “linker” sequence (AGCGGAAGTGGTAGCGGAAGTGGC) to lengthen the distance between the two proteins and decrease probability of incorrect folding. The Rluc2-linker sequence was sub-cloned at the 5’-terminus of the pMT-SRC plasmid, to create pMT- Rluc2-linker-SRC (Rluc2-SRC).

A similar procedure was used to construct the pMT-SRC-linker-Rluc2 (SRC-Rluc2), with some exceptions. Primers harboring Xho1 (forward) and BstB1 (reverse) sites was used to amplify SRC, while primers with BstB1 (forward containing linker sequence) and Age1 (reverse) sites were used to amplify Rluc2.

All four fusion proteins, Rluc2-SRC, SRC-Rluc2, mAme-Met, and Met-mAme, were sequenced (Eton Bioscience, San Diego, CA) to ensure the fluorescent/luminescent proteins were in frame with the respective MfR subunit protein. All fusion constructs were successfully sub-cloned without amino acid substitutions.
**BRET Assay**

BRET assays were performed using methods similar to those described by Powell and Xu 2008 (15). Assays were performed in *Drosophila* Schneider (S2) cells (Invitrogen). Cells were grown in Schneider’s medium, containing 10% heat inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA), 50 mg/ml penicillin G and 50 mg/ml streptomycin sulfate (Fisher Scientific, Pittsburgh, PA), and incubated at 23°C under ambient air atmosphere. Cells were seeded at a density of 3 x 10^6 in 35 mm in 6-well plates and transfected 24 hours after plating.

The relevant plasmids were transfected into cells by calcium phosphate DNA precipitation. Total DNA concentration remained constant across all experiments at 2.83 ng/µL, while the photon donor: protein acceptor ratio was held at an optimized 1:6 ratio (produced highest energy transfer). Transcription was induced by exposing cells to 500 µM CuSO₄ for 24 hours. Cells were treated with test chemical or vehicle control for 1 hour in 1x phosphate-buffered saline medium. The Rluc2 substrate, coelenterazine 400A (Biotium, Inc.), was then added to each well (5 µM), and light emission was measured immediately at 410 ± 40nm (emission produced by Rluc2) and 535 ± 15nm (fluorescence emission produced by mAmetrine) using a FluoroStar fluorimeter (BMG Labtech). The ratio of light emitted at 535nm/410nm (corrected for basal level donor emission of Rluc2 (15, 25)) is termed a BRET ratio. The BRET ratio provided a quantitative measure of the degree of MfR protein association.
Luciferase Reporter Gene Assay

Luciferase-based transcription reporter gene assays were conducted for comparison to BRET with respect to specificity and sensitivity. S2 cells were transfected with daphnid *met* full open reading frame fused to the Gal4 DNA binding domain (22), daphnid SRC, *Renilla Luciferase* (pRL-CMV, internal transfection control, Promega) and the firefly luciferase reporter gene vector (pGL5-Luc, Promega) that contained five upstream GAL4 binding sites. Following transfection, transcription of Met-Gal4 and SRC was induced with CuSO₄ (100 µM for 24 hours). Cells then were treated with test chemical in Ex-cellTM 420 insect serum-free medium with L-glutamine (SAFC Biosciences, Sigma, St. Louis, MO). Cells were harvested after 24 hours incubation. Emissions from the firefly luciferase and *Renilla* luciferases were measured using the Dual-Glo® luciferase system (Promega). Firefly luciferase emission was normalized to *Renilla* luciferase emission, and each chemical treatment group was normalized to DMSO control treated cells.

Male Sex determination

*D. magna* were cultured under parthenogenetic rearing conditions described previously (29). Hydroprene and diofenolan were used in *in vivo* exposure assays to determine their potency in stimulating male sex determination. Gravid adult female daphnids of the same age were selected from cultures, and placed individually in 50 mL beakers containing 40 mL daphnid media. The daphnids were exposed to serial dilutions of the evaluated chemicals, hydroprene and diofenolan, where each treatment was replicated ten times. Solutions were renewed daily. Animals were fed 0.20 mg (dry wt) fish food and 7 x 10⁶ algae cells (*Pseudokirchneriella subcapitata*) daily and daphnids were assessed for brood production.
and molting daily. The percentage of males in the second brood was used to determine chemical potency. Experiments were terminated after female released second brood. The ability of methoprene, kinoprene, pyriproxyfen and fenoxycarb to stimulate male sex determination was evaluated previously (22, 30).

**Statistical Analysis**

Comparisons of two means were evaluated for significance (p<0.05) using Student’s t test. Equal variance between multiple treatment groups was confirmed with Brown-Forsythe’s test. One-way analysis of variance (ANOVA), followed by Tukey’s multiple comparison procedure, was used to evaluate significant differences between the control and multiple treatments. EC$_{50}$ values were determined using the logistic equation and fixed asymptotes at minimum and maximum chemical treatment concentrations. All statistics were performed using Origin software (OriginLab Corp., Northhampton, MA).

**Results**

*MfR Subunit Interaction*

Fusion proteins were constructed (Fig. 1) in different configurations and used in BRET assays to determine which configuration provided the strongest BRET ratio. Significant increases in BRET ratio upon the addition of methyl farnesoate occurred with all fusion protein configurations (Fig. 2). However, the greatest increase in BRET ratio between the control and hormone treatment occurred in cells expressing both subunit proteins fused at
the N-terminus with the fluorophore or luciferase proteins: mAme-Met and Rluc2-SRC (Fig. 2). All subsequent BRET assays were performed with these two protein constructs.

Twenty-nine environmental chemicals were screened at 100 µM for their ability to stimulate MfR subunit association (Table 1). Seven compounds, including the positive control methyl farnesoate, significantly (p<0.05) stimulated MfR subunit association compared to controls (Fig. 3). These chemicals, methoprene, kinoprene, pyriproxyfen, fenoxycarb, diofenoaln, and hydroprene, were all insect growth regulating (IGR) insecticides. The maximum BRET ratios among the IGRs ranged from 1.3 (kinoprene) to 6.6 (diofenolan).

The seven compounds that screened positive were assayed over a range of chemical concentrations to determine relative potency in stimulating MfR association (EC$_{50}$). The positive control, methyl farnesoate, significantly activated subunit association at 3 µM, and has an EC$_{50}$ value of 2.7 µM (Fig. 5A). Methoprene (EC$_{50}$ = 6.06 µM) (Fig. 5B) and kinoprene (EC$_{50}$ = 31.6 µM) (Fig. 5C) were both less potent at stimulating subunit assembly compared to methyl farnesoate. The remaining compounds, pyriproxyfen (EC$_{50}$ = 0.6 µM) (Fig. 5D), hydroprene (EC$_{50}$ = 1.5 µM) (Fig. 5E), diofenolan (EC$_{50}$ = 0.025 µM) (Fig. 5F), and fenoxycarb (EC$_{50}$ = 0.0022 µM) (Fig. 5G), were all more potent than the hormone at MfR subunit association.

The same suite of twenty-nine environmental chemicals (Table 1) also were screened for their ability to stimulate MfR-initiated gene transcription (Fig. 4). Only six compounds, including the positive control, methyl farnesoate, significantly stimulated gene transcription of the reporter gene at 100 µM compared to controls. Despite activating MfR subunit assembly at 100 µM (Fig. 5C), kinoprene did not activate gene transcription (Fig. 6C).
Concentration-response analyses was performed with respect to reporter gene transcriptional activation using methyl farnesoate and the same suite of seven compounds that were active in the BRET assay. Methyl farnesoate significantly stimulated transcription at 30 µM and had an EC$_{50}$ value of 10.0 µM (Fig. 6A). Less potent than the hormone in the reporter gene assay was methoprene (EC$_{50}$ = 18.1 µM) (Fig. 6B) and kinoprene (Fig. 6C), which did not activate gene transcription. Pyriproxyfen (EC$_{50}$ = 0.30 µM) (Fig. 6D), hydroprene (EC$_{50}$ = 0.26 µM) (Fig. 6E), diofenolan (EC$_{50}$ = 1.3 µM) (Fig. 6F), and fenoxycarb (EC$_{50}$ = 0.0034 µM) (Fig. 6G) were all more potent than MF in activating gene transcription.

Male Sex Determination

Lastly, we evaluated the ability of the novel BRET MfR subunit association assay to predict male sex determination in *D. magna*. Two of the most potent chemicals in generating BRET signal, hydroprene and diofenolan, also produced the male offspring phenotype at relatively low concentrations. The daphnids exposed to chemical produced 100% male offspring at 0.001 nM diofenolan (Fig. 7A), and 0.03 nM hydroprene (Fig. 7B). The ability of methoprene, pyriproxyfen and fenoxycarb to stimulate male sex determination was evaluated previously (22, 30).

Discussion

We have constructed a sensitive and rapid approach to assess chemical toxicity using bioluminescence resonance energy transfer (BRET) technology. BRET boasts utility in...
measuring various protein-protein interactions, including but not limited to cytosolic and nuclear protein interactions, transmembrane and soluble protein interactions, as well as agonist and inhibitor receptor interactions (31, 32). However, our application of the protein-protein interaction monitoring technology as an approach to environmental chemical toxicity screening for invertebrates is the first of its kind.

We paired the recently developed version of Renilla luciferase, Rluc2, which emits luminescent signals fifty times greater than the wild type gene (33), with the yellow fluorescent light protein, mAmetrine. This protein pair was recently identified as a novel configuration for BRET protein analysis despite the sub-optimal quantum yield of mAmetrine (27). However, the high luminescence yield of Rluc2 and mAmetrine’s excitation peak in the dark blue region of the light spectrum make the two a proper pair for resonance energy transfer (27).

The sequential orientation of our receptor subunit proteins, relative to the fluorescent and bioluminescent BRET protein tags, were optimized to produce the greatest difference between the control- and methyl farnesoate-treated cells (Fig. 2). To produce significant energy transfer, the BRET proteins must be in close proximity to one another (Fig. 1). The ideal distance, known as the Förster radius, or fluorophore separation distance corresponding to 50% of the maximum resonance energy transfer, is 1-10 nm. Dacres et al. reported a Förster radius values of 4.40 ±0.02 nm in a similar BRET system (34).

Since the binding confirmation of the methyl farnesoate receptor subunits is currently unknown, we constructed and tested four total fusion proteins with the receptor subunit proteins either N- or C-terminally fused to the luminescent or fluorescent proteins, as per the recommendation of other research groups (25). The largest difference between the control-
and methyl farnesoate-treated cells was produced by cells transfected with receptor subunit proteins fused at the N-terminus to photon emitting proteins (Fig. 2). The BRET ratio increased 4.1-fold when cells in this treatment group were exposed to the hormone, while the BRET ratio from the other treatment groups increased by 2.3, 3.1 and 2.2-fold, respectively (Fig. 2). Often, this “N-terminal orientation” produces the highest energy transfer because the N-terminal of the luciferase and fluorescent proteins are minimally restricted (34).

We selected twenty-nine environmental compounds to screen in the assay based on the likelihood of exposure or toxicity. A considerable number of these compounds have been named by the Environmental Protection Agency as High Production Volume compounds, or compounds produced or imported into the United States in quantities equal to or greater than one million pounds per year (35, 36). These were included due to high probability of exposure. None of these compounds exhibited discernable activity with the MfR.

In contrast, all of the IGR insecticides evaluated exhibited some level of activity with the MfR in the BRET assay, and five of the six IGRs were active in the luciferase reporter gene assay. We selected these compounds because precedence exists for some IGRs stimulating male sex determination in branchiopod crustaceans (5, 22, 37-39). A few of the remaining screened compounds were selected based upon structural similarity to the IGRs. Structural similarity was determined using the National Center for Biotechnology Information’s PubChem Compound search tool (www.ncbi.nlm.nih.gov).

Positively tested compounds included kinoprene, methoprene, pyriproxyfen, hydroprene, diofenolan and fenoxy carb (Fig. 3), some of which have been evaluated for their ability to activate this endocrine signaling pathway. However, to our knowledge hydroprene has never been evaluated for MfR activation, namely ligand-mediated transcriptional
activation, but stimulated MfR dimerization at 0.3 µM (Fig. 5E). Iguchi et al. reports an EC$_{50}$ value of 2.9 µg/L for hydroprene to stimulate male sex determination (39). Although none of the tested compounds have been evaluated for ligand-mediated receptor assembly, pyriproxyfen (22), diofenolan (37), and hydroprene (23) have all been reported to stimulate transcriptional activation and male sex determination in daphnids. Further, gene ontology studies suggest diofenolan activates carbohydrate catabolism, cellular macromolecule biosynthesis, nitrogen compound metabolism, cellular component localization, NADH dehydrogenase gene networks and alters expression of JH-responsive genes e.g., hemoglobin, glutathione, cuticle protein, serine protease and vitellogenin-related genes in daphnids after neonatal exposure (40).

All of the compounds that tested positive in the single-concentration BRET screen were confirmed to be active using more definitive concentration-response analysis. Further, all but the weakest agonist in the BRET assay, kinoprene, stimulated male sex determination in vivo. These results suggest that the BRET assay is accurate in the detection of MFR-active compounds, and may be overly sensitive in detecting weak-acting compounds that are inactive in vivo. Further, all compounds that were inactive in the more traditional luciferase reporter gene assay were also inactive in the BRET assay (Figs. 3 and 4). Ligand-binding affinity and fusion protein subunit conformation upon ligand-binding may contribute to the differences between tested compounds in relative BRET ratio (Fig. 3), or relative luciferase units (Fig. 4), as described by Powell et al. (15).

A major advantage of the BRET assay over the reporter gene assay is it functions on the premise that MfR agonist exposure results in ligand-binding to the Met protein resulting in immediate association of the Met and SRC. The resulting fluorescence endpoint can be
measured immediately in the intact cells. In contrast, the reporter gene assay requires transcription of the reporter gene, translation of the resulting mRNA, and accumulation of the reporter protein before the assay results can be measured. Thus, a BRET assay can be conducted with extant cells in less than 90 min, while a comparable reporter gene assay would require at least a day. The BRET methodology is also highly conducive to high-throughput formatting.

Agonist-stimulated receptor dimerization occurs in several nuclear receptors including the estrogen (15), peroxisome proliferator-activated, and thyroid receptors (16). Conceivably, these receptors also could be screened for chemical interaction using a BRET assay. This would significantly increase the efficiency of screening large numbers of chemicals for activity towards a suite of nuclear receptors.
References


32. C. Couturier, B. Deprez, Setting Up a Bioluminescence Resonance Energy Transfer High throughput Screening Assay to Search for Protein/Protein Interaction Inhibitors in Mammalian Cells. *Front Endocrinol* **3**, 100 (2012).


35. USEPA, in *High Production Volume Information System (HPVIS)*.


**Figure 1. The detection of MfR agonists using BRET**

A) SRC is fused to Rluc2, which metabolizes coelenterazine 400A to emit light at 410nm. B) In the presence of an agonist (Ag), dimerization with Met is stimulated and the light emitted at 410nm excites the proximal yellow fluorescent protein, mAmetrine (mAme), that is fused to Met to emit a secondary light at 535nm.
Figure 2. BRET signaling using MfR subunits with different bioluminescent/fluorescent protein configurations Cells were transfected with four different sets of fusion proteins, where Met was fused with a mAmetrine and SRC was fused with Renilla luciferase 2 at either the N- or C- terminus. White bars indicate cells treated with vehicle (DMSO) control and grey bars indicate cells treated with 100 µM methyl farnesoate. Data are presented as mean with standard deviation (n = 3) and * denotes a significant increase in Met: SRC binding compared to control (p<0.05).
### Table 1. Chemicals screened in the MfR BRET assay

<table>
<thead>
<tr>
<th>CHEMICAL NAME</th>
<th>IUPAC NAME</th>
<th>COMMERCIAL USE(S) OR ACTIVITY</th>
<th>SCREENING RATIONALITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>methyl farnesoate</td>
<td>methyl (2E,6E)-3,7,11-trimethyldodeca-2,6,10-trienoate</td>
<td>crustacean hormone</td>
<td>endogenous hormone</td>
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<td>1,2,4-triazole pyrrodiazole dimethylpropanoic acid (dmpa)</td>
<td>1H-1,2,4-triazole</td>
<td>antifungal, fluconazole</td>
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<td>trizma base</td>
<td>2-amino-2-(hydroxymethyl)-propane-1,3-diol</td>
<td>industrial adhesives, sealant and lubricant</td>
<td>1</td>
</tr>
<tr>
<td>4-vinlylcyclohexene</td>
<td>4-ethenylcyclohexene</td>
<td>fuels/additives, solvents</td>
<td>1</td>
</tr>
<tr>
<td>isophthalonitrile</td>
<td>benzene-1,3-dicarbonitrile</td>
<td>agricultural products</td>
<td>1</td>
</tr>
<tr>
<td>2-nitropropane</td>
<td>2-nitropropane</td>
<td>industrial organic solvent for coatings, dyes, adhesives</td>
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<tr>
<td>Resorcinol</td>
<td>benzene-1,3-diol</td>
<td>industrial adhesives/sealants, textiles, and plastic</td>
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</tr>
<tr>
<td>acetyltributyl citrate</td>
<td>tributyl 2-acetoxynoxypropane-1,2,3-tricarboxylate</td>
<td>industrial plasticizers, adhesives, and food packaging</td>
<td>1</td>
</tr>
</tbody>
</table>

---

1) EPA high production volume chemical 2) Chemical is known to stimulate male sex determination in daphnids 3) Chemical has similar structure to known stimulators of male sex determination in daphnids 4) Chemical mimics the action of 20-hydroxyecdysone in *Lepidopterous*
Table 1 continued.

<table>
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<th>Structure/Name</th>
<th>Category and/or Uses</th>
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<td>4-hydroxyanisole</td>
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<td>2-ethenylpyridine</td>
<td>industrial intermediate</td>
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<tr>
<td>benzoyl chloride</td>
<td>Benzoyl chloride</td>
<td>industrial intermediate</td>
<td>1</td>
</tr>
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<td>triclocarban</td>
<td>1-(4-chlorophenyl)-3-(3,4-dichlorophenyl)urea</td>
<td>antibacterial for personal care products</td>
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<tr>
<td>1,3,5-trioxane</td>
<td>1,3,5-trioxane</td>
<td>industrial intermediates</td>
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</tr>
<tr>
<td>1,5-cyclooctadiene</td>
<td>cycloocta-1,5-diene</td>
<td>industrial intermediates and solvents</td>
<td>1</td>
</tr>
<tr>
<td>anethole</td>
<td>1-methoxy-4-[(E)-prop-1-ethyl]benzene</td>
<td>industrial intermediates and personal care products</td>
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</tr>
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<td>3,3',5,5'-tetrabromobisphenol A</td>
<td>2,6-dibromo-4-[2-(3,5-dibromo-4-hydroxyphenyl)propan-2-yl]phenol</td>
<td>flame retardants, electronics and plastics</td>
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<td>triclosan</td>
<td>5-chloro-2-(2,4-dichlorophenoxy)phenol</td>
<td>antibacterial/fungal for personal care products</td>
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<td>methoprene</td>
<td>propan-2-yl (2E,4E)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate</td>
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<td>pyriproxyfen</td>
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<td>dieldrin</td>
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<td>organochlorine insecticide</td>
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<tr>
<td>endrin</td>
<td>(1R,2S,3R,6S,7R,8S,9S,11R)-3,4,5,6,13,13-hexachloro-10-oxapentacyclo[6.3.1.1(^{3,6}).0(^{2,7}).0(^{9,11})]tridec-4-ene</td>
<td>organochlorine insecticide</td>
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<td>atrazine</td>
<td>6-chloro-4-N-ethyl-2-N-propan-2-yl-1,3,5-triazine-2,4-diamine</td>
<td>triazine herbicide</td>
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<td>kinoprene</td>
<td>prop-2-ynyl (2E,4E,7S)-3,7,11-trimethylidodeca-2,4-dienoate</td>
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<tr>
<td>methyl geranate</td>
<td>methyl (2E)-3,7-dimethylocta-2,6-dienoate</td>
<td>3</td>
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<td>heptanoic acid</td>
<td>Heptanoic acid</td>
<td>industrial intermediate and lubricant</td>
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<tr>
<td>diofenolan</td>
<td>2-ethyl-4-[(4-phenoxyphenoxy)methyl]-1,3-dioxolane</td>
<td>insect growth regulator insecticide</td>
<td>3</td>
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<tr>
<td>hydroprene</td>
<td>ethyl (2E,4E)-3,7,11-trimethylidodeca-2,4-dienoate</td>
<td>insect growth regulator insecticide</td>
<td>3</td>
</tr>
<tr>
<td>farnesol</td>
<td>(2E,6E)-3,7,11-trimethylidodeca-2,6,10-trien-1-ol</td>
<td>plant/animal acyclic sesquiterpenoid, pesticide</td>
<td>3</td>
</tr>
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</table>
Figure 3. Chemical screen for MfR activation using BRET assay

White bar indicates DMSO vehicle control, light grey bar indicates endogenous hormone ligand positive control, and dark grey bars indicate screened chemicals. Data are presented as means with standard deviations (n = 3) and * denotes significant increase in Met-SRC dimerization compared to DMSO control (p<0.05).
Figure 4. Chemical screen for MfR activation using luciferase reporter gene assay

White bar indicates DMSO vehicle control, light grey bar indicates endogenous hormone ligand positive control, and dark grey bars indicate screened chemicals. Data are presented as means with standard deviations (n = 3) and * denotes significant increase in Met-SRC dimerization compared to DMSO control (p<0.05).
Figure 5. MfR BRET assay: concentration-responses for MfR activators in initial screen

Cells containing the MfR BRET fusion proteins were treated with increasing concentrations of positively screened compounds. The red circle on the y-axis represents the control group where cells were treated with the DMSO vehicle control. Data are presented as mean with standard deviation (n = 3) and * denotes significant increase in Met: SRC dimerization compared to control (p<0.01).
Figure 6. Reporter gene assay: concentration responses for MfR activators in initial screen

Cells expressing MfR subunits and a luciferase reporter gene were treated with increasing concentration of positively screened compounds in the BRET assay. The red circle on the y-axis represents the control group where cells were treated with the DMSO vehicle control. Data are presented as means with standard deviations (n = 3) and * denotes significant increase in transcription of the luciferase reporter gene compared to control (p<0.05).
Figure 7. Percent of male containing broods produced in response to diofenolan and hydroprene exposure. Adult female daphnids were exposed to diofenolan (A) and hydroprene (B). The red circle on the y-axis represents the control group animals that were treated with the ethanol vehicle control. Data are presented as percent total males from each treatment concentration (n = 9-10) and red
CHAPTER FOUR: A SEARCH FOR A CRUSTACEAN METHYL FARNESOATE RECEPTOR RESPONSE ELEMENT

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Abstract

Nuclear receptor proteins typically mediate cellular signals by binding respective agonists, and subsequently initiating gene transcription that contributes to many different biological processes, e.g., homeostasis, metabolism, reproduction and development. These proteins typically are characterized by distinctive DNA-binding domains. The crustacean methyl farnesoate receptor (MfR) contains two proteins each with basic helix-loop-helix (bHLH) DNA-binding domains. The two subunits, the methoprene-tolerant transcription factor (Met) and the steroid receptor coactivator (SRC), are activated via dimerization by methyl farnesoate and initiate downstream gene transcription. We hypothesized that the DNA sequence to which the MfR docks, the methyl farnesoate response element (MfRE), was a short conserved nucleotide sequence, upstream of genes regulated by MfR agonists in Daphnia sp. Three searches of the Daphnia pulex genome were conducted to identify putative response elements, using Biopython and previously defined response elements of orthologous receptor complexes in insects. Regions up to 5kb upstream of transcription start sites, including genes regulated by MfR agonist exposure, were screened for sequences with homology to GGCTCCACGTG, CACG⁻¹cG, and CACGYGRWG. Of the 159 putative MfREs identified, nine double-stranded sequences were biotinylated to assess MfR binding in electrophoretic mobility shift assays (EMSA). Putative MfREs were assayed in the presence and absence of methyl farnesoate using protein fractions from cells transfected with daphnid Met and SRC fused to V5 protein sequence for immunochromical detection. Western blot analysis confirmed protein expression of both Met-V5 and SRC-V5 in nuclear and cytosolic fractions. No shift indicative of protein:DNA binding occurred with all nine
putative MfREs, suggesting the oligonucleotides screened were not the MfR DNA binding sequence.
**Introduction**

Many members of the bHLH-PAS protein family, named for their characteristic binding domains, function as transcription factors that modulate gene transcription of genes involved in development (1), homeostasis (2) and environmental sensing (3, 4). These proteins often form heterodimeric complexes (5-7) when employed as transcription factors.

Each member of this protein family contains canonical domains that permit their mediatory function in the various signaling pathways. The most N-terminal domain, basic helix-loop-helix domain (bHLH), positions the transcription factor complex for optimal major groove-binding on respective DNA response elements, as well as participates in protein-protein binding (8-10). The Per-Arnt-Sim (PAS) domain is a conserved region with homology to three founding members for which it is named: period protein, aryl hydrocarbon receptor nuclear translocator protein, and single-minded protein. These domains act in protein hetero-dimerization (6), binding cellular chaperones (11), and binding ligands (7, 12).

Steroid receptor coactivator proteins (SRC) represent a sub-category of bHLH-PAS proteins that are involved in reproductive, cancer and metabolic signaling (13). Often recognized as transcription enhancers for ligand-binding receptors, SRCs have also been shown to serve as DNA-binding partners to other bHLH-PAS proteins (6, 14). Li et al. revealed that the bHLH domain of SRC from *Aedes aegypti* was necessary for DNA binding of both subunits in a heterodimeric receptor (6). Members of this protein family are distinguished by unique C-terminal domains including: LxxLL motifs (where “L” is leucine and “x” is any amino acid) for ligand-bound receptor protein binding, a glutamine (Q)-rich
region, and activation domains that have histone methylation and chromatin remodeling capabilities (13, 15).

The bHLH family of proteins regulate gene transcription by binding small consensus sequences called “E-box,” or enhancer box, motifs. These conserved palindromic hexa-nucleotide motifs (CANNTG) provide a docking site for the dimeric transcription factors (16-18). The basic nucleotides contained within the larger bHLH domains interact with the major groove of the DNA double-helix (10, 17, 18). For example, Ellenberger et al. reported a crystal structure of the Drosophila E47 transcription factor homodimer binding the DNA E-box with amino acids in its N-terminal helix and loop regions. Each amino acids contained basic side chains and interacted with the E-box half-sites through hydrogen binding and Van der Waals forces (17).

The crustacean methyl farnesoate receptor (MfR) was recently identified in Daphnia sp. (5, 19) and is composed of the methoprene-tolerant transcription factor (Met) and the steroid receptor coactivator (SRC) (5, 19). Based on structural similarity, both daphnid subunits possessed bHLH and PAS domains characteristic of the protein family, while SRC has several LxxLL motifs (5, 19, 20). Ligand-mediated MfR activation was confirmed using a luciferase reporter gene transcription activation assay in the presence of the methyl farnesoate homrone (5).

The methyl farnesoate signaling pathway regulates various aspects of reproduction and development in crustaceans (21-23). However, some environmental chemicals have been shown to disrupt this signaling pathway leading to gynadromorphism in offspring (24) and increased number of male offspring (25). In fact, many compounds known as insect growth regulating insecticides (IGRs) directly interact with MfR subunit assembly (26) and initiate...
MfR transcriptional activation (5, 26). Neonatal daphnids exposed to IGRs, methoprene or fenoxycarb, exhibited altered expression of genes including juvenile hormone epoxide hydrolase (methyl farnesoate synthesis), vitellogenin (precursor to egg yolk protein) fused with superoxide dismutase, and hemoglobin-related genes (27). Similarly, hemoglobin-related genes were induced in adult female daphnids exposed to fenoxycarb and pyriproxyfen, other potent IGRs (28). While the expression of vitellogenin-related genes were inhibited after exposure to the same compounds (29).

An endogenous MfR-docking DNA sequence that initiates downstream gene expression (a methyl farnesoate response element (MfRE)), remains elusive, despite the highly characterized receptor interactions (5, 20) and gene expression networks (27) of the MfR endocrine signaling pathway. Response elements in insects with orthologous receptors have been identified. The insect juvenile hormone receptor (JHR) initiates gene transcription of Kruppel homolog 1, a suppressor of insect metamorphosis, by binding a short consensus “E-box” sequence (CACGTG) found in the promoter region of many insect species (30). The JHR also binds a different short sequence (CCACACGCAGAAG) found upstream of the early trypsin gene, a sex-specific mid-gut protease, in Drosophila (31).

We hypothesized that the daphnid MfRE, was a short conserved nucleotide sequence, upstream of genes differentially expressed after in vivo exposure of daphnids to methyl farnesoate or its analogs. Herein, we provide a thorough description of the search methods and results of several attempts in identifying an endogenous MfR response element.
Methods

Genome-wide MfRE searches

Three genome-wide searches were conducted using the published *Daphnia pluex* genome (www.fleabase.org), three consensus sequence/motifs, and other variable parameters. All calculations were performed in Biopython (http://biopython.org/wiki/Documentation).

In the first search, the *D. pulex* genome was screened for candidate MfREs by searching for sequences with homology to *Bombyx mori* kJHREc (GGCCTCCACGTG), a functionally validated JHRE that regulates *Kruppel homolog 1* gene expression (30). Promoter regions 5-kb upstream from transcription or translation start sites in the *D. pulex* genome were scanned for similarity to a kJHREc consensus sequence using a 12-bp sliding window. The kJHREc consensus sequence was generated from sequences with similarity to kJHREc that were found in the promoter regions of Kr-h1 in the *B. mori*, *T. castaneum*, *A. mellifera*, *N. vitripennis*, *A. pisum*, and *D. melanogaster* gnomes. A position-specific scoring matrix (PSSM) was calculated using the kJHREc consensus sequence. A preliminary list of candidate MFREs and corresponding regulated genes was generated by scanning all *D. pulex* promoter regions, and their reverse complements, using the PSSM and a false-positive rate of $10^{-6}$.

A second *D. pulex* genome-wide MfRE search was conducted using the six-base palindromic canonical E-box motif (CACGTG) or the non-palindromic E-box-like motif (CACGCG); sequences known to bind bHLH protein domains (30, 32). Promoter regions, and their reverse compliments, 5-kb upstream from transcription start sites were scanned for
similarity to the binding motif sequences using a 9-bp sliding window and false-positive rate of $10^{-6}$.

The third and final MfRE search identified sequences with homology to the nine-base consensus sequence CACGYGRWG (32). This sequence is inclusive of experimentally proven Met-binding motif in *Aedes aegypti*, and contains the CACGTG and CACGCG E-box motifs, a JHRE in *B. mori* (30, 32). Promoter regions, and their reverse compliments, up to 5-kb upstream from transcription start sites were scanned for similarity to the consensus sequence using a 9-bp sliding window and false-positive rate of $10^{-6}$. The preliminary list of candidate MfREs was filtered by removing all sequences that did not contain either the E-box motif in the first six bases.

Final candidate MfREs were generated by finding the overlap between the preliminary list of MfREs from each search with a list of genes differentially expressed in response to MfR agonists.

*Western Blotting*

Western blot analysis was performed for Met and SRC to ensure cells were producing desired daphnid MfR subunit proteins (for use in electrophoretic mobility shift assays). *Drosophila Schneider* (S2) cells (Invitrogen) were grown in Schneider’s medium (Gibco, Carlsbad, CA, USA), containing 10% heat inactivated fetal bovine serum (Gibco), 50 mg/ml penicillin G (Fisher Scientific, Pittsburgh, PA), 50 mg/ml streptomycin sulfate (Fisher Scientific) and incubated at 23°C under ambient air atmosphere. Cells were seeded at a density of $3 \times 10^6$ in 35 mm in 6-well plates. Twenty-four hours after plating, cells were transfected with either empty plasmid vectors, or plasmid vectors containing the entire open
reading frame of daphnid *Met* (5) or *SRC* (20) genes fused with the V5 epitope sequence for antibody recognition. Transfections were performed by calcium phosphate DNA precipitation and total DNA transfected was constant across treatments. Transcription of the transfected genes was induced with CuSO₄ (500 µM) and twenty-four hours later proteins were fractionated into sub-cellular compartments (cytoplasmic and nuclear) using NE-PER™ nuclear and cytoplasmic extraction kit and manufacturer’s protocol (ThermoFisher Scientific). For each protein sample, absorbance was measured at 595 nm on a Spectronic Gensys 2 spectrometer and protein concentrations were determined using a bovine serum albumin standard curve.

Approximately 60 ng total protein and 5 µL 4x SDS loading buffer in a total volume of 20 µl was loaded in each well of a NuPAGE® Tris-Acetate polyacrylamide gel (Life Technologies). The Novex® Sharp pre-stained protein standard (Invitrogen) and protein samples were run at 150 V for 1 hr. Proteins were transferred to a nitrocellulose membrane by electrophoresis and non-specific protein binding was blocked with a 5% dry milk in tris-buffered saline with Tween 20 (Sigma-Aldrich, St. Louis, MO) (TBST) wash. The membrane was then washed in TBST with the primary antibody, AntiV5-IgG (Invitrogen) for 1 hr and then the secondary antibody, AntiIgG-HRP (Invitrogen) for 1 hr. The membrane was washed in a 1:1 luminol: peroxide solution (Pierce™) and exposed to autoradiography film for 10 sec.

**Electrophoretic Mobility Shift Assays**

Binding of the MfR to putative MfREs was tested using electrophoretic mobility shift assays (EMSAs). Double-stranded biotinylated oligonucleotide sequences representing the
candidate MfREs were synthesized (Integrated DNA Technologies, Inc., Coralville, Iowa) for use in electrophoretic mobility shift assays.

At least one putative MfRE was screened for MfR binding from each of the three distinct searches. A total of nine putative MfRE were screened. Methyl farnesoate (Echelon Biosciences Inc., Salt Lake City, Utah) was dissolved in 95% ethanol for delivery to the assays. At least 16 µg Met-V5 and SRC-V5- containing protein fractions were incubated with a putative MfRE probe and 2.5 mM methyl farnesoate for 20 min at room temperature. Each 20 µL binding reaction contained 2 µL 10x binding buffer, 0.5 µM double stranded biotin-labeled MfRE oligonucleotide, 2.5 % glycerol, 5mM MgCl2, 50 ng/µl Poly dI·dC, and 0.05%NP-40. Epstein-Barr nuclear antigen (EBNA) positive control binding reactions (ThermoFisher Scientific) consisted of 1 U EBNA protein extract, 20 fmol biotin-labeled EBNA control DNA, and 4 pmol unlabeled EBNA DNA.

Binding reactions were loaded onto a 6% polyacrylamide DNA retardation gel and electrophoresed in Tris/Borate/EDTA buffer at 100 V for 70 min. DNA was transferred by electrophoresis onto a nylon membrane at 100 V for 40 min, then UV-crosslinked to the membrane. Blocking buffer from the Lightshift™ Chemiluminescent RNA EMSA Kit (ThermoFisher Scientific) was used to block non-specific binding for 30 min. A streptavidin-HRP antibody wash followed by a 1:1 solution luminol: peroxide wash enabled probe visualization. Autoradiography film was exposed to membrane for no more than 30 sec.
Results

We sought to identify the sequence of the MfRE in *Daphnia pulex*. Three separate queries were preformed to identify putative DNA-binding sites within the *D. pulex* genome. The first search was conducted using the *Bombyx mori* kJHRe (GGCCTCCACGTG). Only one sequence with homology to kJHRe was located upstream of hemoglobin, a gene known to be regulated by methyl farnesoate analogs (Table 1).

The second search for putative MfREs was conducted using either the palindromic canonical E-box motif (CACGTG) and the non-palindromic E-box-like motif (CACGCG), known to bind bHLH domain-containing proteins (32). There were 82 and 33 sequences, respectively, with homology to the queried sequence (Table 1) and upstream of transcription start sites of genes known to be regulated by methyl farnesoate analogs. These sequences were located upstream of the ecdysone receptor, probable nuclear hormone receptor HR3, nuclear hormone receptor Ftz-f1β, nuclear hormone receptor Ftz-f1α, vitellogenin putative, vitellogenin receptor precursor, vitellogenin fused with superoxide dismutase, heat shock protein 90, hemoglobin, di-domain hemoglobin precursor genes (Table 2).

The third and final search to locate putative MfREs in the *D. pulex* genome was conducted using the *A. aegypti* nine-base consensus sequence CACGYGRWG (32). The query resulted in 43 putative MfRE sequences with homology to the *A. aegypti* consensus sequence (Table 1) and upstream of transcription start sites of genes known to be regulated by methyl farnesoate analogs. Our three separate queries produced varying quantities of putative MfREs, depending on the queried sequence and search parameters. However, the total putative sequences provided us with ample MfREs to screen for MfR binding.
Western blot analysis confirmed the transcription and translation of Met and SRC. Both Met and SRC proteins were localized in both cytoplasmic (Fig. 1, Lanes 3 and 4) and nuclear (Fig. 1, Lanes 7 and 8) fractions. Therefore, we utilized both cellular and nuclear fractions from cells transfected with Met and SRC to analyze DNA: protein binding in EMSAs.

Nine putative MfREs were screened for MfR protein binding using EMSAs (Tables 3A-F). Regardless of the MfR protein subunit included and the presence of methyl farnesoate, none of the screened putative MfREs resulted in a shifted band characteristic of protein-bound DNA (Fig. 2A-F). These results suggest that none of the nine putative MfREs screened in this study contain the MfRE.

We also conducted a set of positive control (EBNA) binding reactions to confirm all EMSA experimental conditions were suitable for specific protein:DNA binding (Fig. 2A). The EBNA biotin-labeled probe (Fig. 2, Lane 1) shifted with the addition of EBNA protein extract (Fig. 2, Lane 2) indicating successful protein and DNA binding. The binding is mitigated upon the addition of unlabeled-EBNA DNA, confirming that the protein and DNA binding interaction was specific. Positive controls for each successive MfRE EMSA resulted in the same shift (Data not shown). Taken together with the negative MfRE: MfR binding results, we can conclude none of the screened putative MfR DNA binding sequences are the endogenous MfRE.
Discussion

Over the past several decades many groups have contributed to the elucidation of the daphnid methyl farnesoate signaling pathway (5, 21, 25, 33-36). The more recent identification of the daphnid methyl farnesoate receptor complex (5) and gene networks differentially regulated post-methyl farnesoate ortholog exposure (36) prompted this search for an endogenous DNA docking site. Although our search for the MfRE was unsuccessful, a solid foundation was established for future the efforts toward MfRE discovery.

Western blot analysis confirmed the receptor subunits were not confined to one cellular compartment, but that both Met and SRC exist in cytoplasmic and nuclear fractions. Our previous work indicates that multimeric Met dissociation and MfR subunit assembly only occur in the presence of methyl farnesoate (20), yet the western blot analyses were conducted in the absence of the hormone. Conceivably, multimeric Met and SRC exist in the cytoplasm while cellular methyl farnesoate levels are negligible. Then, in the presence of the hormone, Met exchanges its homo-multimeric form for its heterodimeric partner, SRC, and translocates into the nucleus. This is similar to the well-established aryl hydrocarbon receptor (AhR): aryl hydrocarbon receptor translocator (ARNT) heterodimer activation paradigm (7).

Our strategic and thorough search for putative response elements provided us with many testable sequences. We were unable to test all possibilities due to the large number of putative sequence results. Ultimately, none of the screened sequences produced a band shift in the EMSAs. However, we can confidently conclude the reaction environment of the experimental binding reactions were conducive to protein-DNA binding since our positive EBNA control resulted in a distinct band shift.
We propose a continuation of this study using a new strategic approach moving forward. We could search the promoter region up to 5 kb upstream of a single differentially regulated gene. We could systematically delete portions of the promoter region to determine which sequence(s) are important for MfR binding. Some research groups have had success in determining orthologous receptor response elements by focusing on a promoter region upstream of a single gene differentially regulated by the endogenous hormone (6, 31).

Identifying the MfRE would be an invaluable step towards completing this crustacean environmental endocrine signaling pathway. Many groups have contributed to identifying environmental cues (25, 34), neurological signaling (35), hormone synthesis (37), receptor identification (5) and activation (20), and gene expression networks (36) involved in daphnid reproduction and development. However a gap between receptor activation and gene expression remains. Knowledge of an endogenous response element may even support the development of a more accurate screening tool for chemicals that activate MfR-mediated gene transcription, as well as the elucidation of other developmental signaling pathways since they have been linked mechanistically (38).
References


35. K. Toyota, H. Miyakawa, K. Yamaguchi, S. Shigenobu, Y. Ogino, N. Tatarazako *et al*., NMDA receptor activation upstream of methyl farnesoate signaling for short day-induced male offspring production in the water flea, *Daphnia pulex*. *BMC Genom* 16, (2015).


**Table 1. Summary of the variable parameters for MfRE searches** Five parameters varied between the first, second and third *D. pulex* genome-wide search for putative MfRE(s); the nucleotide sequence search, rationale for each sequence, how initial search results were filtered, number of final putative sequences, and the number of results assayed by EMSA binding analysis.

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Table 2. Genes that are modulated by MfR agonist exposure Modulated genes used to filter the initial sequence results of search 1 and 2 (27).

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Figure 1. Expression of Met and SRC proteins in cells transfected with the *Met* and *SRC* open reading frames. Lanes 1-4 contain cytoplasmic proteins from untransfected cells (UNT), cells transfected with the empty vector (pMT-B), cells transfected with vectors containing Met (*Met*), and cells transfected with vector containing SRC (*SRC*). Lanes 4-8 are from the same respective cells but contain proteins from the nuclear fractions. Bands appearing near the 80kDa marker represent Met-V5 proteins and bands appearing near the 260kDa marker represent SRC-V5 proteins. Proteins were detected by western blotting.
Table 3. EMSA reaction components Positive control reagents, MfR subunits, assayed putative sequence, and presence of methyl farnesoate are described for each respective EMSA binding reactions shown in Figure 2A-F. “Labeled” indicates positive control DNA and putative MfREs that are biotinylated. A) Reactions for EMSA binding reactions in Fig. 1A B) Reactions for EMSA binding reactions in Fig. 1B C) Reactions for EMSA binding reactions in Fig. 1C D) Reactions for EMSA binding reactions in Fig. 1D E) Reactions for EMSA binding reactions in Fig. 1E F) Reactions for EMSA binding reactions in Fig. 1F.

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Figure 2. MfR: MfRE EMSA binding results Nine different endogenous *D. pulex* nucleotide sequences, containing putative MfREs were assayed for binding to Met, SRC, and Met: SRC. A) EBNA positive control and MfRE1 B) MfRE6 and MfRE7 C) MfRE8 and MfRE9 D) MfRE10 E) MfRE11 and F) EBNA positive control, MfRE13 and MfRE14. Specific proteins, putative MfRE, and other reagents included in each binding reaction/lane are detailed in Table 2A-F.
CONCLUSIONS

Many existing environmental chemicals have been shown to adversely affect humans and wildlife (1). However, the growing number of unevaluated environmental chemicals has presented risk assessors with the daunting task of evaluating potential toxicity at unattainable speeds using current screening methods. Current in vivo methods are time-consuming and require numerous resources for assessment of apical endpoints, such as a clinical sign or pathological state.

In 2007, the National Research Council proposed a new paradigm for toxicity testing; a paradigm aimed at reducing costs, animal usage, and time (2). Evaluation of perturbations in defined toxicity pathways, rather than apical endpoints, without compromising the hazard evaluation process, was an essential component to the Council’s vision. Meaning, suites of biochemical assays, like receptor-binding or reporter gene expression assays primarily conducted in cells or cell lines, would greatly reduce, if not eliminate, the need for the apical animal testing (2).

This pathway paradigm implies that a xenobiotic-mediated perturbation at the inception of a series of biological signaling events causes an adverse phenotypic outcome, and therefore the initial molecular interaction can serve as an alternative endpoint for accurate chemical toxicity assessment (3). However, as described by the Council in 2007, the development of novel chemical toxicity testing regimes requires the elucidation of applicable signaling pathways (2).

Daphnid species remain a widely used aquatic toxicology model organism with established in vivo toxicity screening and testing methods (4, 5). They are abundant and
widespread in many freshwater habitats and provide a key link between primary producers and consumer species in many aquatic food webs. They have even been referred to as keystone species (6). The methyl farnesoate signaling pathway orchestrates aspects of reproduction and development e.g. male sex determination and differentiation in daphnids (7). However, a thorough study of the molecular initiating event within this toxicity pathway was lacking.

We hypothesized that methyl farnesoate activates a methyl farnesoate receptor complex, composed of the methoprene-tolerant transcription factor (Met) and steroid receptor coactivator (SRC). Further, we proposed that receptor activation is initiated with the dissociation of Met multimers and subsequent heterodimerization of Met and SRC subunits. Finally, we hypothesized the activated methyl farnesoate receptor regulates downstream expression of reproductive and developmental genes by binding short consensus sequences in the gene promoter regions. We proposed that one or more of these activities associated with receptor activation would be suitable for use in the high-throughput screening of chemicals for interaction with this pathway.

The elucidation of this pathway began with the search for and discovery of the crustacean methyl farnesoate receptor (8). In our first sub-hypothesis, we proposed that methyl farnesoate activates the methyl farnesoate receptor complex, composed of the methoprene-tolerant transcription factor (Met) and steroid receptor coactivator (SRC). Several candidate receptor complexes were tested, but as we hypothesized the Met and SRC heterodimer was the only transcription factor combination activated by methyl farnesoate in a luciferase reporter gene assay (8). Miyakawa et al. confirmed methyl farnesoate-mediated
activation of the receptor complex in multiple daphnid species using a similar reporter gene assay (9).

Our discovery of the hormone receptor provided a molecular target for the endogenous hormone, as well as some endocrine disrupting compounds. Numerous environmental chemicals disrupt the reproductive strategies in daphnids (10-14). We, and others, have shown that some of these compounds interact directly with the methyl farnesoate receptor complex, similar to the endogenous hormone (8, 9, 11).

In our second sub-hypothesis, we proposed that activation of the methyl farnesoate receptor activation is initiated with the dissociation of Met multimers and subsequent assembly of Met and SRC subunits. We utilized various technical methods to measure specific intra-molecular interactions. Our results indicated the molecular initiating event of this particular adverse outcome pathway occurs upstream of transcriptional activation, and upstream of the endpoint used in previous endocrine disruption studies (8, 9, 11). We correctly predicted the hormone-mediated dissociation of daphnid Met multimers. Other groups have shown similar molecular interactions in in phylogenetically similar species with orthologous hormone receptor complexes (15, 16). Surprisingly, the methyl farnesoate hormone activated receptor subunit assembly in the absence of a respective DNA docking site, or response element.

Aside from building upon a specific adverse outcome pathway, our receptor assembly model will benefit the study of basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) proteins. The daphnid methyl farnesoate receptor is composed of two members of the bHLH-PAS protein class, the methoprene-tolerant transcription factor (Met) and a steroid receptor coactivator (SRC). Many other research groups have defined the specificity of receptor
protein DNA-binding domains (17-19) and the role receptor protein dimerization domains play in transcriptional activation (17, 20). Our model implied that proteins within this class of transcription factors dimerize prior to DNA docking. Additionally, our model suggested that SRC binds ligand-bound receptor subunits prior to the receptor binding a DNA response element, therefore adding to their already broad range of molecular functions (21).

The final part of our hypothesis proposed that the activated methyl farnesoate receptor regulates downstream expression of reproductive and developmental genes by binding short consensus sequences in the gene promoter regions. Identification of an endogenous methyl farnesoate receptor response element would aid in further elucidating this crustacean adverse outcome pathway (3). Mounting evidence suggests orthologous receptors, in phylogenetically similar species, bind short nucleotide sequences upstream of relevant developmental genes (16, 20, 22, 23). We targeted some of these specific short conserved sequences in our genomic searches, which provided ample putative response element sequences for protein-binding testing. However, our experimental binding assays were unproductive in identifying an endogenous methyl farnesoate receptor response element. Therefore, we are unable to conclude if this portion of our hypothesis was correct. However, we did provide experimental evidence of receptor subunit cellular localization.

As recommended by the National research council, we elucidated a molecular initiating event in an important toxicity pathway. Our final aim was to identify the optimal method with which to measure receptor activation. We proposed this method ultimately would be employed as a part of future ecological risk assessments.

Many approaches exist to detect endocrine activity within a single signaling pathway (24), but current in vitro endocrine toxicity testing trends favor ligand-mediated
transcriptional activation as a representative molecular endpoint, with a reporter gene to quantify relative activation (24-26). Essentially, an engineered stable cell line contains a reporter gene, most often the firefly luciferase, under the regulation of an endocrine receptor-specific response element. Upon ligand binding, the receptor activates gene transcription and relative activation quantified by light emitted by the luciferase enzyme.

Our discovery of hormone-mediated methyl farnesoate receptor subunit assembly prompted a comparison study. We hypothesized that receptor activation was a suitable endpoint for a high-throughput chemical toxicity screening approach. Therefore we sought to determine if our newly constructed methyl farnesoate receptor assembly assay, which used a relatively new technique called bioluminescence resonance energy transfer (BRET), was comparable to the “gold standard” of in vitro toxicity testing, the luciferase reporter gene assay.

In recent years, BRET methods have proven both sensitive and useful in measuring protein-protein interactions (27-29). We concluded that our BRET assembly assay was not only equally as sensitive to our reporter gene assay in detecting receptor activation, but considerably more rapid. However, the value of this novel assay far exceeds detecting potential reproductive and developmental toxicity in crustaceans. We have created a novel approach to high-throughput chemical toxicity screening. Conceivably, the receptor protein components can be exchanged with different ligand-mediated endocrine receptor subunits, therefore making the assay applicable other endocrine signaling pathways in any number of species.

The studies described herein detail the activation and disruption of an environmental-endocrine signaling pathway involved in crustacean reproduction and development, with a
general focus on the daphnid methyl farnesoate hormone-mediated receptor. We identified the receptor complex and established a molecular initiating event upstream of gene transcription within this adverse outcome pathway. As a result, we created and validated a novel approach to high-throughput chemical toxicity screening that is as sensitive but more rapid than current approaches. Undoubtedly, this work has advanced our understanding of reproductive and developmental endocrine signaling in an ecologically important species and afforded a novel approach to endocrine disrupting chemical detection.
References


