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NR1L: A NOVEL NUCLEAR RECEPTOR GROUP ACTIVATED BY JUVENILE HORMONE ANALOGS

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NR1L: A NOVEL NUCLEAR RECEPTOR GROUP ACTIVATED BY JUVENILE
HORMONE ANALOGS

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ABSTRACT

Annotation of the *Daphnia pulex* nuclear receptors revealed a novel group of three receptors designated NR1L, and named HR97a/b/g because of their similarity to the HR96 receptors involved in xenobiotic detection. We cloned and sequenced the three receptors from a related species often used in aquatic toxicology studies, *Daphnia magna*, and then analyzed their genomic structure and conducted phylogenetic studies. Phylogenetic studies confirmed that the HR97s do form a distinct group with HR97g being the precursor of HR97a and b. They also confirmed that the HR97 receptors are related to the HR96 (NR1J) receptors, and the VDR/CAR/PXR (NR1I) group. Mining other arthropod genomes revealed that *Ixodes* also has a NR1L member (IsHR97). Gal4-chimeric plasmids that contain the ligand binding domain of HR97a/b/g were constructed for transactivation assays. Transactivation assays demonstrated that HR97a and HR97g repress basal transcription in the absence of a activator; however, HR97b has constitutive activity. We hypothesized that the HR97's are involved in xenobiotic detoxification. Contrary to our hypothesis, the HR97s are not activated by multiple toxicants. We found that pyriproxyfen and methyl farnesoate activate HR97g. Dose-response studies found that pyriproxyfen and methyl farnesoate have EC50's of 3.4 and 2.2 uM, respectively. The two chemicals are juvenile hormone analogs that have been found to induce the production of males in the otherwise female parthenogenic *Daphnia*. HR97a and b are also activated by pyriproxyfen. Further, although NR1L is phylogenetically related to NR1I and NR1J, the HR97s do not share significant similarity with the NR1I and NR1J

members. We conclude that HR97s are not xenobiotic sensors, but may be involved in juvenile hormone signaling and potentially male production.

DEDICATION

I dedicate this work to my parents, Li Ning and Li Yunxiu.

ACKNOWLEDGMENTS

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CHAPTER ONE

BACKGROUND

The multi-level regulation of gene expression has become the center of modern molecular biology, providing insights into physiology, pathology and developmental biology (Evans, 2005). In this regulatory process, lipophilic hormones which act as ideal messengers, pass the phospholipid bilayer of the cell membrane and interact with proteins within the cells (Mangelsdorf et al., 1995). The development of radiolabeled ligands demonstrated that these hormones bind to protein receptors and translocate from the cytoplasm to the nucleus, suggesting their function in transcriptional regulation (Jensen et al., 1966). Later steroids were found to target certain tissues with the presence of high affinity receptor proteins and hormonally responsive genes were identified within these tissues (Yamamoto, 1985). These studies provided the classic model of steroid hormone action where the binding of hormonal ligands to a receptor induces a conformational change that allows the ligand-receptor complex to bind to the response element of a gene and regulate transcription.

After the cloning of the glucocorticoid receptor (Hollenberg et al., 1985), estrogen receptor (Green et al., 1986) and thyroid hormone receptor (Sap et al., 1986), in 1987 the discovery of the retinoic acid receptor confirmed the idea of a nuclear receptor superfamily (Giguere, Ong, Segui, & Evans, 1987; Petkovich, Brand, Krust, & Chambon, 1987). Since then, new receptors for known hormones as well as orphan receptors with ligands yet to be identified have been constantly added to the nuclear receptor

superfamily with the various sequencing and annotation projects, providing valuable

information on transcriptional regulation and evolution of the animal kingdom (Evans,

2005). The known nuclear receptors can be divided into seven subfamilies (Nuclear

Receptors Nomenclature Committee, 1999) with diverse functions ranging from

embryonic development, reproduction, nutrient allocation, detoxification, and in general

the maintenance of homeostasis (Benoit et al., 2006; Evans, 2005; Mota, Hernandez, &

Baldwin, 2010).
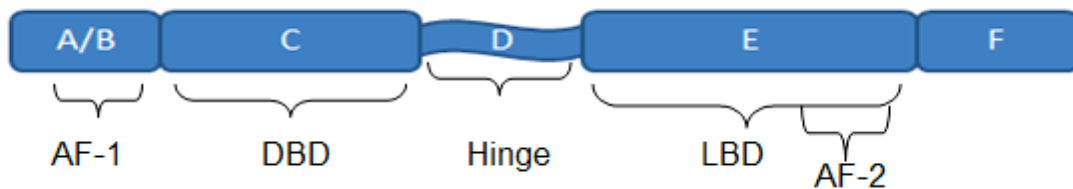
Figure 1.1 Five-domain structure of a nuclear receptor. At the N-terminal is the A/B

domain that functions in ligand-independent transcriptional activation (AF-1). C is the

DNA binding domain. D is the hinge domain. E is the ligand binding domain and has a

ligand-dependent activation function (AF-2). At the C-terminal is the F domain.

Nuclear receptors share a distinct five-domain structure (Laudet & Gronemeyer, 2002)

(Figure 1.1). At the N-terminus is the A/B domain that functions in ligand-independent

transcriptional activation (AF-1), meaning this is where the coactivators can bind in order

to activate the basal transcriptional complex. The C domain, featuring two Cys rich Zinc

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finger motifs in tandem, is the DNA binding domain (DBD), which targets the specific DNA sequences known as the response elements. The C domain is involved in both DNA binding and dimerization as one zinc finger recognizes the half-site in the response element and the other zinc finger functions in homodimerization or heterodimerization. The nuclear receptor RXR is a common heterodimerization partner (Benoit et al., 2006). The D domain is the hinge domain between the C and E domains. This hinge region adds to the protein flexibility thus makes possible the simultaneous receptor dimerization and DNA binding. The E domain is the ligand binding domain (LBD). Binding of a ligand, which is usually a small lipophilic compound (Kozlova, Lam, & Thummel, 2009), leads to the dissociation of co-repressors and recruitment of co-activator proteins. The E domain also mediates dimerization and has a ligand-dependent activation function (AF-2). The AF-2 site interacts with the LXXLL motif (where X can be any amino acid) in co-regulators (Centenera, Harris, Tilley, & Butler, 2008). The F domain is not present in all nuclear receptors and is highly diverse. Its functional significance is not fully understood.

The binding of nuclear receptors and co-regulators to the response elements can either regulate the recruitment of RNA polymerase II or stimulates the phosphorylation of pre-loaded RNA polymerase II. Co-regulators can be enzymes that modify histones and other transcription factors, or build links to histone acetylases and other enzymes to the receptors (Kininis & Kraus, 2008) (Figure 1.2).
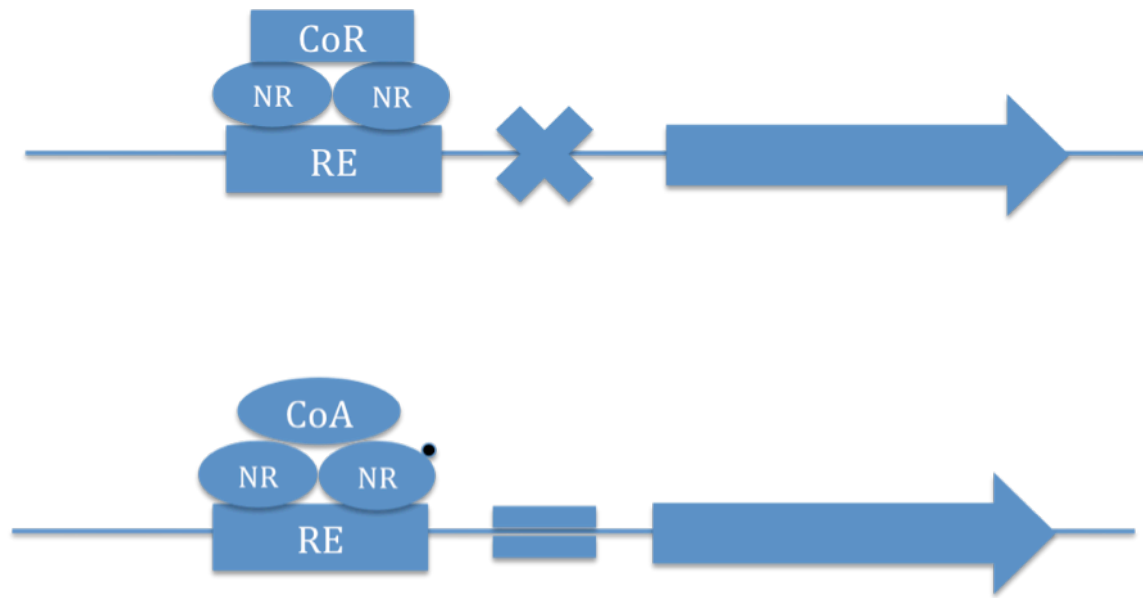
Figure 1.2 Regulation of transcription by nuclear receptors. Nuclear receptors usually form homo- or heterodimers and recognize the response elements. In the absence of a ligand the nuclear receptors could be bound by co-repressors that recruit multiple histone deacetylases to the gene, resulting in a compact chromatin that cannot be transcribed. Ligand (•) binding releases the co-repressors and recruites co-activators that could be histone acetyl transferases or chromatin remodelers and leads to transcription.

Of the five domains, the C and E domains are the most conserved domains of nuclear receptors. The domain swap technique, where the LBD of a receptor is linked to the DBD of a known receptor, makes it possible for researchers to screen for ligands by activation of a target gene while the physiologic functions of a receptor is still unknown (Eads, Andrews, & Colbourne, 2008). The first set of domain swap experiments also demonstrated the function of the conserved C and E domains (Giguere et al., 1987;

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Petkovich et al., 1987). The domain swap technique commonly used nowadays involves

linking the LBD of a receptor in frame to the DNA binding domain of GAL4. The

reporter plasmid usually contains the GAL4 response element UAS (Upstream Activation

Aequence) followed by the luciferase gene. Thus, transcriptional activation can be

detected through luminescence with the aid of luciferase substrate (Baker, Warren,

Thummel, Gilbert, & Mangelsdorf, 2000) (Figure 1.3)
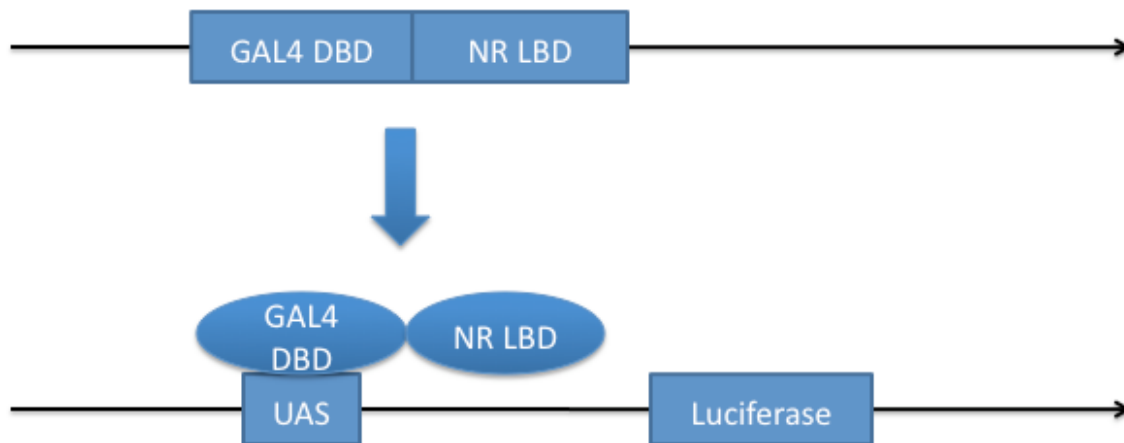
Figure 1.3 Illustration of the domain swap technique. The chimeric plasmid is

constructed by linking the LBD of a receptor in frame to the DNA binding domain of

GAL4. This plasmid expresses the chimeric protein that contains the GAL4 DBD and

nuclear receptor LBD. The reporter plasmid contains the GAL4 response element UAS

followed by the luciferase gene. And transcriptional activation could be detected through

luminescence with the aid of luciferase substrate.

The cladoceran crustacean *Daphnia magna*, commonly known as the water flea, is a crucial component of the aquatic food web because it is a major prey object for fish and invertebrate predators. It is also important in water quality control because of its feeding on algae (Tatarazako & Oda, 2007). Daphnids have several well-known stress responses, such as the formation of neckteeth in response to predator kairomone (Laforsch et al., 2004), hemoglobin synthesis in response to oxygen deprivation (Gorr, 2004), male production in response to specific unfavorable environments (Tatarazako & Oda, 2007) such as overcrowding, changes in photoperiod, poor food quality, and the presence of juvenile hormone analogs (Eads et al., 2008).

Daphnia magna is best known for its use as an aquatic toxicology test organism. In part, it is often used a bioindicator species and in aquatic toxicology tests because it is amenable to culture and inexpensive to use. In turn, eight percent of ECOTOX data for aquatic organisms are from *Daphnia magna* (http://daphnia.cgb.indiana.edu/people/news/47) making *Daphnia* species the most popular organisms for testing the toxicity of common aquatic pollutants.

Although the ecology of *Daphnia magna* is well understood, genomic studies on this organism have only recently been started. While the *Daphnia magna* genome project is currently underway, the genome sequencing project of *Daphnia pulex*, a related cladoceran species, has been recently completed, making *Daphnia* the first aquatic

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arthropod and the first crustacean fully sequenced (Colbourne et al., 2011). This provides

biologists with greater tools with which to study evolution of arthropods and how

Daphnia deal with environmental stressors, including seasonal changes, food allocation,

aquatic toxicants, and to determine the mechanisms that guides the development of

Daphnia through their life cycle; of which male production is a long unsolved puzzle

(Eads et al., 2008).

Daphnia magna is a cyclical parthenogen. When resources are abundant, female

daphnids asexually produce female offspring. Under environmental changes, such as

shorter day length, food depletion, and overcrowding, females produce male daphnids

and the population switch to sexual reproduction (Tatarazako & Oda, 2007). It has been

proved that the sesquiterpenoid hormone methyl farnesoate, the unepoxidated form of

insect Juvenile Hormone III, induces the production of male broods in *Daphnia magna*

(Olmstead & Leblanc, 2002). The juvenile hormone analog pyriproxyfen has been shown

to induce male broods with even higher potency (Tatarazako, Oda, Watanabe, Morita, &

Iguchi, 2003). However the molecular events behind these observations are still unclear.

Phylogenetic comparisons of the nuclear receptors from *Daphnia pulex* to other species,

indicate that *Daphnia* have a novel group of three nuclear receptors putatively placed in

the newly formed NR1L group. We named them HR97a, HR97b, and HR97g because of

their similarity to the NR1J group of nuclear receptors that includes HR96 found in

Daphnia pulex and *Drosophila melanogaster*. The phylogenetic data suggest that HR97g

is the evolutionary precursor to the other two HR97 receptors that are found in tandem repeat (Thomson et al., 2009).

While the function of the NR1L group is unknown, the similarity of these receptors to HR96 and the NR1I subfamily (HsVDR, HsCAR, HsPXR) suggests that this group might play a role in the regulation of metabolic enzymes involved in the metabolism of xenobiotics or endobiotics. The fact that this group exists in crustaceans but not insects suggests the possibility of a unique pathway of detoxification in crustaceans, or an endocrine process unique to crustaceans.

The goal of this study is to determine potential activators for the HR97 receptors. The objectives of this study are:

Objective 1: Cloning, sequencing, and characterization of magnaHR97g

The aim of objective 1 is to clone and sequence HR97g from *Daphnia magna*, compare it to *Daphnia pulex*, and identify potential activators for magnaHR97g. We hypothesize that HR97g from *Daphnia magna* will have similar sequence identity and genomic structure to *Daphnia pulex* HR97g. Furthermore, we hypothesize that HR97g is activated by multiple toxicants because previous studies showed that it is related to the multi-toxicant sensors HR96, CAR, and PXR.

We will amplify fragments of magnaHR97g by regular PCR using short primers designed based on the DappuHR97g sequence and RACE PCR. After sequencing the PCR products we will clone the whole magnaHR97g into pCR 2.1 vector using TA cloning. Phylogenetic studies will be conducted to confirm its evolutionary history and relatedness to the HR96 receptors. Then we will construct a chimeric pBIND-GAL4-97gDEF plasmid with the D, E and F domains of magnaHR97g linked in frame to the GAL4 DNA binding domain. This plasmid will express the chimeric protein consisting of the GAL4 DNA binding domain and the magnaHR97g D, E and F domains. This plasmid will be used in transactivation assays in combination with the reporter plasmid pG5*luc* which contains five GAL4 binding sites upstream of the firefly luciferase gene. Transactivation assays are to be conducted to measure HR97g activity under different conditions and in the presence of different chemicals. Lastly, the dose-dependent induction of magnaHR97g will be measured following treatment with different concentrations of putative activators.

Objective 2: Cloning, sequencing, and characterization of magnaHR97a and HR97b. The aim of objective 2 is to clone and sequence the two duplicated HR97 receptors from *Daphnia magna*, HR97a and HR97b, that are found in tandem repeat. Potential activators will be identified using a transactivation assay. We hypothesize that HR97a and HR97b from *Daphnia magna* will have similar sequence identity and genomic structure to *Daphnia pulex* HR97a/b. Furthermore, we hypothesize that these nuclear receptors are activated by multiple toxicants because previous studies showed that they

are related to the multi-toxicant sensors HR96, CAR, and PXR. *Daphnia pulex* is the first aquatic arthropod sequenced. *Daphnia* contain considerably more genes that *Drosophila melanogaster* or even *Homo sapiens*, and we consider it likely that the amplification of genes was necessary for adapting to an ever changing aquatic environment that contains and harbors multiple toxins and toxicants. Therefore, multiple receptors may be needed.

Fragments of magnaHR97a/b will be amplified through regular PCR and RACE PCR. After sequencing the PCR products, we will clone the whole magnaHR97a and b receptors into pCR 2.1 vectors using TA cloning. The genomic structure of magnaHR97a/b will be determined and compared to DappuHR97a/b. Then we will construct chimeric pBIND-GAL4-97a/bDEF plasmids with the D, E and F domains of magnaHR97a/b linked in frame to the GAL4 DNA binding domain and carry out transactivation assays in combination with the reporter plasmid pG5*luc*. Transactivation assays are to be conducted to measure magnaHR97a/b activity under different conditions and in the presence of different chemicals. Dose-response studies will be carried out with different concentrations of putative HR97a/b activators.

The characterization of the three novel nuclear receptors in *Daphnia magna* has two purposes. First, it will broaden our current knowledge of nuclear receptor functions and evolution. Second, it will reveal some new molecular basis for *Daphnia* physiology, potentially providing novel genetic support to some long-established toxicity tests.

References

Baker, K. D., Warren, J. T., Thummel, C. S., Gilbert, L. I., & Mangelsdorf, D. J. (2000). Transcriptional activation of the drosophila ecdysone receptor by insect and plant ecdysteroids. *Insect Biochemistry and Molecular Biology, 30*(11), 1037-1043.

Benoit, G., Cooney, A., Giguere, V., Ingraham, H., Lazar, M., Muscat, G., et al. (2006). International union of pharmacology. LXVI. orphan nuclear receptors. *Pharmacological Reviews, 58*(4), 798-836.

Centenera, M. M., Harris, J. M., Tilley, W. D., & Butler, L. M. (2008). Minireview: The contribution of different androgen receptor domains to receptor dimerization and signaling. *Molecular Endocrinology, 22*(11), 2373-2382.

Colbourne, J. K., Pfrender, M. E., Gilbert, D., Thomas, W. K., Tucker, A., Oakley, T. H., et al. (2011). The ecoresponsive genome of *Daphnia pulex. Science, 331*(6017), 555-561.

Eads, B. D., Andrews, J., & Colbourne, J. K. (2008). Ecological genomics in *Daphnia*: Stress responses and environmental sex determination. *Heredity, 100*(2), 184-190.

Evans, R. M. (2005). The nuclear receptor superfamily: A rosetta stone for physiology *Molecular Endocrinology, 19*(6), 1429-1438.

Giguere, V., Ong, E. S., Segui, P., & Evans, R. M. (1987). Identification of a receptor for the morphogen retinoic acid. *Nature, 330*(6149), 624-629.

Gorr, T. A. (2004). Hypoxia-induced synthesis of hemoglobin in the crustacean *Daphnia magna* is hypoxia-inducible factor-dependent. *Journal of Biological Chemistry, 279*(34), 36038-36047.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J. M., Argos, P., & Chambon, P. (1986). Human oestrogen receptor cDNA: Sequence, expression and homology to v-erb-A. *Nature, 320*(6058), 134-139.

Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., et al. (1985). Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature, 318*(6047), 635-641.

Jensen, E.V., Jacobson, H.I., Flesher, J.W., Saha, N.N., Gupta, G.N., Smith, S., Colucci, V., Shiplacoff, D., Neuman, H.G., Desombre, E.R., & Jungblut, P. W. (1966). Estrogen receptors in target tissues. In Steroid Dynamics, G. Pincus, T. Nakao, and J.F. Tait, eds. (NewYork: Academic Press), pp. 133-156.

Kininis, M., & Kraus, W. L. (2008). A global view of transcriptional regulation by nuclear receptors: Gene expression, factor localization, and DNA sequence analysis. *Nuclear Receptor Signaling, 6*, e005.

Kozlova, T., Lam, G., & Thummel, C. S. (2009). *Drosophila* DHR38 nuclear receptor is required for adult cuticle integrity at eclosion. *Developmental Dynamics : An Official Publication of the American Association of Anatomists, 238*(3), 701-707.

Laforsch, C., Ngwa, W., Grill, W., & Tollrian, R. (2004). An acoustic microscopy technique reveals hidden morphological defenses in *Daphnia. Proceedings of the National Academy of Sciences of the United States of America, 101*(45), 15911-15914.

Laudet, V., & Gronemeyer, H. (2002). *The Nuclear Receptor Facts Book.* New York: Academic Press.

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., . . . Evans, R. M. (1995). The nuclear receptor superfamily: The second decade. *Cell, 83*(6), 835-839.

Mota, L. C., Hernandez, J. P., & Baldwin, W. S. (2010). Constitutive androgen receptor-null mice are sensitive to the toxic effects of parathion: Association with reduced cytochrome p450-mediated parathion metabolism. *Drug Metabolism and Disposition: The Biological Fate of Chemicals, 38*(9), 1582-1588.

Nuclear Receptors Nomenclature Committee. (1999). A unified nomenclature system for the nuclear receptor superfamily. *Cell, 97*(2), 161-163.

Olmstead, A. W., & Leblanc, G. A. (2002). Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna. The Journal of Experimental Zoology, 293*(7), 736-739.

Petkovich, M., Brand, N. J., Krust, A., & Chambon, P. (1987). A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature, 330*(6147), 444-450.

Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., et al. (1986). The c-erb-A protein is a high-affinity receptor for thyroid hormone. *Nature, 324*(6098), 635-640.

Tatarazako, N., & Oda, S. (2007). The water flea *Daphnia magna* (crustacea, cladocera) as a test species for screening and evaluation of chemicals with endocrine disrupting effects on crustaceans. *Ecotoxicology (London, England), 16*(1), 197-203.

Tatarazako, N., Oda, S., Watanabe, H., Morita, M., & Iguchi, T. (2003). Juvenile hormone agonists affect the occurrence of male *Daphnia*. *Chemosphere, 53*(8), 827-833.

Thomson, S. A., Baldwin, W. S., Wang, Y. H., Kwon, G., & Leblanc, G. A. (2009). Annotation, phylogenetics, and expression of the nuclear receptors in *Daphnia pulex*. *BMC Genomics, 10*, 500.

Yamamoto, K. R. (1985). Steroid receptor regulated transcription of specific genes and gene networks. *Annual Review of Genetics, 19*, 209-252.

CHAPTER TWO

HR97G: A NOVEL NUCLEAR RECEPTOR ACTIVATED BY JUVENILE
HORMONE ANALOGS

ABSTRACT

The recently sequenced *Daphnia pulex* genome revealed three novel nuclear receptors related to the CAR/PXR/VDR group and were named HR97a/b/g. We cloned HR97g from *Daphnia magna*, a commonly used sentinel species in toxicity testing, and constructed a Gal4-chimeric nuclear receptor that contains the LBD of HR97g for transactivation assays. Three putative activators were identified in transactivation assays: cortisol, pyriproxyfen, and methyl farnesoate. Both methyl farnesoate and pyriproxyfen are juvenile hormone analogs that are male sex determinants in the otherwise female parthenogenic *Daphnia*. Concentration-response studies found that pyriproxyfen and methyl farnesoate have EC50's of 3.4 and 2.2 μM, respectively. In summary, HR97g is a candidate juvenoid receptor in the crustacean, *Daphnia magna*, because it is activated by juvenoids. However, the lack of pharmacological inhibitors or targeted mutations limits our ability to conclude that HR97g is a JHA receptor involved in environmental sex determination.

INTRODUCTION

Nuclear receptors constitute a superfamily of transcription factors with a distinct five-domain structure (Laudet & Gronemeyer, 2002). Of the five domains, the DNA binding domain (DBD, C domain) and the ligand binding domain (LBD; E domain) are the most

conserved domains. Ligand binding induces the interaction of the C domain with the response element of a gene, and typically results in an increase in transcriptional activity (Mangelsdorf et al., 1995).

Since the cloning of the glucocorticoid receptor in 1985 (Hollenberg et al., 1985), new members have been constantly added to the nuclear receptor superfamily with the various sequencing and annotation projects providing valuable information on transcriptional regulation and evolution of the animal kingdom (Evans, 2005; Bridgham et al., 2010). The known nuclear receptors can be divided into seven subfamilies (Nuclear Receptors Nomenclature Committee, 1999) with diverse functions ranging from embryonic development, reproduction, nutrient allocation, detoxification, and in general the maintenance of homeostasis (Benoit et al., 2006; Evans, 2005; Hernandez, Mota, & Baldwin, 2009).

Daphnia magna, commonly known as the waterflea, is a crustacean in the Branchiopod class. It is a crucial component of the aquatic food web and is best known for its use as an aquatic toxicology test organism as it is easy to maintain and inexpensive to use. Eight percent of ECOTOX data for aquatic organisms are from *Daphnia magna* (http://daphnia.cgb.indiana.edu/people/news/47) making *D. magna* one of the most widely used aquatic toxicity test organisms. Although the ecology of *Daphnia magna* is well understood, genomic studies on this organism have only recently been started. The *Daphnia magna* genome project is currently underway, and the *Daphnia pulex* genome

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sequencing project was recently completed. *Daphnia* are the first aquatic arthropod and the first crustacean fully sequenced (Colbourne et al., 2011), providing biologists with genomic information on the unique survival requirements of aquatic arthropods, and greater tools for studying arthropod evolution.

Phylogenetic comparisons of the nuclear receptors from *Daphnia pulex* to other species demonstrate that *Daphnia pulex* contain three novel nuclear receptors placed in the newly formed NR1L group. All three of these receptors are orphan receptors in that their ligands are not known. We named them HR97a, HR97b, and HR97g because of their similarity to the NR1J group of nuclear receptors that includes HR96 found in *Daphnia pulex* and *Drosophila melanogaster*. Phylogenetic data suggest that HR97g is the evolutionary precursor to the other two HR97 receptors found in tandem repeat (Thomson et al., 2009).

While the ligands and function of the NR1L group is unknown, the similarity of these receptors to HR96 and the NR1I subfamily (HsVDR, HsCAR, HsPXR) suggests that this group might play a role in responding to environmental cues such as common xenobiotics or dietary endobiotics (Baskin-Bey et al., 2007; Finn et al., 2009; Hernandez et al., 2009; Horner et al., 2009; King-Jones et al., 2006; Sieber & Thummel, 2009; Sonoda et al., 2005). The fact that this group exists in the crustacean *Daphnia* but not insects suggests the possibility of a unique adaptive pathway of detoxification in crustaceans, or an

endocrine process unique to crustaceans. Conversely, insects may have lost the HR97 group of nuclear receptors.

Genomic data from the *Daphnia* genome projects are currently being used to determine how aquatic arthropods acclimate or adapt to environmental stressors including seasonal changes, food allocation, and aquatic toxicants (Miyakawa et al., 2010; Schwarzenberger, Courts, & von Elert, 2009; Schwerin et al., 2009; Scoville & Pfrender, 2010; Shaw et al., 2007; Shaw et al., 2008; Spanier et al., 2010; Tollrian & Leese, 2010; Weber & Pirow, 2009; Zeis et al., 2009). This includes the role of stress in environmental sex determination and sexual reproduction, as parthenogenic *Daphnia* produce males for sexual reproduction when under specific stressful situations, and the molecular mechanisms behind male production and release is a key unsolved puzzle (B. D. Eads, Andrews, & Colbourne, 2008; B. D. Eads, Colbourne, Bohuski, & Andrews, 2007). The Juvenoid methyl farnesoate (MF), and insecticidal juvenile hormone analogs (JHAs) such as fenoxycarb and pyriproxyfen appear to control male production (Olmstead & Leblanc, 2002; Olmstead & LeBlanc, 2000; Tatarazako et al., 2003). A recent study has found a *Doublesex* gene (*Dsx*) in *Daphnia* that controls sexual dimorphism (Kato et al., 2011). However, the link between MF and *Dsx* is missing. In other words, the receptor that responds to MF and induces expression of the *Doublesex* gene in *Daphnia* is not known.

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Below we describe the partial characterization of the novel *Daphnia* nuclear receptor HR97g from *Daphnia magna*. The study of the HR97g group will serve two purposes. First, it will broaden our current knowledge of nuclear receptor function and evolution. Second, it may reveal some new molecular basis for *Daphnia* physiology, potentially providing novel genetic support to some long-established toxicity tests. In this study magnaHR97g was cloned, sequenced and compared genomically and phylogenetically with similar receptors in the NR1 subfamily. A GAL4-97gDEF chimeric plasmid was constructed with the magnaHR97g D, E and F domains (Thomson et al., 2009). Transactivation assays were conducted to measure HR97g activity under different conditions and in the presence of different chemicals, including the juvenile hormone analogs (JHA), methyl farnesoate and pyriproxyfen, involved in male production. This is the first study to examine the putative functions of HR97g.

MATERIALS and METHODS

D. magna:

Our colony of *D. magna* has been cultured at Clemson University for over 15 years and was provided by Dr. Steve Klaine (Clemson University). Daphnids were maintained at 15 individuals/L in 22°C deionized water reconstituted with $MgSO_4 \bullet 7H_2O$ 123 mg/L, $CaSO_4$ 60 mg/L, KCl 4 mg/L and $NaHCO_3$ 96 mg/L under a 16:8 light:dark photoperiod in an environmental chamber. Daphnids were fed twice daily with 6 X 10^6 cells of *Celenastrum capricornutum* and 10μL (~ 0.1 mg dry weight) Tetrafin fish food suspension (Tetra Holding Inc, VA) per adult daphnid.

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Nucleotide extraction:

RNA was extracted from fresh female *Daphnia magna* of a variety of ages (2-14 days old). RNA was extracted with PureZol (BioRad, Hercules, CA) according to the manufacturer's directions followed by digestion of residual genomic DNA with DNAse (Promega, Madison, WI). Complementary DNA was synthesized from 2μg of RNA with Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT), a dNTP mixture, and random hexamers (Promega).

Cloning Daphnia magna HR97g (magnaHR97g):

The *Daphnia* HR97g transcript was cloned by a combination of using primer sets designed from highly conserved regions of nuclear receptors and rapid amplification of cDNA ends (RACE). Primer sets (F = forward; R = reverse) F5/R4, F1/R1, and F97g-GL/R-97g-GL (kindly provided by Gerald LeBlanc, North Carolina State University) were designed based on the *D. pulex* HR97g sequence (Thomson et al., 2009) (*http://genome.jgi-psf.org/cgi-bin/browserLoad?db=Dappu1&position=scaffold_40:123499-125619*). F5: 5'-GAAGATGTCCAGCGTCTTCT-3'; R4: 5'-TCAATTTGCGCCAGTTC-3'; F1:5'-ATGGATGACAGCAACAGTTCT-3'; R1:5'-CGAAGCCATCCTTTCTCCAT-3'; F97g-GL: 5'-ACATGGCCAAACATGTGTCA-3'; R97g-GL: 5'-TGTCTTCAAAGCTTGGTTCG-3'. PCR products were ligated into pCR 2.1 by TA cloning (Invitrogen, Carlsbad, CA). Fm2/Rm2 primer pair (Fm2: 5'-

CCAATTGGTGCAACACTCCTAG-3'; Rm2: 5'-GCTCGATCGGGCGTAAACAT-3')

was designed after sequencing the PCR products from the previous reactions and used to

determine the unknown areas within the middle of the gene. The 3' end of magnaHR97g

was cloned following 3'-RACE (Invitrogen, Carlsbad, CA) with the following primers:

F-GSP1: 5'-CCTGGAAGATGTCCAGCGCCTTCT-3'; nested primer F-GSP2: 5'-

GCCCGATCGAGCAGATCTTGTTGCTCGT-3'. DNA sequencing was performed by

MacrogenUSA (Rockville, MD).

Genomic structure:

Genomic sequencing of *D. magna* clone Xlnb3 from Finland (Fin-magna) is currently

underway. We were able to use the partial genomic data available to compare the

magnaHR97g genes in different strains. Our Clemson magnaHR97g sequence was

blasted to the *D. magna* genome. The genomic structure of magnaHR97g was

determined by the position, length, and phase of each intron (*D. magna* genome). Protein

sequence identities of each nuclear receptor domain was compared between

magnaHR97g, Fin-magnaHR97g, *D. pulex* HR97g (DappuHR97g), *D. pulex* HR97a

(DappuHR97a), *D. pulex* HR97b (DappuHR97b), *D. pulex* HR96 (DappuHR96),

Drosophila melanogaster HR96 (DHR96), *C. elegans* DAF12, and *Homo sapiens*

constitutive androstane receptor (CAR) using ClustalW

(http://www.ebi.ac.uk/Tools/clustalw2/). Appendix A contains the accession numbers of

each receptor used in the analysis. In addition, intron-exon junctions and intronic size

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were compared between the *D.magna* strain Fin-magnaHR97g, and *D. pulex* HR97g (DappuHR97g).

Phylogenetics:

Phylogenetic analyses of HR97g and the related NR1I group of receptors was performed using methods described previously (Hannas et al., 2010; Thomson et al., 2009) with some modification. HR97g from *D. magna* was compared to those of other species available in GenBank, including *Drosophila melanogaster*, *Homo sapiens*, *Danio rerio*, *C. elegans*, and *Daphnia pulex*. NCBI accession numbers for the receptors used are available in Appendix A. The DNA binding domain (DBD) and the ligand binding domain (LBD) of each receptor were identified using the conserved domain database (CDD) (Marchler-Bauer et al., 2005). Zf-C4 (pfam00105) was used to identify the DBD and Hormone Recep (pfam00104) was used to identify the LBD. Analysis was performed with the DBD alone, LBD alone, and DBD and LBD joined. ClustalX default parameters were used to align the domains (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). Phylogenetic analysis using the combined LBDs and DBDs of the nuclear receptors is provided in the manuscript, while the phylogenetic analysis of the DBDs only, and the LBDs only are presented as Appendices B and C.

Trees were constructed using Bayesian Inference with MrBayes software version 3.1.2 (Ronquist & Huelsenbeck, 2003) on the Computational Biology Service Unit of Cornell University (http://cbsuapps.tc.cornell.edu/mrbayes.aspx). Phylogenetic trees were

constructed using the "mixed-model" approach in which the Markov chain Monte Carlo sampler explores nine different fixed-rate amino acid substitution models implemented in MrBayes. We used 4 chains with runs of 5 million generations, chains sampled every 100 generations, a burnin of 10,000 trees with the *WAG* model (Whelan & Goldman, 2001). The *Daphnia magna* ecdysone receptor was used as an outgroup.

Maximum Parsimony and distance parameters were used to provide additional support for the phylogenetic relationships observed. Distance parameters were measured using PAUP 4.0b10 with default characteristics (mean character difference and among site rate variation), and full heuristic searches. Branch support was measured by bootstrap analysis with 1000 replicates. Parsimony was constructed using PAUP version 4.0b10 with heuristic searches, tree-bisection-reconnection, topological constraints not enforced, and multiple tree option in effect with an initial maximum tree setting at 100,000. Branch support was measured by bootstrapping with 10,000 replicates. Trees were visualized with FigTree (http://tree.bio.ed.ac.uk/software).

Chimeric expression plasmids:

The GAL4-97gDEF chimeric plasmid was constructed according to the Clontech In-Fusion Dry-Down PCR Cloning Kit protocol (Clontech Laboratories, Mountain View, CA). The D, E and F domains of magnaHR97g were amplified using primer pair F-clontech-97g-BamHI (5'-GAA TTC CCG GGG ATC GAA GAA AAT GTG AAA ATG AGA GAG GCC AAG-3') and R-clontech-97g-XbaI (5'-CTG CGG CCG CTC

TAGATTA AAC TGG GTT ATC TGT TTC CAT TTG TTG ACT-3'). The PCR product was inserted into the pBIND vector (Promega CheckMate Mammalian Two-hybrid system) in frame after the GAL4 DNA binding domain. This plasmid expresses a chimeric protein consisting of the GAL4 DNA binding domain and HR97g hinge (D), ligand binding (E), and F domains.

Transactivation Assays:

HEPG2 human hepatoma cells (ATCC, Rockville MD) were cultured in phenol red free Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% L-glutamine (Invitrogen, Carlsbad, CA) and 1% Penicillin/Streptomycin (Invitrogen, Carlsbad, CA) at 37 °C under 5% CO_2.

HepG2 cells were plated in 12-well plates at 200,000 cells per well. Typically, cells were transfected with 0.02 µg of the GAL4-97gDEF chimeric plasmid and 0.1 µg of the reporter plasmid pG5*luc* that contains five GAL4 binding sites upstream of the firefly luciferase gene (Promega CheckMate Mammalian Two-hybrid system) using Effectene Transfection Reagent according to the manufacturer's recommendations (Qiagen, Valencia, CA). On the third day cells were either left untreated or treated with the chemicals of interest dissolved in 0.1% DMSO. Control wells also received 0.1% DMSO. Luciferase activity was measured 24 hours later with the Steady-Glo Luciferase

Assay System (Promega, Madison, WI) in a Turner Biosystems 20/20^n luminometer (Promega, Madison, WI).

Several hormones, fatty acids, and xenobiotics were used to test whether they activated HR97g. These include 4-nonylphenol (CAS 84852-15-3), bisphenol A (80-05-7), chlorpyrifos (2921-88-2), cortisol (50-23-7), dihyroandrosterone (1852-53-5), ethyinyl estradiol (57-63-6), β-estradiol (50-28-2), endosulfan (115-29-7), parathion (56-38-2), methyl farnesoate (10485-70-8), palmitic acid (57-10-3), and stearic acid (57-11-4).

Data are presented as the mean of triplicate assays $\pm$ standard error. Statistical significance was determined by ANOVA followed by Dunnett's test as the post hoc test using the GraphPad Prizm 4.0 statistical and graphing package (La Jolla, CA). For the dose-response studies, activities were normalized as a percent of the maximal activation and concentrations were log transformed. EC50 values and Hillslopes of each chemical were derived from the sigmoid dose-response curves generated by GrapPad Prizm 4.0 (Baldwin & Roling, 2009).

RESULTS

Genomic Structure:

The position, length, and phase of each intron and exon of Fin-magnaHR97g was determined and compared with DappuHR97g (Figure 2.1). The intron phases are identical between DappuHR97g and magnaHR97g, and the exon sizes are nearly the

same with the exception of the fourth exon that contains the highly variable F-domain. In addition, the protein sequence of each domain of magnaHR97g was aligned with the corresponding domain of several other NR1 subfamily members including Fin-magnaHR97g, DappuHR97g, DappuHR97a, DappuHR97b, DappuHR96, CeDAF12, DmDHR96, and HsCAR and percent identity was determined (Table 2.1). Our Clemson magnaHR97g sequence agrees well with only minor variances from the HR97g from the Finnish strain of *D. magna* (Fin-magnaHR97g) whose genome is currently being sequenced. The five domains of HR97g are also similar between *D. magna* and *D. pulex*, especially in the DNA and ligand binding domains (Appendix D contains a ClustalX document comparing *D. pulex* and *D. magna* HR97g). However, HR97g varies considerably from the other nuclear receptors including the highly related HR97a/b receptors as well as the HR96 receptor. This suggests that each of the HR97 receptors evolved to accommodate different ligands and probably activate different enhancer elements on DNA.
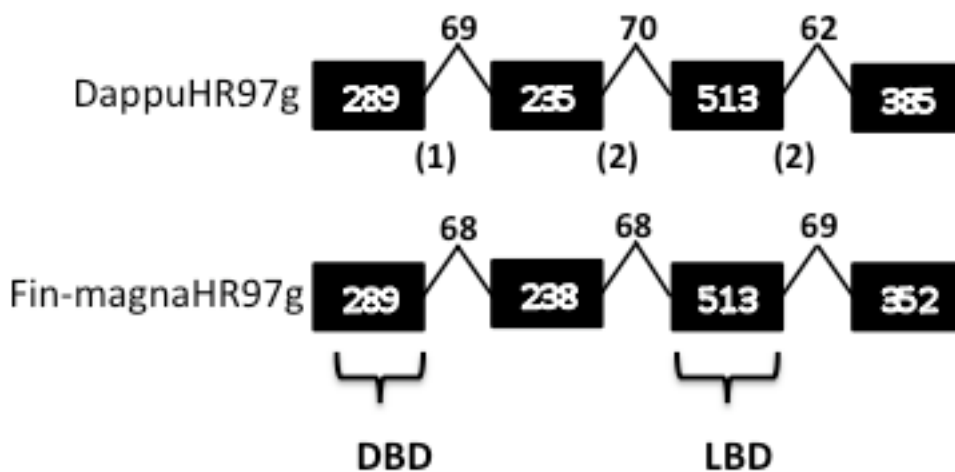
Figure 2.1 Comparison of the gene structure of HR97g from *D. magna and D. pulex*.

The blocks represent exons, the numbers indicate the size of the introns and exons, and the numbers in parentheses represent the intron phases. The inron phases were identical between the two species as was the placements of the DBD and LBD within the first and third exons, respectively.

Table 2.1 Domain comparison between magnaHR97g and other NR1 subfamily members. Data shown as identity scores to magnaHR97g derived from ClustalW.

NR	A/B	C	D	E	F
magnaHR97g	100	100	100	100	100
Fin-magnaHR97g	100	97	99	99	100
DappuHR97g	83	84	70	90	76
DappuHR97a	25	63	32	40	10
DappuHR97b	12	63	33	38	10
DappuHR96	8	51	23	28	33
CeDAF12	12	48	14	11	0
DmDHR96	25	5	16	22	50
HsCAR	25	44	5	17	50

Phylogenetics:

The HR97 group (putative NR1L group) of nuclear receptors was first discovered as part of the *Daphnia pulex* genome project (Thomson et al., 2009). *Daphnia* species are

currently the only species known to contain HR97 members; however, *Daphnia pulex* are the only crustacean species fully sequenced. The NR1L group is related to the NR1J group that contains HR96 (Thomson et al., 2009), which in turn is related to the NR1I group containing CAR, PXR, and VDR (Lin, Kozaki, & Scott, 2010). Therefore, the NR1I, NR1J, and NR1L receptors were compared phylogenetically. In addition, the knirps that are members of the NR0A group but phylogenetically related to HR96 and HR97 (Thomson et al., 2009) were also examined.

The DBD/LBD combined phylogenetic tree separates into four distinct clades; one containing the HR97 group (NR1L), one containing the HR96 group (NR1J), one containing CAR/PXR/VDR (NR1I), and one containing the knirps involved in fruitfly development (NR0A) (Figure 2.2). This data verifies that the HR97s are deserving of their own distinct nuclear receptor group. In addition, the phylogenetic tree confirms that magnaHR97g is highly related to DappuHR97g, substantiating that HR97g is found in another cladoceran species. Phylogenetic analysis also confirms that the HR97 group is closely related to the NR1I and NR1J groups with a closer relationship to the NR1J (HR96) group than the NR1I (VDR/PXR/CAR) group (Figure 2.2).

Interestingly, distance methods show some disagreement as they estimate that NR1L(HR97)/NR1J(HR96) are more closely related to the knirps than they are to the NR1I group (CAR/PXR/VDR) with a bootstrap value of 61.

Because the knirps lack a LBD, we also did the phylogenetic analysis using only the

DBDs or LBDs (Appendices B and C). The DBD only phylogenetic tree suggested a

different relationship between the receptors than the LBD containing trees. DBD-only

trees show four distinct groups divided into two parent clades; one clade contains the

NR1I and NR1J groups, and the other clade contains the NR1L (HR97) and knirps

(NR0A) using Bayesian analysis. This indicates that the DBDs of the HR97 receptors are

more closely related to the knirps than the other receptors, and suggests a potential role of

the HR97 receptors in transcriptional activation of genes involved in development.

However, neither NJ nor Maximum Parsimony agrees with this conclusion and the

posterior probabilities are not significant.
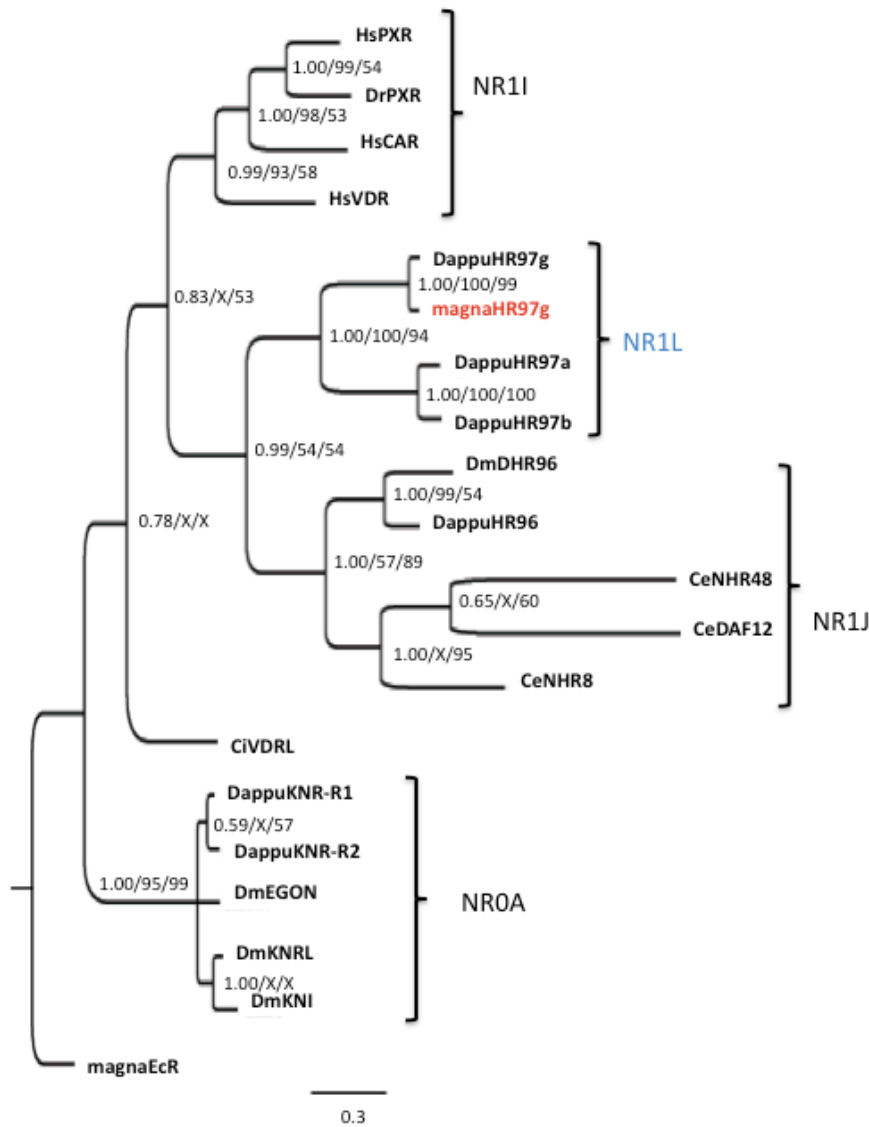
Figure 2.2 Phylogenetic analysis of HR97g. The nuclear receptors within the 0A

(knirps), NR1I, 1J, and 1L groups from several different species including *Homo sapiens*

(Hs), *D. magna* (magna), *D. pulex* (Dappu), *Drosophila melanogaster* (Dm), *C. elegans*

(Ce), Ciona intestinalis (Ci) were subjected to phylogenetic analysis. Bayesian Inference,

Neighbor-Joining, and Maximum Parsimony were used to determine the relationship of

HR97g to the NR1J and NR1I groups. The Bayesian tree is shown with posterior

probabilities from the Bayesian tree, and bootstrap support values (frequency of

occurrence) from the Neighbor-Joining and Maximum Parsimony trees provided in order

from left to right, respectively as confirmatory analysis of the Bayesian analysis.

Posterior probability values are separated by forward slashes at each corresponding node;

an X indicates an area of disagreement from the Bayesian tree. *D. magna* EcR was

chosen as an outgroup. Accession numbers of the analyzed nuclear receptors are

provided in Appendix A.

HR97g Transactivation

Cells were transfected with GAL4-97gDEF chimeric expression plasmid and the pG5*luc*

to determine if HR97g has constitutive activity in HepG2 cells. Control cells were

transfected with 0.1 μg of the pG5*luc* reporter plasmid only. The luciferase assay shows

that HR97g represses transcription in the absence of an activator (Figure 2.3A).

Therefore, we transfected cells with increasing concentrations of the GAL4-97gDEF

chimeric plasmid to test whether there is a dose-dependent pattern. To ensure that each

well received equal amounts of plasmid, the empty pBIND plasmid was co-transfected

into each well so that the plasmid concentration was equal (0.08 μg) in all of the wells.

HR97g exerted a weak, but discernable dose-dependent repression on transcriptional

activity in the absence of an activator (Figure 2.3B), suggesting that HR97g is a

transcriptional repressor in the absence of activators. Other nuclear receptors, such as

PXR, have also been found to repress gene expression in the absence of ligands because of their recruitment of co-repressors (Ourlin et al., 2003; Takeshita, Taguchi, Koibuchi, & Ozawa, 2002).

Transactivation assays were performed in the presence of several different chemicals to test whether HR97g acts as a promiscuous xenobiotic/endobiotic sensor similar to the some of the NR1I and NR1J receptors (Baldwin & Roling, 2009; Hernandez et al., 2009; Kliewer et al., 1998; Lin et al., 2010). Cells were treated with 4-nonylphenol, chlorpyrifos, endosulfan, parathion, bisphenol A, stearic acid, palmitic acid, β-estradiol, 1,3,5 (10)-estratrien-17α ethynyl-3,17β-diol (ethynyl estradiol), cortisol, and pyriproxyfen at 10 μM. Of the chemicals tested, only the stress hormone, cortisol and the JHA, pyriproxyfen significantly activated transcription (Figure 2.4). Pyriproxyfen increased luciferase activity 54% and cortisol increased activity 32%.
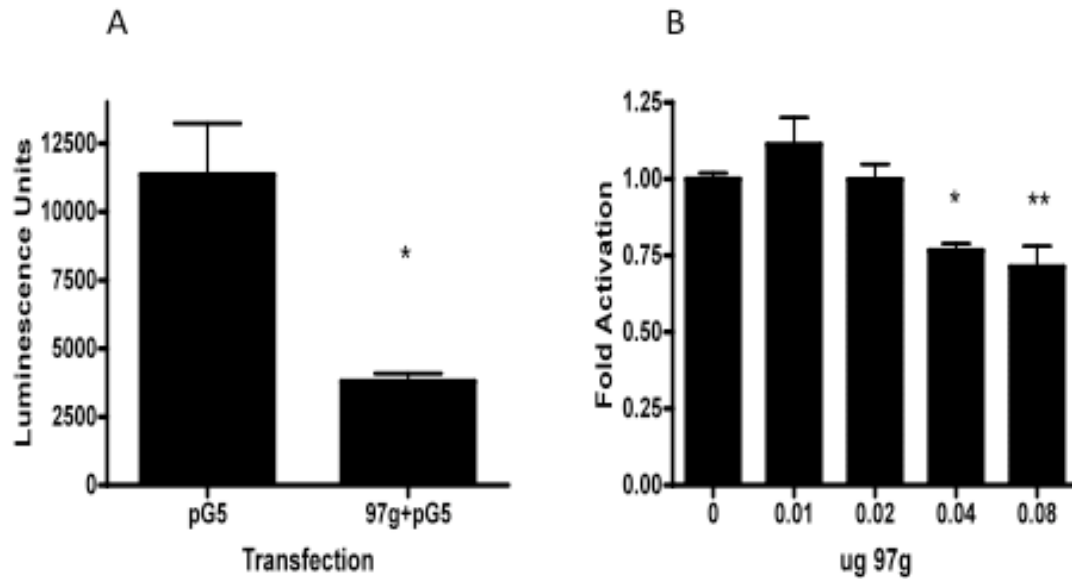
Figure 2.3 HR97g acts as a transcriptional repressor in the absence of activators. A) HepG2 ells were transfected with pBIND-GAL4-97gDEF chimeric plasmid and the reporter plasmid, pG5*luc*. Control cells were transfected with pG5*luc* only, and transactivation activity was measured as described in the Materials and Methods. B) Cells were transfected with 0, 0.01, 0.02, 0.04 and 0.08 μg pBIND-GAL4-97gDEF chimeric plasmid supplemented with empty pBIND-GAL4 vector to ensure all wells contained 0.08μg of vector. The assay was repeated two times. An asterisk or two asterisks indicates statistical significance at $p < 0.05$ or $p < 0.01$ as determine by Student's t-test (A) or ANOVA followed by Dunnett's multiple comparison test (B) with GraphPad Prizm 4.0 (La Jolla, CA) (n = 3).
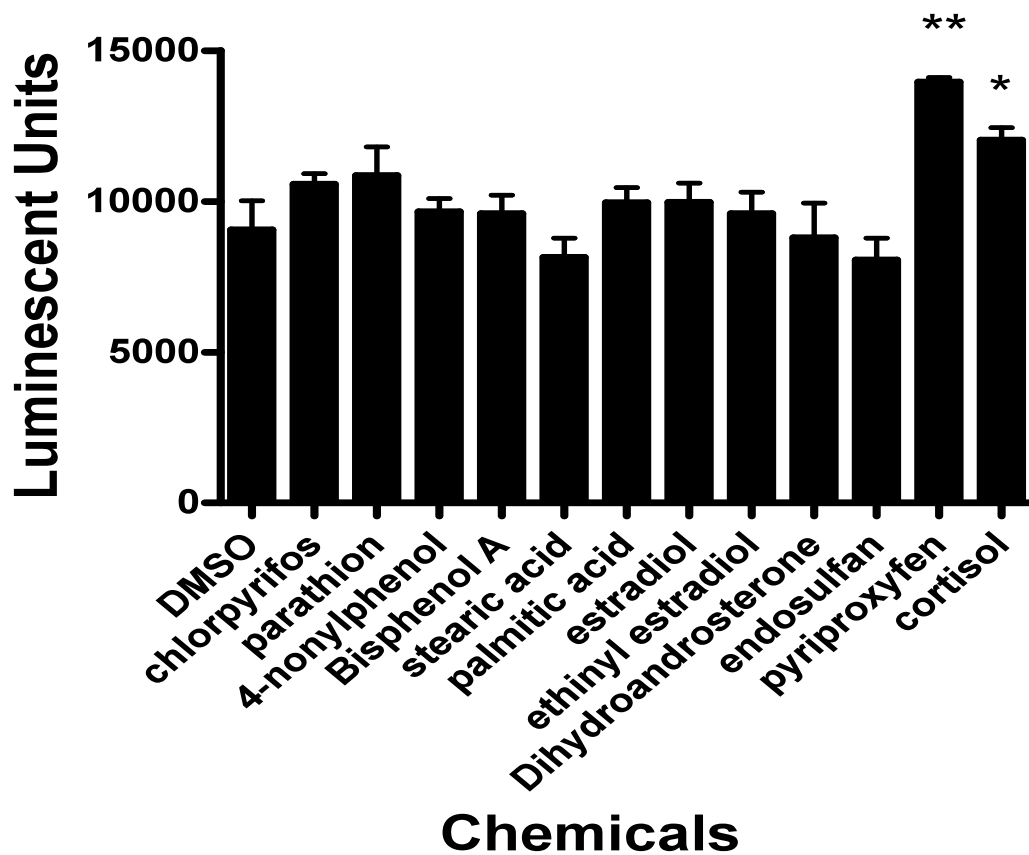
Figure 2.4 Chemical activation of HR97g. Transactivation assays were performed with several chemicals to determine if they activate HR97g. Only cortisol and pyriproxyfen significantly increased activation as determined by ANOVA followed by Dunnett's multiple comparison test as the post-hoc test. An asterisk indicates $p < 0.05$ and two asterisks indicate $p < 0.01$ (n = 3).

Pyriproxyfen and methyl farnesoate dose response assays

To determine whether HR97g contributes to the regulation of male sex determination, dose-response transactivation assays were performed with both the juvenile hormone

analog pyriproxyfen, and the endogenous juvenoid hormone methyl farnesoate (MF). Pyriproxyfen increased transcriptional activation in a dose-dependent fashion with peak activation at 10 μM and a significant decrease at 30 μM, presumably because of cell toxicity (Figure 2.5A). Dose-response curves (Figure 2.5B) determined that the EC50 of pyriproxyfen is 3.36 μM (Table 2.2). MF also activates HR97g. Luciferase activity was increased in the presence of MF in a dose-dependent manner with 30 μM MF exerting maximal transcriptional effects (Figure 2.5C). The EC50 of MF is 2.22 μM (Figure 2.5D) demonstrating that both JHAs activate HR97g transcriptional activity in the low micromolar range (Table 2.2).
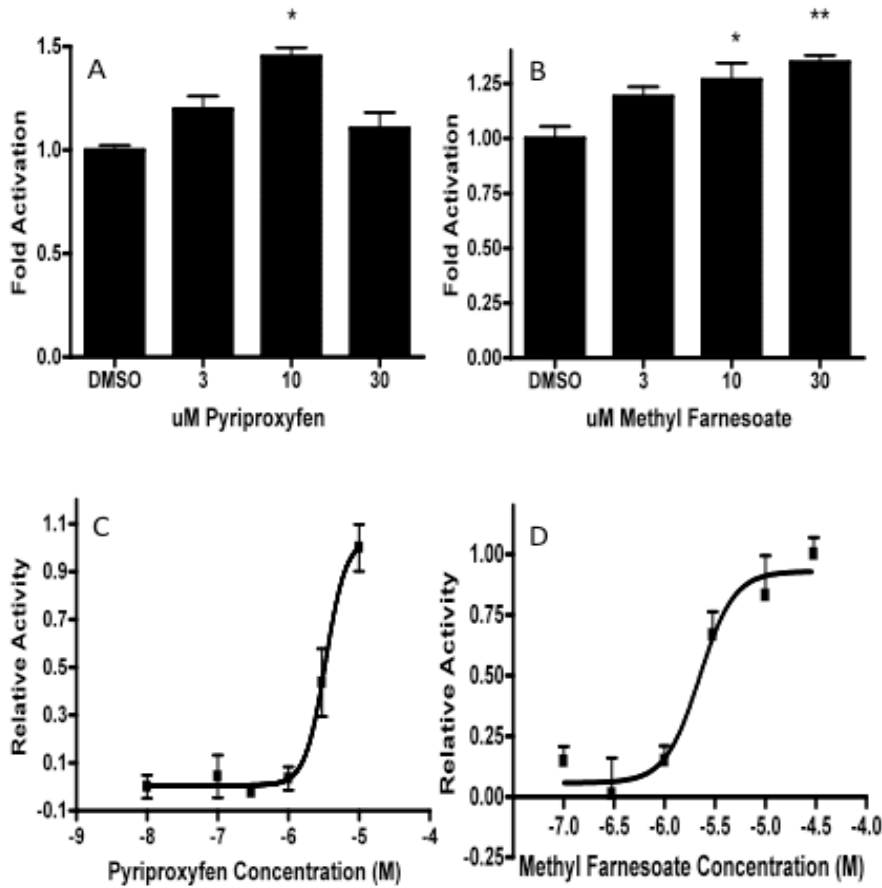
Figure 2.5 Pyriproxyfen and methyl farnesoate increase luciferase activity in transactivation assays in a dose-dependent manner. Cells transfected with the GAL4-97gDEF and the pG5*luc* reporter were treated with pyriproxyfen (A) at concentrations of 3, 10, and 30 μM and luciferase activity measured as described in the Materials and Methods. B) Sigmoidal dose-response for pyriproxyfen at concentrations ranging from 0.01 μM to 10 μM. C) Cells transfected with GAL4-97gDEF and pG5*luc* were treated with methyl farnesoate at concentrations of 3, 10, and 30 μM and luciferase activity measured. D) Sigmoidal dose-response curve of methyl farnesoate at concentrations

ranging from 0.1 μM to 30 μM. Dose-reponse curves and EC50s were determined with

GrapPad Prizm 4.0 (n = 3).

Table 2.2 EC50 and 95% Confidence Interval (CI) of the HR97g activators,

pyriproxyfen and methyl farnesoate.

JHAs	EC50 (μM)	95% CI (μM)
Pyriproxyfen	3.36	2.00-5.64
MF	2.22	1.23-4.00

DISCUSSION:

Few of the chemicals tested in transactivation assays increased HR97g activity. All three

of the chemicals that induced luciferase activity are stress hormones or analogs of stress

hormones. Two of the three activators of HR97g are the pesticide and juvenile hormone

analog, pyriproxyfen, and the endogenous crustacean juvenile hormone, methyl

farnesoate. The discovery that magnaHR97g is activated by JHAs is interesting because

most JHAs induce the production of males with high efficacy (Olmstead & Leblanc,

2002; Tatarazako et al., 2003), and the production of males is the first step in the switch

from parthenogenic reproduction to sexual reproduction.

Because HR97g and the other HR97 receptors (NR1L group) are a novel group of nuclear

receptors, we investigated their relationship to other nuclear receptor groups.

Phylogenetically, the HR97 receptors are related to the NR1J group that contains HR96 and DAF12. Both of these groups are related to the NR1I group of nuclear receptors that contain CAR, PXR, and VDR. The function of magnaHR96 is unknown, but *Drosophila* HR96 is activated by phenobarbital and induces a number of detoxification enzymes similar to CAR and PXR (King-Jones et al., 2006). Therefore, we hypothesized that HR97g may be a promiscuous toxicant receptor activated by a plethora of environmental chemicals; however our data does not support this hypothesis as only cortisol and JHAs activated HR97g. Furthermore, analysis of the percent identities of the different domains from CAR, DappuHR96, and Drosophila HR96 (Table 2.1) suggests that the HR97 receptors probably bind different chemicals and activate the transcription of different genes.

Daphnia switch from parthenogenic cloning of females to sexual reproduction because of several different environmental signals such as short day length, food shortage, and high population density (Tatarazako & Oda, 2007). The key step in the response of *Daphnia* to these stressful conditions is producing males (Hebert, 1978). Males are required for mating with adult females to produce resting eggs covered with an ephippium that is capable of enduring adverse conditions for long periods of time (Hebert, 1978). Juvenile hormones such as MF induce male production (Olmstead & Leblanc, 2002). However the MF receptor is not known. Both MF (sesquiterpenoid) and all-trans-retinoic acid (diterpenoid) are terpenoid signaling molecules that mediate development processes. Therefore, speculation has revolved around RXR as it is a retinoid binding hormone in

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mammals and its insect homolog, USP/RXR, is bound and activated by JH at low micromolar concentrations (Jones & Sharp, 1997). However, recent research demonstrated that crustacean RXR is not activated by MF, and activation of RXR by other ligands such as tributyltin does not increase male production (Wang & LeBlanc, 2009), indicating that RXR is not a crucial receptor in male production.

Here our data show that HR97g may be a candidate MF receptor. However, receptor activation was not potent. This could be caused by the assay, high constitutive activity, or the lack of key *Daphnia* cofactors. The development of a *Daphnia* cell line would be helpful. Furthermore, the EC50's we observe here are usually considered pharmacological-type and not the high affinity EC50's observed for the steroid receptors; methyl farnesoate has an EC50 of 2.2μM and pyriproxyfen has an EC50 of 3.4μM for HR97g. Nevertheless, these EC50's are within the physiological concentration range of typical adopted orphan receptors. For example, the EC50 of 9-cis-retinoic acid for RXR is 0.5 μM (Schwimmer et al., 2004), while the EC50 of the putative endogenous RXR ligand docosahexaenoic acid is > 50 μM (Calderon & Kim, 2007). Therefore, we consider MF a putative HR97g ligand.

In addition, *in vivo* activity and in vitro transactivation are at comparable doses. Pyriproxyfen activates 100% male production in a daphnid's third brood at about 0.3 μg/L (934 pM) (Tatarazako et al., 2003). Pyriproxyfen's bioconcentration factor (BCF) in fish is 1397-1495 (Report, 2009). Using this calculated BCF from fish estimates a

body burden of pyriproxyfen after 934 pM exposure in *Daphnia* of approximately 1.4

µM or close to the EC50 of pyriproxyfen for HR97g in transactivation assays. MF

causes 100% male production at about 100 µg/L (400 nM) (Olmstead & Leblanc, 2002).

MF's BCF has not been tested to our knowledge, but if it has a bioconcentration factor of

100, which is reasonable, then it would reach concentrations of approximately 40 µM in

the *Daphnia*, well above the EC50 of MF for HR97g. Therefore, the EC50s of

pyriproxyfen and methyl farnesoate are within the range necessary to cause male

production *in vivo*.

Currently, there is not enough evidence to conclude that HR97g is the MF receptor

involved in male production. HR97g may work in combination with another receptor to

enhance MF binding. For example, HR97g may interact with another receptor in a

manner similar to how several receptors interact with RXR (Amoutzias et al., 2007).

Activation may also be enhanced by co-transfection of the proper co-activators (Fleming

et al., 2004; Kamei et al., 2003). It is also possible that HR97g is only one of several JH

receptors in *Daphnia*, in which case it would not necessarily be the specific receptor

involved in male production.

It is not unusual for an organism to have multiple receptors responding to one chemical

signal. For example, there are three groups of receptors activated by retinoic acids in

humans with different developmental and tissue-expression. These include receptors

from the NR1B (RARa, RARb, RARg), NR2B (RXRa, RXRb, RXRg) (Chambon, 1996)

and NR2F (COUP-TFI, COUP-TFII, EAR2) groups, although in the NR2F group only COUP-TFII has been shown to bind retinoids (Kruse et al., 2008). Therefore, there may be several MF receptors in crustacea; each one with a different function or expression pattern so that juvenile development and environmental sex determination may be controlled by one hormone reducing the need for new hormone synthesis pathways. Juvenile hormone also functions in juvenile development and reproductive maturation (Riddiford, 2008). Therefore, we must consider that HR97g may be influencing these functions in addition to or instead of environmental sex determination.

Determining the exact function of HR97g *in vivo* may be difficult as knockout technologies are not currently available in *Daphnia*. Preliminary reports suggest that RNAi may be available soon; however this technology has only been used successfully for a short period of time in developing embryos and not in reproductive adults (Kato et al., 2011). Furthermore, the proper vectors for long-term repression of expression have not been explored in *Daphnia* species. Thus, the physiological function of HR97g may need to be estimated based on pharmacological studies, different *Daphnia* ecotypes, and circumstantial evidence until these techniques are available.

The purpose of our research was to characterize HR97g, a novel nuclear receptor found in *Daphnia* species related to the NR1I and NR1J groups. Unlike some of the receptors found in the NR1I (CAR/PXR) or NR1J (HR96/CeNHR8/CeNHR48) groups, HR97g is not promiscuous. Instead it is activated by the juvenile hormone analogs MF (the

endogenous hormone) and pyriproxyfen (pesticide). The data provide intriguing

evidence that HR97g may be a MF receptor.

References

Amoutzias, G. D., Pichler, E. E., Mian, N., De Graaf, D., Imsiridou, A., Robinson-

Rechavi, M., et al. (2007). A protein interaction atlas for the nuclear receptors:

Properties and quality of a hub-based dimerisation network. *BMC Systems Biology,*

1, 34.

Baldwin, W. S., & Roling, J. A. (2009). A concentration addition model for the activation

of the constitutive androstane receptor by xenobiotic mixtures. *Toxicological*

Sciences : An Official Journal of the Society of Toxicology, 107(1), 93-105.

Baskin-Bey, E. S., Anan, A., Isomoto, H., Bronk, S. F., & Gores, G. J. (2007).

Constitutive androstane receptor agonist, TCPOBOP, attenuates steatohepatitis in the

methionine choline-deficient diet-fed mouse. *World Journal of Gastroenterology:*

WJG, 13(42), 5635-5641.

Benoit, G., Cooney, A., Giguere, V., Ingraham, H., Lazar, M., Muscat, G., et al. (2006).

International union of pharmacology. LXVI. orphan nuclear receptors.

Pharmacological Reviews, 58(4), 798-836.

Bridgham, J. T., Eick, G. N., Larroux, C., Deshpande, K., Harms, M. J., Gauthier, M. E., et al. (2010). Protein evolution by molecular tinkering: Diversification of the nuclear receptor superfamily from a ligand-dependent ancestor. *PLoS Biology, 8*(10), e1000497.

Calderon, F., & Kim, H. Y. (2007). Role of RXR in neurite outgrowth induced by docosahexaenoic acid. *Prostaglandins, Leukotrienes, and Essential Fatty Acids, 77*(5-6), 227-232.

Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. *The FASEB Journal, 10*(9), 940-954.

Colbourne, J. K., Pfrender, M. E., Gilbert, D., Thomas, W. K., Tucker, A., Oakley, T. H., et al. (2011). The ecoresponsive genome of *Daphnia pulex*. *Science, 331*(6017), 555-561.

Eads, B. D., Andrews, J., & Colbourne, J. K. (2008). Ecological genomics in *Daphnia*: Stress responses and environmental sex determination. *Heredity, 100*(2), 184-190.

Eads, B. D., Colbourne, J. K., Bohuski, E., & Andrews, J. (2007). Profiling sex-biased gene expression during parthenogenetic reproduction in *Daphnia pulex. BMC Genomics, 8*(1), 464.

Evans, R. M. (2005). The nuclear receptor superfamily: A rosetta stone for physiology. *Molecular Endocrinology, 19*(6), 1429-1438.

Finn, R. D., Henderson, C. J., Scott, C. L., & Wolf, C. R. (2009). Unsaturated fatty acid regulation of cytochrome P450 expression via a CAR-dependent pathway. *The Biochemical Journal, 417*(1), 43-54.

Fleming, F. J., Hill, A. D., McDermott, E. W., O'Higgins, N. J., & Young, L. S. (2004). Differential recruitment of coregulator proteins steroid receptor coactivator-1 and silencing mediator for retinoid and thyroid receptors to the estrogen receptor-estrogen response element by beta-estradiol and 4-hydroxytamoxifen in human breast cancer. *The Journal of Clinical Endocrinology and Metabolism, 89*(1), 375-383.

Hannas, B. R., Wang, Y. H., Baldwin, W. S., Li, Y., Wallace, A. D., & LeBlanc, G. A. (2010). Interactions of the crustacean nuclear receptors HR3 and E75 in the regulation of gene transcription. *General and Comparative Endocrinology, 167*(2), 268-278.

Hebert, P. D. N. (1978). The population biology of *Daphnia* (crustacea, daphnidae). *Biological Reviews, 53*(3), 387- 426.

Hernandez, J. P., Mota, L. C., & Baldwin, W. S. (2009). Activation of CAR and PXR by dietary, environmental and occupational chemicals alters drug metabolism, intermediary metabolism, and cell proliferation. *Current Pharmacogenomics and Personalized Medicine, 7*(2), 81-105.

Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., et al. (1985). Primary structure and expression of a functional human glucocorticoid receptor cDNA *Nature, 318*(6047), 635-641.

Horner, M. A., Pardee, K., Liu, S., King-Jones, K., Lajoie, G., Edwards, A., . . . Thummel, C. S. (2009). The drosophila DHR96 nuclear receptor binds cholesterol and regulates cholesterol homeostasis. *Genes & Development, 23*(23), 2711-2716.

Jones, G., & Sharp, P. A. (1997). Ultraspiracle: An invertebrate nuclear receptor for juvenile hormones. *Proceedings of the National Academy of Sciences of the United States of America, 94*(25), 13499-13503.

Kamei, Y., Ohizumi, H., Fujitani, Y., Nemoto, T., Tanaka, T., Takahashi, N., et al. (2003). PPARgamma coactivator 1beta/ERR ligand 1 is an ERR protein ligand, whose expression induces a high-energy expenditure and antagonizes obesity. *Proceedings of the National Academy of Sciences of the United States of America, 100*(21), 12378-12383.

Kato, Y., Kobayashi, K., Watanabe, H., & Iguchi, T. (2011). Environmental sex determination in the branchiopod crustacean *Daphnia magna*: Deep conservation of a doublesex gene in the sex-determining pathway. *PLoS Genetics, 7*(3), e1001345.

King-Jones, K., Horner, M. A., Lam, G., & Thummel, C. S. (2006). The DHR96 nuclear receptor regulates xenobiotic responses in drosophila. *Cell Metabolism, 4*(1), 37-48.

Kliewer, S. A., Moore, J. T., Wade, L., Staudinger, J. L., Watson, M. A., Jones, S. A., . . . Lehmann, J. M. (1998). An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell, 92*(1), 73-82.

Kruse, S. W., Suino-Powell, K., Zhou, X. E., Kretschman, J. E., Reynolds, R., Vonrhein, C., et al. (2008). Identification of COUP-TFII orphan nuclear receptor as a retinoic acid-activated receptor *PLoS Biology, 6*(9), e227.

Laudet, V., & Gronemeyer, H. (2002). *The Nuclear Receptor Facts Book.* New York: Academic Press.

Lin, G. G., Kozaki, T., & Scott, J. G. (2010). Hormone receptor-like in 96 and broad-complex modulate phenobarbital induced transcription of cytochrome P450 CYP6D1 in drosophila S2 cells. *Insect Molecular Biology.*

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., et al. (1995). The nuclear receptor superfamily: The second decade. *Cell, 83*(6), 835-839.

Marchler-Bauer, A., Anderson, J. B., Cherukuri, P. F., DeWeese-Scott, C., Geer, L. Y., Gwadz, M., et al. (2005). CDD: A conserved domain database for protein classification. *Nucleic Acids Research, 33*(Database issue), D192-6.

Miyakawa, H., Imai, M., Sugimoto, N., Ishikawa, Y., Ishikawa, A., Ishigaki, H., et al. (2010). Gene up-regulation in response to predator kairomones in the water flea, *Daphnia pulex. BMC Developmental Biology, 10*, 45.

Nuclear Receptors Nomenclature Committee. (1999). A unified nomenclature system for the nuclear receptor superfamily. *Cell, 97*(2), 161-163.

Olmstead, A. W., & Leblanc, G. A. (2002). Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna. The Journal of Experimental Zoology, 293*(7), 736-739.

Olmstead, A. W., & LeBlanc, G. A. (2000). Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna. Environmental Toxicology and Chemistry, 19*(8), 2107-2113.

Ourlin, J. C., Lasserre, F., Pineau, T., Fabre, J. M., Sa-Cunha, A., Maurel, P., et al. (2003). The small heterodimer partner interacts with the pregnane X receptor and represses its transcriptional activity. *Molecular Endocrinology (Baltimore, Md.), 17*(9), 1693-1703.

Report, C.A. (2009) Pyriproxyfen: Product-type 18 (Insecticides, acaricides and products to control other arthropods). *Directive 98/8/EC concerning the placing of biocidal products on the market: Inclusion of active substances in Annex I to Directive 98/8/EC.* The Netherlands, pp. 49.

Riddiford, L. M. (2008). Juvenile hormone action: A 2007 perspective. *Journal of Insect Physiology, 54*(6), 895-901.

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models *Bioinformatics (Oxford, England), 19*(12), 1572-1574.

Schwarzenberger, A., Courts, C., & von Elert, E. (2009). Target gene approaches: Gene expression in *Daphnia magna* exposed to predator-borne kairomones or to microcystin-producing and microcystin-free microcystis aeruginosa. *BMC Genomics, 10*, 527.

Schwerin, S., Zeis, B., Lamkemeyer, T., Paul, R. J., Koch, M., Madlung, J., et al. (2009). Acclimatory responses of the *Daphnia pulex* proteome to environmental changes. II. chronic exposure to different temperatures (10 and 20 degrees C) mainly affects protein metabolism. *BMC Physiology, 9*, 8.

Schwimmer, L. J., Rohatgi, P., Azizi, B., Seley, K. L., & Doyle, D. F. (2004). Creation and discovery of ligand-receptor pairs for transcriptional control with small molecules. *Proceedings of the National Academy of Sciences of the United States of America, 101*(41), 14707-14712.

Scoville, A. G., & Pfrender, M. E. (2010). Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators. *Proceedings of the National Academy of Sciences of the United States of America.*

Shaw, J. R., Colbourne, J. K., Davey, J. C., Glaholt, S. P., Hampton, T. H., Chen, C. Y., et al. (2007). Gene response profiles for *Daphnia pulex* exposed to the environmental stressor cadmium reveals novel crustacean metallothioneins. *BMC Genomics, 8*(1), 477.

Shaw, J. R., Pfrender, M. E., Eads, B. D., Klaper, R., Callaghan, A., Sibly, R. M., et al. (2008). *Daphnia* as an emerging model for toxicological genomics. *Advances in Experimental Biology*, *2*, 165-328.

Sieber, M. H., & Thummel, C. S. (2009). The DHR96 nuclear receptor controls triacylglycerol homeostasis in *Drosophila*. *Cell Metabolism, 10*(6), 481-490.

Sonoda, J., Chong, L. W., Downes, M., Barish, G. D., Coulter, S., Liddle, C., et al. (2005). Pregnane X receptor prevents hepatorenal toxicity from cholesterol metabolites. *Proceedings of the National Academy of Sciences of the United States of America, 102*(6), 2198-2203.

Spanier, K. I., Leese, F., Mayer, C., Colbourne, J. K., Gilbert, D., Pfrender, M. E., & Tollrian, R. (2010). Predator-induced defences in *Daphnia pulex*: Selection and evaluation of internal reference genes for gene expression studies with real-time PCR. *BMC Molecular Biology, 11*, 50.

Takeshita, A., Taguchi, M., Koibuchi, N., & Ozawa, Y. (2002). Putative role of the orphan nuclear receptor SXR (steroid and xenobiotic receptor) in the mechanism of CYP3A4 inhibition by xenobiotics. *The Journal of Biological Chemistry, 277*(36), 32453-32458.

Tatarazako, N., & Oda, S. (2007). The water flea *Daphnia magna* (crustacea, cladocera) as a test species for screening and evaluation of chemicals with endocrine disrupting effects on crustaceans. *Ecotoxicology (London, England), 16*(1), 197-203.

Tatarazako, N., Oda, S., Watanabe, H., Morita, M., & Iguchi, T. (2003). Juvenile hormone agonists affect the occurrence of male *Daphnia. Chemosphere, 53*(8), 827-833.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 25*(24), 4876-4882.

Thomson, S. A., Baldwin, W. S., Wang, Y. H., Kwon, G., & Leblanc, G. A. (2009). Annotation, phylogenetics, and expression of the nuclear receptors in *Daphnia pulex. BMC Genomics, 10*, 500.

Tollrian, R., & Leese, F. (2010). Ecological genomics: Steps towards unraveling the

genetic basis of inducible defenses in *Daphnia*. *BMC Biology, 8*(1), 51.

Wang, Y. H., & LeBlanc, G. A. (2009). Interactions of methyl farnesoate and related

compounds with a crustacean retinoid X receptor. *Molecular and Cellular*

Endocrinology, 309(1-2), 109-116.

Weber, A. K., & Pirow, R. (2009). Physiological responses of *Daphnia pulex* to acid

stress. *BMC Physiology, 9*, 9.

Whelan, S., & Goldman, N. (2001). A general empirical model of protein evolution

derived from multiple protein families using a maximum-likelihood approach.

Molecular Biology and Evolution, 18(5), 691-699.

Zeis, B., Lamkemeyer, T., Paul, R. J., Nunes, F., Schwerin, S., Koch, M., et al. (2009).

Acclimatory responses of the *Daphnia pulex* proteome to environmental changes. I.

chronic exposure to hypoxia affects the oxygen transport system and carbohydrate

metabolism. *BMC Physiology, 9*, 7.

CHAPTER THREE

CHARACTERIZATION OF NUCLEAR RECEPTORS HR97A AND HR97B IN

DAPHNIA MAGNA

ABSTRACT

Annotation of the *Daphnia pulex* nuclear receptors revealed a novel group of receptors designated NR1L, with its three members named HR97a/b/g. We cloned and sequenced the two duplicated forms found in tandem repeat, HR97a and HR97b, from *Daphnia magna*. We analyzed their genomic structure, and conducted phylogenetic studies that demonstrate their similarity to each other, but indicate significant differences from their ancestor HR97g. Gal4-chimeric plasmids were constructed that contain the LBD of HR97a/b for transactivation assays. Interestingly, HR97a represses transcription in the absence of a activator similar to many other nuclear receptors; however, HR97b has constitutive activity similar to the constitutive androstane receptor (CAR), a related NR1I member. Phylogenetic studies show that the HR97s do form a distinct group, and the newly-discovered *Ixodes* IsHR97 is also a member of this group. Although NR1L is phylogenetically related to NR1I and NR1J, the HR97s share little amino acid sequence identity with the NR1I and NR1J members, many of which are xenosensors (Fin-magnaHR96, DmDHR96, CeDAF12 or HsCAR). Further, transactivation assays found pyriproxyfen, the juvenile hormone analog that induces the production of males in the otherwise female parthenogenic *Daphnia,* activates HR97a and b.

INTRODUCTION

Nuclear receptors are a superfamily of transcription factors involved in diverse physiological functions ranging from reproduction, development, homeostasis and detoxification through direct or indirect transcriptional regulation (Benoit et al., 2006; Evans, 2005; Hernandez, Mota, & Baldwin, 2009). The regulatory function of nuclear receptors is made possible by their unique five-domain structure (Laudet & Gronemeyer, 2002). From the N-terminal to the C-terminal there is the A/B domain that functions in ligand-independent transcriptional activation (AF-1), the C domain that binds to the response element of a gene (DNA binding domain; DBD), D domain that is the hinge domain between the C and E domain, E domain that is the ligand binding domain (LBD), and the highly diverse F domain. Of the five domains, C is highly conserved and E is moderately conserved. The C domain is the most conserved domain because it binds to DNA at well-conserved response elements. Binding of a small lipophilic compound (ligand) to the E-domain, (Kozlova, Lam, & Thummel, 2009), usually leads to a series of conformational changes in the receptor, typically resulting in translocation to the nucleus, DNA binding, and ultimately transcriptional activation (Mangelsdorf et al., 1995).

Studies on nuclear receptor function and phylogenetics are crucial to understanding metazoan evolution as nuclear receptors have gone through numerous duplication events during evolution. In the recent sequencing of the *Daphnia pulex* genome, which is also the first full genome sequencing project on crustaceans (http://wFleaBase.org), 25 nuclear receptor genes with a conserved DBD have been identified (Thomson et al., 2009). It has

been estimated that there are 48 nuclear receptors in *Homo sapiens*, 21 in *Drosophila melanogaster*, and over 270 in *Caenorhabditis elegans* (Adams et al., 2000; Robinson-Rechavi, Carpentier, Duffraisse, & Laudet, 2001; Sluder & Maina, 2001). This diversity is interesting because it is likely to reveal the time and roles of gene duplications in evolution and facilitate phylogenetic reconstruction (Escriva Garcia, Laudet, & Robinson-Rechavi, 2003). For example, a recent study, using genomic, biochemical, functional, structural, and phylogenetic analyses, has shown that modern nuclear receptors evolved through subtle tinkering of a ligand-dependent ancestral receptor (Bridgham et al., 2010).

Comparisons of the nuclear receptors from *Daphnia pulex* to other species indicate that *Daphnia* have a novel group of nuclear receptors. This group is designated the NR1L group. Its three members are named HR97a, HR97b, and HR97g because of their similarity to HR96 (NR1J) found in both in *Daphnia pulex* and *Drosophila melanogaster* (Thomson et al., 2009). In Chapter two we described our characterization of HR97g, the evolutionary precursor to the HR97a and HR97b receptors. Here we will focus on HR97a and HR97b, which are found in tandem repeat in the *D. pulex* genome (Thomson et al., 2009). We used *D. magna* in this study because it is widely used in aquatic toxicicity tests as a surrogate organism for other pelagic aquatic invertebrates, and the *D. magna* genome project is currently underway. We hope this study will provide mechanistic support to some long-established toxicity tests as well as broaden our current understanding of nuclear receptor functions and phylogenetics.

In this study, *D. magna* HR97a and HR97b (magnaHR97a and magnaHR97b) were cloned using 5'- and 3'-RACE, sequenced, analyzed and compared phylogenetically with other receptors in different species. GAL4-97a/b DEF chimeric plasmids were constructed with the magnaHR97a/b D, E and F domains (Thomson et al., 2009), and transactivation assays were performed to determine HR97a/b activity under different conditions and in the presence of different chemicals, including pyriproxyfen, the juvenile hormone analog that has been discovered to activate magnaHR97g (Chapter Two).

MATERIALS and METHODS

Daphnia culture: Our colony of *D. magna* was kindly provided by Dr. Steve Klaine (Clemson University). The moderately hard water used in *Daphnia* culture was deionized water reconstituted with $MgSO_4 \bullet 7H_2O$ 123 mg/L, $CaSO_4$ 60 mg/L, KCl 4 mg/L and $NaHCO_3$ 96 mg/L. The population was maintained at 22^oC under a 16:8 light:dark photoperiod in an environmental chamber with 15 adult daphnids/L. Daphnids were fed twice daily with 6×10^6 *Selenastrum capricornutum* algal cells per adult daphnid and 0.15 mL Tetrafin fish food suspension per liter of media (Tetra Holding Inc, VA) (Chapter Two).

RNA isolation and cDNA synthesis: RNA was extracted from fresh female *Daphnia magna* with Tri-Zol (BioRad, Hercules, CA) according to the manufacturer's directions.

DNA digestion using DNAse (Promega, Madison, WI) was subsequently performed, and cDNA synthesized by reverse transcription of 2μg of RNA with 200 units Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT), a 10mM dNTP mixture, and 0.05 mg random hexamers (Promega Corporation, Madison, WI) .

Cloning HR97a from D. magna: A small piece of *magna* HR97a gene was isolated using a pair of primers provided by Dr. Gerald LeBlanc at North Carolina State University: F-97a-GL: 5'-CCAACTCGAGTGGAGGAGAG-3'; R-97a-LeBlanc: 5'-GCCACCTTTGAGCAGGATAC-3'. The PCR product was ligated into pCR 2.1 by TA cloning (Invitrogen, Carlsbad, CA). DNA sequencing was performed by MacrogenUSA (Rockville, MD). The Invitrogen 3' RACE kit (Invitrogen, Carlsbad, CA) was used to amplify the 3' half of the gene. The forward primer used in the first 3' RACE PCR is F-97a-GL. F-GSP-97a (5'- TAC GCT CGC TTG ATG GCC GAC -3') was used in the nested PCR. After that, PCR was performed using primer pair 97a-F2/R-97a-GL. 97a-F2 (5'-CAAGAGTTACCATTTCGGCG -3') was designed based on *D. pulex's* sequence. The cloning of 97a was completed with the Invitrogen 5' RACE kit (Invitrogen, Carlsbad, CA). The steps of 5' RACE include first strand cDNA synthesis, TdT tailing of cDNA and PCR of dC-tailed cDNA. Two reverse primers were designed based on the known *magna*HR97a sequence determined from the work described above. Primer sequence information: R-GSP6-97a: 5'- CACACTGGCCACGGTGACAGCAC – 3'; R-GSP7-97a: 5' – CGGAACGACGAAAGAAAGCCTTGC – 3'. The former was used in the first 5' RACE PCR and the latter was used in the nested PCR.

Cloning HR97b from D. magna: A small piece of *D. magna* HR97b gene was isolated

using a pair of primers provided by Dr. Gerald LeBlanc from the *D. pulex* genome

project: F-97b-GL (5'-GAG CTG CCT TCT GAA AGG TG-3') and R-97b-GL (5'-GCG

TGA ACA GAA CGA TCA AG-3'). The 3'-end of the HR97b gene was cloned via 3'-

RACE (Invitrogen, Carlsbad, CA). Forward primer used in the first 3'-RACE PCR is F-

97b-GL. F-GSP-97b (5'- GGG AGT CGA CGA ACC GAC CAT CAT -3') was used in

the nested PCR. Two rounds of 5'-RACE (Invitrogen, Carlsbad, CA) was performed to

isolate and determine the 5'-sequence of HR97b with gene specific and nested primers

(5' – GGACATGTTGGCGTTTGGCCAGCG – 3'; nested: 5' –

ACGGGTCGTAGACTAGCGCTCCTC – 3') (5' –

GCTCGAAAAGTCGGCCATCAGCCG – 3'; nested: 5' –

GGCGAAACGACGAATCAGAGTCCC -3').

Genomic structure: We blasted our Clemson magnaHR97a and HR97b sequences to

the genomic sequences from *D. magna* clone Xlnb3 (Fin-magnaHR97a/b). Xlnb3 is the

strain of *D. magna* isolated from a pond in Finland of which the genomic sequencing is

currently underway. We were able to find the corresponding HR97a and HR97b

sequences in the scaffolds and use this information to construct the genomic model of

magnaHR97a and b. The genomic structure of magnaHR97a/b was analyzed in terms of

position, length, and phase of each intron. We also compared the intronic parameters and

protein sequence homology of the two receptors between *D. magna* and *D. pulex* using

ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/).

Expression plasmids: Chimeric plasmids containing the D, E (LBD), and F domains of the magnaHR97a (GAL4-97aDEF) and magnaHR97b (GAL4-97bDEF) receptor attached to the DNA binding domain (DBD) of Gal4 were assembled for the purpose of performing transactivation assays. The chimeric plasmids were constructed using the Clontech In-Fusion Dry-Down PCR Cloning Kit (Clontech Laboratories, Mountain View, CA) as described previously (Chapter Two). Primers used in amplifying the D, E and F domains of the two receptors include: F-clonetech-97a-BamHI (5'- GAA TTC CCG GGG ATC GAT GAA CGC AAA GCC CTG ATG AAA GCA CGT -3'), R-clonetech-97a-XbaI (5'- CTG CGG CCG CTC TAGA TCA TTG GAG CTT GTT GGT ATC TTT GGC TGG TCG -3'), F-clonetech-97b-BamHI (5'- GAA TTC CCG GGG ATC GAT GAA CGC AAA GCT TTA ATG AAA GCT CGA GC -3'), and R-clonetech-97b-XbaI (5'- CTG CGG CCG CTC TAGA TCA GTG GGC GTC GTA AAG CTC TGA ATA TTC TTC -3'). The PCR products were inserted into the pBIND vector (Promega CheckMate Mammalian Two-hybrid system, Promega, Madison, WI) in frame after the GAL4 DNA binding domain.

Transactivation Assays: HEPG2 human hepatoma cells (ATCC, Rockville MD) were used for transactivations. The cells were cultured in phenol red free Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% L-glutamine (Invitrogen, Carlsbad, CA) and 1% Penicillin/Streptomycin (Invitrogen, Carlsbad, CA) at 37 °C under 5% CO_2.

For transactivation assays HepG2 cells were plated in 12-well plates at 200,000 cells per well. Transfection was carried out the day after plating using Effectene Transfection Reagent (Qiagen, Valencia, CA) with 0.02 μg expression plasmid GAL4-97aDEF or GAL4-97bDEF and 0.1 μg the pG5*luc* (Promega, Madison, WI) reporter plasmid that contains five GAL4 binding sites upstream of the firefly luciferase gene. On the third day cells were either left untreated or treated with the chemicals of interest dissolved in 0.1% DMSO. Control cells received 0.1% DMSO. Luciferase activity was measured as luminescence with the Steady-Glo Luciferase Assay System (Promega, Madison, WI) 24 hours after chemical treatment.

Results of the transactivation assays are presented as the mean of triplicate assays $\pm$ standard error. Statistical analysis was carried out using GraphPad Prizm 4.0 statical and graphing package (La Jolla, CA). Statistical significance was determined by ANOVA followed by Dunnett's test as the post hoc test.

Phylogenetics:

Phylogenetic analyses of HR97a and HR97b and the related receptors was performed using methods described previously (Hannas et al., 2010; Thomson et al., 2009) with some modification. HR97a and HR97b from *D. magna* was compared to *D. pulex* and to receptors of other species available in GenBank such as *Drosophila melanogaster*, *Xenopus laevis, Danio rerio*, *Homo sapiens*, *Ciona intestinalis, Ixodes scapularis,* and

Caenorhabditis elegans. NCBI accession numbers for the receptors used are available in Appendix E. The DNA binding domain (DBD) and the ligand binding domain (LBD) of each receptor were identified using the conserved domain database (CDD) (Marchler-Bauer et al., 2005). Zf-C4 (pfam00105) was used to identify the DBD and Hormone recep (pfam00104) was used to identify the LBD. ClustalX default parameters were used to align the domains (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997).

Phylogenetic trees were were constructed using Bayesian Inference, Neighbor Joining, and Maximum Parsimony, and all other trees are compared to the Bayesian tree. Bayesian Inference was performed with MrBayes software version 3.1.2 (Ronquist & Huelsenbeck, 2003) on the Computational Biology Service Unit of Cornell University (http://cbsuapps.tc.cornell.edu/mrbayes.aspx). Trees were constructed using the "mixed-model" approach in which the Markov chain Monte Carlo sampler explores nine different fixed-rate amino acid substitution models implemented in MrBayes. We used 4 chains with runs of 5 million generations, chains sampled every 100 generations, a burnin of 5,000 trees with the *WAG* model (Whelan & Goldman, 2001). CeNHR-1, a member of the NR1K group, was used as outgroup.

Maximum Parsimony and distance parameters were used to provide additional support for the phylogenetic relationships observed. Distance parameters were measured using PAUP 4.0b10 (Swofford, 2001) with default characteristics (mean character difference and among site rate variation), and Neighbor-joining/UPGMA searches. Branch support

was measured by bootstrap analysis with 1000 replicates. Parsimony was constructed

using PAUP version 4.0b10 with heuristic searches, tree-bisection-reconnection,

topological constraints not enforced, and multiple tree option in effect with an initial

maximum tree setting at 100,000. Branch support was measured by bootstrapping with

50,000 replicates. FigTree (http://tree.bio.ed.ac.uk/software) was used to visualize the

phylogenetic trees.

RESULTS

Genomic Structure

After we cloned the magnaHR97a and HR97b genes using 5'- and 3'-RACE, we were

able to blast them against the newest version of the *D. magna* genome project. Similar to

DappuHR97a and HR97b, these two genes are found in tandem repeat. In addition, the

position, length, and phase of each intron within the magnaHR97a and magnaHR97b

genes was determined and compared to magnaHR97g and the HR97a and b receptors

from *Daphnia pulex* (DappuHR97a/b) (Figure 3.1). There is not much species variation

as to gene structure between *D. magna* and *D. pulex*. HR97a and HR97b have similar

gene structures: both have two introns, and the phases of the first and second introns are 0

and 1, respectively. This is different from HR7g that has three introns and of which the

intron phases are 1, 2 and 2 from the first to the third intron respectively. However,

within the first exon of DappuHR97a, we found a possible intronic site from base pair

426 to 506 that obeys the GT-AG rule. A potential intron site is also found in

magnaHR97a from base pair 416 to 514 (Figure 3.2). The potential intron would make the gene structure of HR97a more similar to HR97g. The genomic structure of HR97a and HR97b further indicates that HR97g is the precursor of HR97a and HR97b. However, the alternative magnaHR97a and DappuHR97a does not share the same intron phases.

In addition, the five domains of magnaHR97a and HR97b were aligned and compared to the corresponding domains in Fin-magnaHR97a/b and DappuHR97a/b (Table 3.1). Our *D. magna* sequences agree very well with the Finish strain of *D. magna* with only minor variances. The five domains of HR97a/b, especially the C and E domains, are very similar between *D. magna* and *D. pulex* with identity scores equal or close to 100. Alignment of the A/B domain of magnaHR97b and DappuHR97b produced the lowest identity score. This domain in magnaHR97b is shorter than DappuHR97b by 32 amino acids, meaning it is 36% shorter than the A/B domain of DappuHR97b. The identity score of the A/B domain of HR97a in both species is only 68, but it is mainly due to the difference in amino acid composition as opposed to the length. The F domain of each receptor varies in the two species both in length and amino acid composition.

The five domains of magnaHR97a were also aligned with the corresponding domains of several other nuclear receptors including magnaHR97b, magnaHR96-Fin, DappuHR96, DmDHR96, HsCAR, magnaKNR-R1, magnaKNR-R2 and magnaRXR. MagnaHR97a is highly similar to magnaHR97b in the C and E domains, but not similar to other receptors,

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including HR97g. This suggests that HR97a and HR97b have different ligands and

different response elements than HR97g.
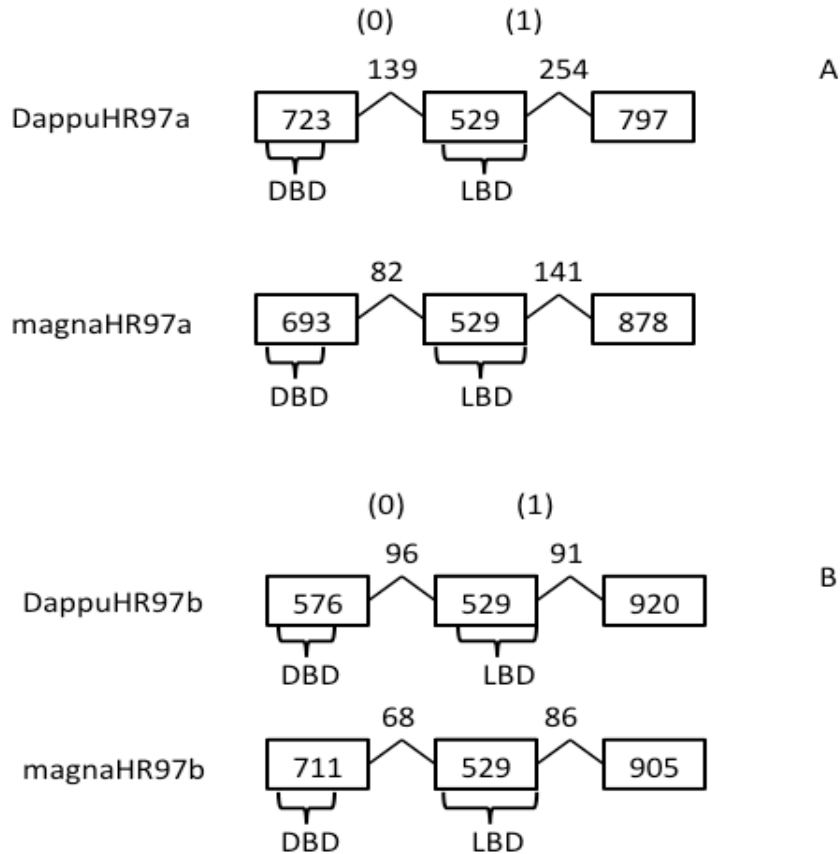
Figure 3.1 Gene structure of HR97a and HR97b in *D. magna and D. pulex*. A) Gene

structure of HR97a. B) Gene structure of HR97b. The blocks represent exons. The

numbers represent the size of the exons and introns. Numbers in parentheses represent the

intron phases.
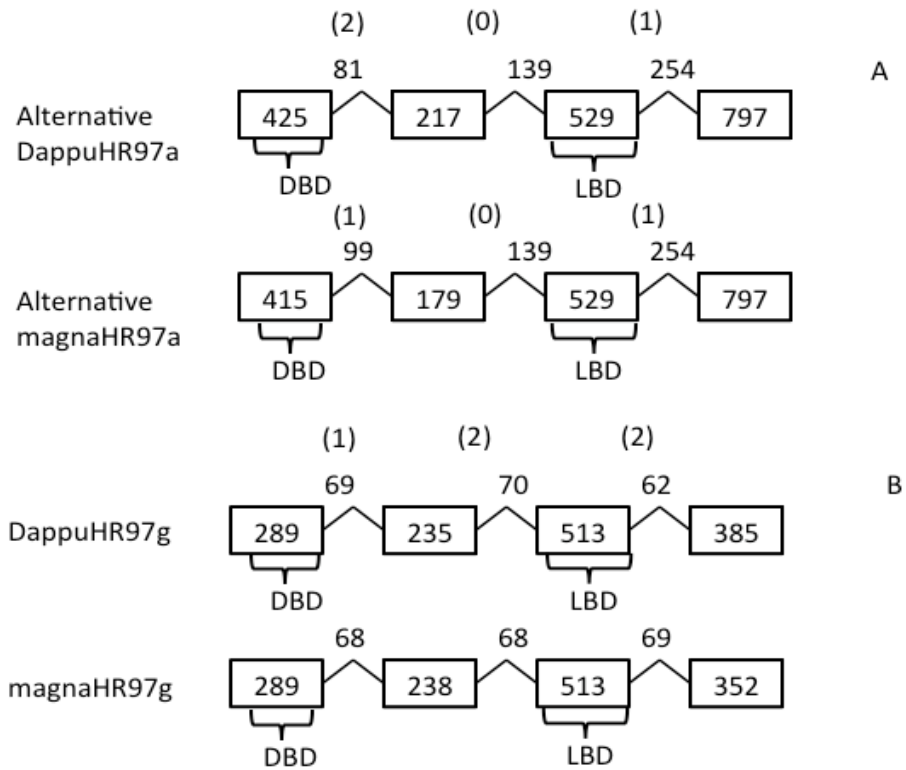
Figure 3.2 Gene structure of alternative HR97a and comparison to HR97g. A) Gene structure of alternative HR97a in *D. magna* and *D. pulex*. B) Gene structure of HR97g in *D. magna* and *D. pulex*. The blocks represent exons. The potential intron splits the first exon into two, which makes the gene structure of the alternative HR97a more similar to HR97g.

Table 3.1 Percent identity[a] of each domain when comparing the cloned HR97a and HR97b genes from the Clemson *D. magna* strain to HR97a and HR97b from the Finland *D. magna* strain (*D. magna*-Fin) and *Daphnia pulex* (Dappu).

A: HR97a

	A/B	C	D	E	F
D. magna-Fin	97	100	100	97	99
D. pulex	68	100	84	90	47

B: HR97b

	A/B	C	D	E	F
D. magna-Fin	98	98	98	100	98
D. pulex	35	98	92	97	60

[a]Data shown as identity scores derived from ClustalW

Table 3.2 Percent identity of magnaHR97a (Clemson strain) to other NRs.

NR	A/B	C	D	E	F
magnaHR97b	24	98	74	76	21
magnaHR97g	20	63	31	40	8
magnaHR96-Fin	12	48	20	29	33
DappuHR96	12	48	23	29	33
DmDHR96	25	44	14	25	33
HsCAR	12	42	11	20	50
magnaKNR-R1	2	44	11	N/A	N/A
magnaKNR-R2	2	44	10	N/A	N/A
magnaRXR	15	42	8	15	0

[a]Data shown as identity scores to magnaHR97a derived from ClustalW.

Hs = *Homo sapiens*, Dm = *Drosophila melanogaster*, Dappu = *Daphnia pulex*, magna = *D. magna*

Phylogenetics

The DBD/LBD combined tree seperates the receptors into three clades—NR1L that contains the HR97s; NR1J that contains HR96s, CeNHR48, CeNHR8 and CeDAF12; NR1I that contains CAR, PXR and VDR (Figure 3.3). In this tree there is no

disagreement between the three programs Bayesian Inference, Maximum Parsimony and Neighbor-Joining. HR97s are more closely related to HR96s than to CAR, PXR and VDR. This tree further confirms that the HR97s constitute a distinct group and that the newly-discovered IsHR97 identified from mining the tick (*Ixodes scapularis*) genome is a member of the HR97s. The arachnid *Ixodes* is within the Chelicerata subphylum of Arthropoda (Regier et al., 2010). We speculate that there has been an HR97 receptor in the ancient arthropods and it was carried on to *Ixodes*. But for the Pancrustacea branch which contains both hexapods and crustaceans (Regier et al., 2010), the land-based hexapods which include insects lost HR97, while the aquatic branchiopod *Daphnia* kept it and eveutually developed three HR97 receptors. The tree indicates that the differentiation between HR97a/b/g occurred before the speciation of *D. magna* and *D. pulex*. The DBD/LBD tree also shows, in agreement with previous studies (Thomson et al., 2009), that HR97g is the precursor of HR97a and HR97b.

The LBD tree agrees very well with the DBD/LBD combined tree with only minor differences between the programs (Appendix F). The DBD Bayesian tree segregates into three clades—the Knirps, the HR97s, and the third clade consists of the NR1J and NR1I groups and is not able to separate the two (Appendix G). The Knirps are not in the other two trees because they lack a LBD. The DBD tree disagrees with the other two trees in that it suggests that *Daphnia* HR97a and HR97b are more closely related to IsHR97 rather than *Daphnia* HR97g, but overall evidence indicates that the DBD/LBD combined tree is more reliable. The DBD tree also fails to differentiate HR97a from HR97b, likely

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due to the high level of similarity in the DBDs of the receptors. The Parsimony DBD tree, like the Bayesian tree, was not able to separate the NR1J and NR1I groups. The NJ DBD tree, though, segregates into four distinct clades: the Knirps, the HR97s, NR1J and NR1I.
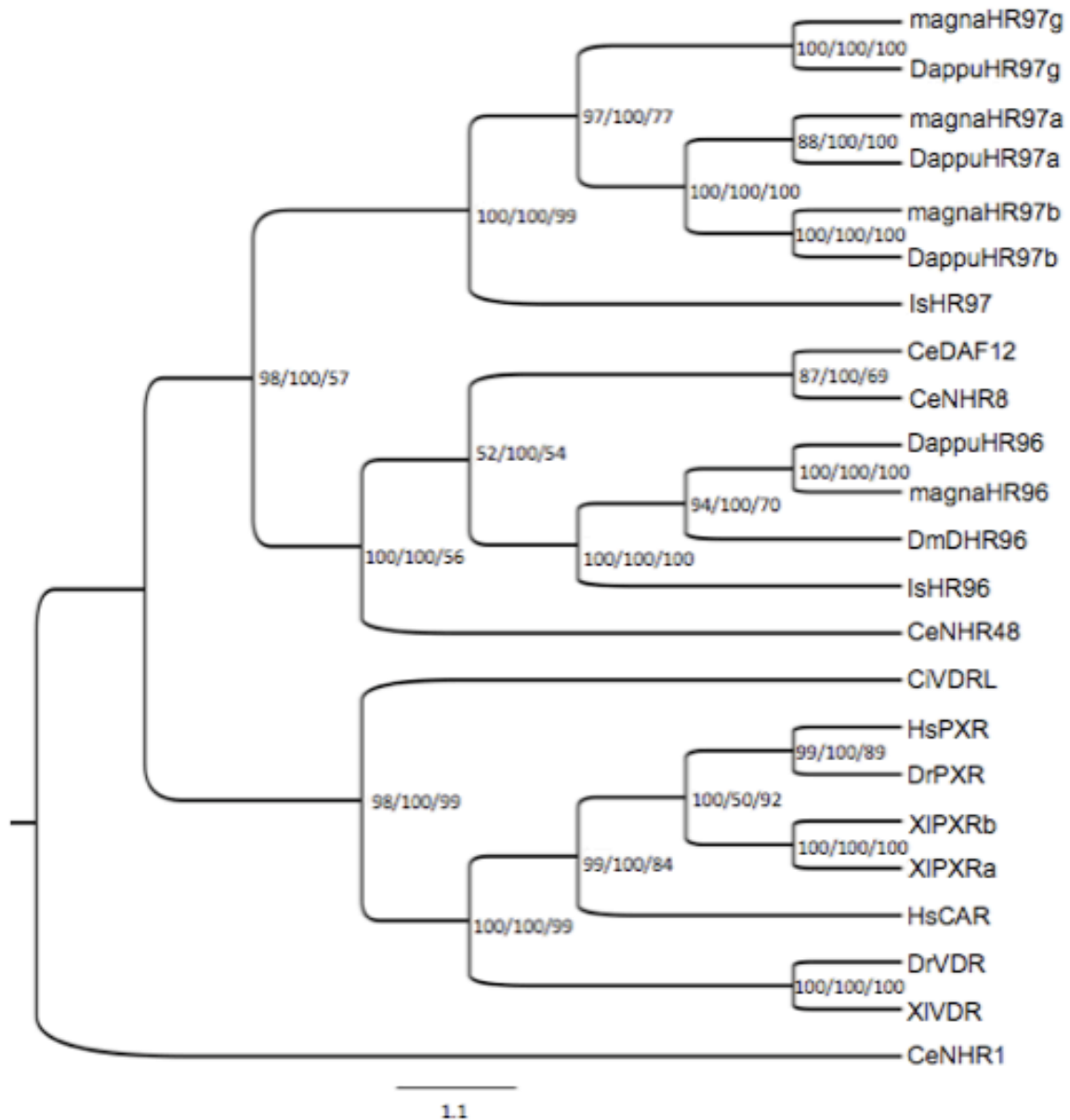
Figure 3.3 Phylogenetic analysis of the NR1L group and similar nuclear receptors.

NRs from several different species were analyzed based on their C and E domains using

Bayesian Inference, Maximum Parsimony and Neighbor-Joining methods. CeNHR1 was chosen as the outgroup. We adoped the tree structure from Bayesian Inference. Probability values were provided from left to right as from Bayesian Inference, Maximum Parsimony and Neighbor-Joining respectively. An X indicates disagreement from the Bayesian tree. Abbreviation of species names are as follows: magna=*Daphnia magna, Dappu=Daphnia pulex,* Is=*Ixodes scapularis,* Ce=*Caenorhabditis elegans,* Dm=*Drosophila melanogaster,* Ci=*Ciona intestinalis,* Dr=*Danio rerio,* Hs=*Homo sapiens,* Xl=*Xenopus laevis.*

HR97a/b basal regulation

To examine the basal regulation of HR97a, we transfected HepG2 cells with the GAL4-97a/bDEF chimeric expression plasmid and the reporter plasmid pG5*luc*. Control cells were transfected with 0.1 μg of the reporter plasmid pG5*luc* only. After treatment, the cells received no further treatment. The luciferase assay shows that HR97a is able to repress transcription in the absence of an activator which is similar to the activity of HR97g described in Chapter two (Figure 3.4). Next we transfected cells with increasing amounts of GAL4-97aDEF chimeric plasmid (0-0.08 ug) supplemented with empty pBIND-GAL4 vectors to make the total amount of pBIND plasmids 0.08 μg. With the exception of the 0.04 μg treatment, HR97a exerts greater repression at higher doses (Figure 3.5A).

The basal regulation of HR97b was tested in the same way as HR97a. However, the luciferase assay result shows that HR97b regulates transcription in a very different manner comparing to HR97a and HR97g: it induces transcription when unbound (Figure 3.4). In the dose response study of HR97b, the presence of empty pBIND-GAL4 vectors seemed to repress the activity of GAL4-97bDEF (Figure 3.5B). So we did another assay with different doses of GAL4-97bDEF but without empty pBIND-GAL4 vectors. The result shows that HR97b up-regulates transcription in the absence of activators with the maximal activation at 0.02 μg (Figure 3.5C).
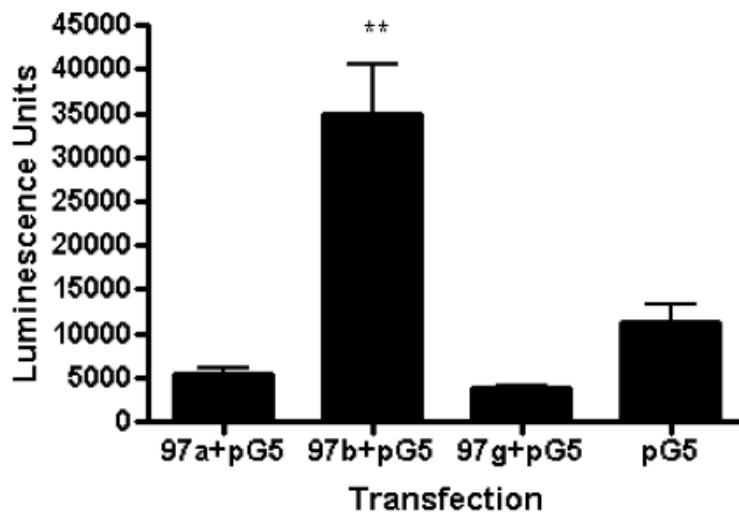
Figure 3.4 Basal activity of the HR97 receptors. Cells were transfected with 0.02 μg GAL4-97a/b/gDEF chimeric plasmid and 0.1 μg reporter plasmid pG5*luc*. Control cells were transfected with 0.1 μg reporter plasmid pG5*luc* only. HR97a and HR97g repress

transcription in the absence of an activator. HR97b significantly up-regulates

transcription (3-fold). Statistical significance was determined using GraphPad Prizm 4.0

statistical and graphing package (La Jolla, CA).
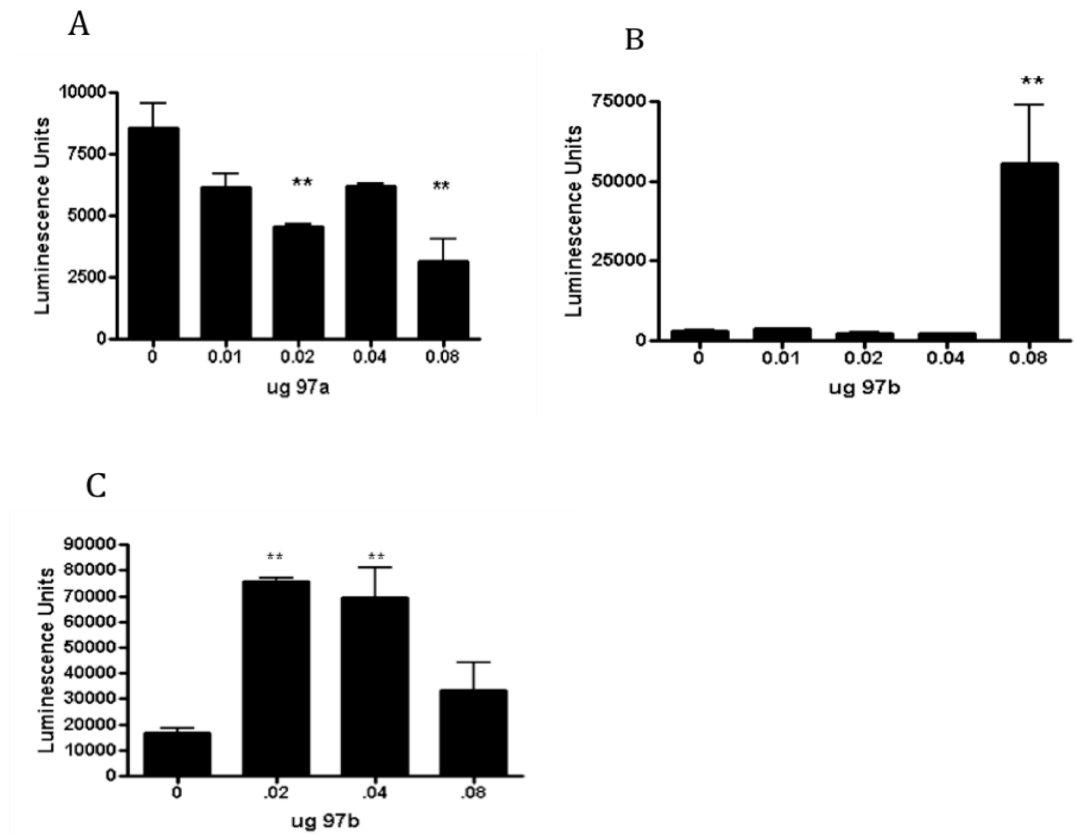
Figure 3.5 Dose response studies of HR97a/b basal regulation. A) Cells were

transfected with 0, 0.01, 0.02, 0.04 and 0.08 µg GAL4-97a DEF chimeric plasmid

supplemented with empty pBIND-GAL4 vectors to make the total amount of pBIND

plasmids 0.08ug. Except for the 0.04 µg treatment, HR97a exerts greater repression at

higher doses. B) Cells were transfected with 0, 0.01, 0.02, 0.04 and 0.08 µg GAL4-97b

DEF chimeric plasmid supplemented with empty pBIND-GAL4 vectors to make the total

amount of pBIND plasmids 0.08 μg . Empty pBIND-GAL4 vectors repress the activity of GAL4-97bDEF. C) Cells were transfected with 0, 0.02, 0.04 and 0.08 μg GAL4-97a DEF chimeric plasmid without adding empty pBIND-GAL4 vectors. HR97b up-regulates transcription with the maximal activation at 0.02 μg in the absence of activators.

Chemical screening and pyriproxyfen activation:

HepG2 cells were transfected with GAL4-97gDEF chimeric plasmid and the reporter plasmid pG5*luc* to determine the potential HR97a and HR97b activators. The next day cells were treated with chemicals at 10 μM, control cells were treated with 0.1% DMSO. None of the chemicals tested activated HR97a or HR97b (Figure 3.6). Next, we treated cells with pyriproxyfen and methyl farnesoate at 10 μM, as these two chemicals have been discovered to activate HR97g, another member of the NR1L group and the evolutionary precursor of HR97a/b. The luciferase assay shows that pyriproxyfen significantly activates HR97a but the activation for HR97b was not statistically significant (Figure 3.7 A B). We subsequently repeated the pyriproxyfen treatment at both 3 μM and 10 μM. This time pyriproxyfen significantly activates HR97b at 10 μM (Figure 3.7 D). However, this time although HR97a was activated in a dose-dependent manner, the activation was not statistically significant (Figure 3.7 C). In all, pyriproxyfen may be a weak activator for both HR97a and HR97b, but is unlikely a potent activator.
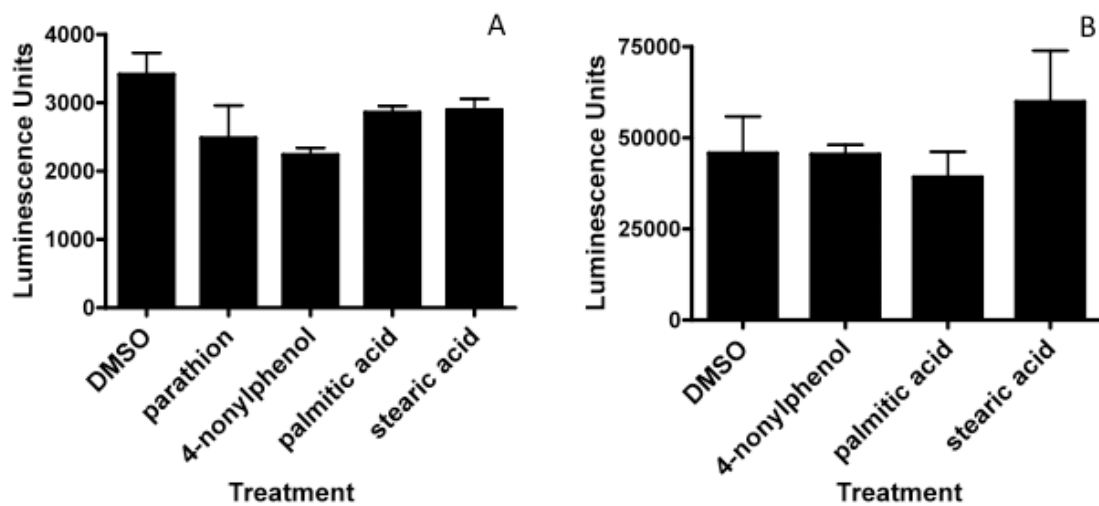
Figure 3.6 HR97a/b multi-chemical tests. Cells transfected with GAL4-97aDEF(A) or GAL4-97bDEF (B) and pG5*luc* were treated with different chemicals at 10 uM. Control cells were treated with 0.1% DMSO. Parathion and 4-nonylphenol caused some cell death in the 97a treatment. None of the chemicals activates either receptor.
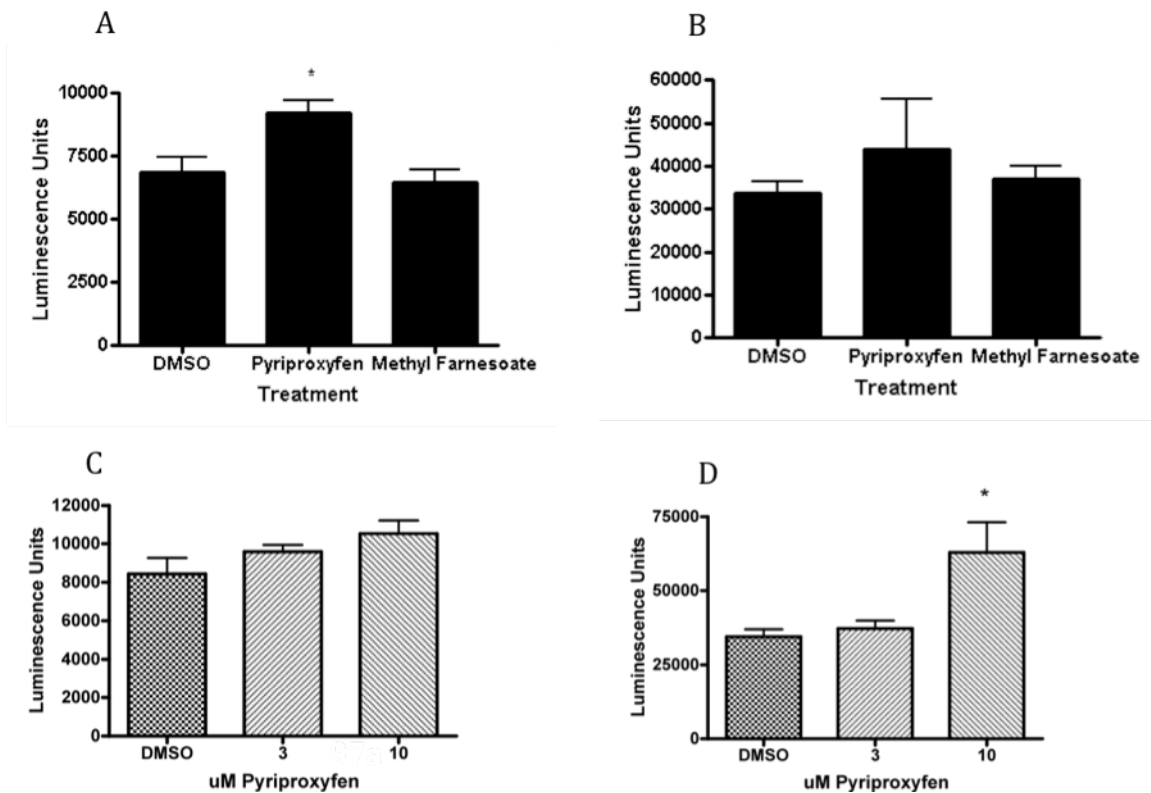
Figure 3.7 Pyriproxyfen and methyl farnesoate test. A) HR97a tansactivation. Cells transfected with GAL4-97aDEF and pG5*luc* were treated with pyriproxyfen and methyl farnesoate at 10 uM. Control cells were treated with 0.1% DMSO. B) HR97b tansactivation. Cells transfected with GAL4-97bDEF and pG5*luc* were treated with pyriproxyfen and methyl farnesoate at 10 uM. C) HR97a pyriproxyfen two-dose test. Cells were treated with pyriproxyfen at 3 uM and 10uM after transfection with GAL4-97aDEF and pG5*luc*. D) HR97b pyriproxyfen two-dose test. Cells were treated with pyriproxyfen at 3 uM and 10uM after transfection with GAL4-97bDEF and pG5*luc*. Pyriproxyfen has weak activation for both receptors at 10 uM.

DISCUSSION

The evident difference in the regulatory functions of magnaHR97a and magnaHR97b provides further evidence that closely related nuclear receptors could develop different roles through changes in amino acid composition during evolution. Without activators HR97a functions as a repressor, while HR97b constitutively up-regulates transcription. The relationship between the two is very much like CAR and PXR, two closely related receptors in the NR1I group. PXR represses gene expression in the absence of ligands (Hernandez et al., 2009; Takeshita et al., 2002). HR97a, like PXR, is likely to recruit co-repressors such as N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors) when unliganded. Ligand binding causes the release of the co-repressors and recruits coactivators (Larsen et all, 2002), which is probably why we see the increased activity when we treat cells with pyriproxyfen. On the other hand, HR97b functions like CAR that has been proven to have constitutive activity (Baes et al., 1994). The constitutive activity of CAR has been attributed to its unique structure that orients the AF-2 helix in the active position (Xu et al., 2004). It is possible that HR97b has a similar structure that could be used to explain its constitutive activity.

In addition, this study provides additional evidence that the LBD of a receptor is not only crucial in functions beyond ligand-binding, but also in its distinct activation functions. There are two motifs in a nuclear receptor that are involved in the recruitment of coactivators—AF-1 in the A/B domain and AF-2 in the LBD (Thomson et al., 2009). Since the A/B domain of the HR97a/b is not included in our chimeric plasmids, the

difference in the functions of the receptors shown by the transactivations in the absence of activators is mostly likely attributed to the AF-2 motif within the LBD.

ClustalW alignment shows that HR97a and HR97b share high similarity in all five domains. They are almost identical in the DNA binding domain, indicating that they might regulate the same genes. Both HR97a and HR97b are activated by pyriproxyfen, like their evolutionary precursor HR97g (Chapter Two). However, contrary to HR97g, they are not activated by MF. The ligand binding domains of HR97a and HR97b are not similar to that of HR97g, meaning the ligand binding pockets in the LBDs of the receptors have different structures, which could explain the different results from transactivations.

The phylogenetic tree confirms that HR97a and HR97b are most closely related and less ancestral than HR97g, agreeing with previous discovery that HR97g is the evolutionary precursor to HR97a and HR97b, which are in tandem repeat (Thomson et al., 2009). The phylogenetic tree also puts IsHR97 in the same clade with the HR97s from *Daphnia*.

NR1L is related to the NR1J and NR1I groups. Although CAR and PXR in the NR1I group as well as HR96 in the NR1J group have been shown to be promiscuous receptors activated by many chemicals (Hernandez et al., 2009; King-Jones et al., 2006), this study and our previous study (Chapter Two) showed that HR97s are not promiscuous receptors.

Furthermore, recent data our research group obtained demonstrated that *Daphnia* HR96 is promiscuous.

In addition, not all receptors related to the HR97s are promiscuous chemical sensors. The NR1J member DAF-12 in *C. elegans*, for example, has been shown to regulate dauer diapause and development (Antebi et al., 2000). The NR0A members KNI and KNRL, closely related to the HR97s in the DBD only tree, regulate the development of the second wing vein in *Drosophila* (Lunde et al., 1998). The juvenile hormone analog pyriproxyfen has been shown to induce male production, which is the first step of *Daphnia*'s defense mechanism against harsh environment, a mechanism that serves the similar purpose as dauer diapause in *C. elegans*, as the male *Daphnia* will turn the population from parthenogenetic reproduction into sexual reproduction. And the eggs from sexual reproduction will go into a diapause until the environmental conditions get better (Tatarazako & Oda, 2007). The fact that HR97a and b are activated by pyriproxyfen would suggest a putative role in juvenile hormone functions, except the endogenous *Daphnia* juvenile hormone methyl farnesoate did not activate HR97a or HR97b in this study. It is possible that these receptors need to pair with a partner receptor in order to have greater activation.

In summary, HR97a/b/g developed from one ancient HR97 receptor that was amplified into three receptors before the speciation of *D. magna* and *D. pulex*. HR97a functions as a repressor in the absence of an activator while HR97b is a constitutive activator. HR97a

and b are activated by pyriproxyfen and we hypothesize that they are involved in development based on their low promiscuity and their phylogenetic relationship to the NR0A and NR1J receptors.

References

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., et al. (2000). The genome sequence of drosophila melanogaster *Science (New York, N.Y.), 287*(5461), 2185-2195.

Antebi, A., Yeh, W. H., Tait, D., Hedgecock, E. M., & Riddle, D. L. (2000). Daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans. Genes & Development, 14*(12), 1512-1527.

Baes, M., Gulick, T., Choi, H. S., Martinoli, M. G., Simha, D., & Moore, D. D. (1994). A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Molecular and Cellular Biology, 14*(3), 1544-1552.

Benoit, G., Cooney, A., Giguere, V., Ingraham, H., Lazar, M., Muscat, G., et al. (2006).
International union of pharmacology. LXVI. orphan nuclear receptors.
Pharmacological Reviews, 58(4), 798-836.

Bridgham, J. T., Eick, G. N., Larroux, C., Deshpande, K., Harms, M. J., Gauthier, M. E.,
et al. (2010). Protein evolution by molecular tinkering: Diversification of the nuclear
receptor superfamily from a ligand-dependent ancestor. *PLoS Biology, 8*(10),
e1000497.

Escriva Garcia, H., Laudet, V., & Robinson-Rechavi, M. (2003). Nuclear receptors are
markers of animal genome evolution. *Journal of Structural and Functional
Genomics, 3*(1-4), 177-184.

Evans, R. M. (2005). The nuclear receptor superfamily: A rosetta stone for physiology
Molecular Endocrinology, 19(6), 1429 -1438.

Hannas, B. R., Wang, Y. H., Baldwin, W. S., Li, Y., Wallace, A. D., & LeBlanc, G. A.
(2010). Interactions of the crustacean nuclear receptors HR3 and E75 in the
regulation of gene transcription. *General and Comparative Endocrinology, 167*(2),
268-278.

Hernandez, J. P., Mota, L. C., & Baldwin, W. S. (2009). Activation of CAR and PXR by
dietary, environmental and occupational chemicals alters drug metabolism,
intermediary metabolism, and cell proliferation. *Current Pharmacogenomics and
Personalized Medicine, 7*(2), 81-105.

King-Jones, K., Horner, M. A., Lam, G., & Thummel, C. S. (2006). The DHR96 nuclear receptor regulates xenobiotic responses in *Drosophila*. *Cell Metabolism, 4*(1), 37-48.

Kozlova, T., Lam, G., & Thummel, C. S. (2009). *Drosophila* DHR38 nuclear receptor is required for adult cuticle integrity at eclosion. *Developmental Dynamics : An Official Publication of the American Association of Anatomists, 238*(3), 701-707.

Larsen, P. R., Kronenberg, H. M., Melmed, S., & Polonsky, K. S. (2002). *Williams textbook of endocrinology* (10th ed.). USA: Saunders.

Laudet, V., & Gronemeyer, H. (2002). *The Nuclear Receptor Facts Book.* New York: Academic Press.

Lunde, K., Biehs, B., Nauber, U., & Bier, E. (1998). The knirps and knirps-related genes organize development of the second wing vein in *Drosophila. Development (Cambridge, England), 125*(21), 4145-4154.

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., et al. (1995). The nuclear receptor superfamily: The second decade. *Cell, 83*(6), 835-839.

Marchler-Bauer, A., Anderson, J. B., Cherukuri, P. F., DeWeese-Scott, C., Geer, L. Y., Gwadz, M., et al. (2005). CDD: A conserved domain database for protein classification. *Nucleic Acids Research, 33*(Database issue), D192-6.

Regier, J. C., Shultz, J. W., Zwick, A., Hussey, A., Ball, B., Wetzer, R., et al. (2010). Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. *Nature, 463*(7284), 1079-1083.

Robinson-Rechavi, M., Carpentier, A. S., Duffraisse, M., & Laudet, V. (2001). How

    many nuclear hormone receptors are there in the human genome? *Trends in*

    Genetics: TIG, 17(10), 554-556.

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference

    under mixed models *Bioinformatics (Oxford, England), 19*(12), 1572-1574.

Sluder, A. E., & Maina, C. V. (2001). Nuclear receptors in nematodes: Themes and

    variations. *Trends in Genetics : TIG, 17*(4), 206-213.

Swofford, D. L. (2001). PAUP*: Phylogenetic Analysis Using Parsimony (*and other

    methods), Version 4.0b10, Sinauer Associates, Sunderland, MA.

Takeshita, A., Taguchi, M., Koibuchi, N., & Ozawa, Y. (2002). Putative role of the

    orphan nuclear receptor SXR (steroid and xenobiotic receptor) in the mechanism of

    CYP3A4 inhibition by xenobiotics. *The Journal of Biological Chemistry, 277*(36),

    32453-32458.

Tatarazako, N., & Oda, S. (2007). The water flea *Daphnia magna* (crustacea, cladocera)

    as a test species for screening and evaluation of chemicals with endocrine disrupting

    effects on crustaceans *Ecotoxicology (London, England), 16*(1), 197-203.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997).

    The CLUSTAL_X windows interface: Flexible strategies for multiple sequence

    alignment aided by quality analysis tools. *Nucleic Acids Research, 25*(24), 4876-

    4882.

Thomson, S. A., Baldwin, W. S., Wang, Y. H., Kwon, G., & Leblanc, G. A. (2009).

    Annotation, phylogenetics, and expression of the nuclear receptors in *Daphnia pulex*.

    BMC Genomics, 10, 500.

Whelan, S., & Goldman, N. (2001). A general empirical model of protein evolution

    derived from multiple protein families using a maximum-likelihood approach.

    Molecular Biology and Evolution, 18(5), 691-699.

Xu, R. X., Lambert, M. H., Wisely, B. B., Warren, E. N., Weinert, E. E., Waitt, G. M., et

    al. (2004). A structural basis for constitutive activity in the human CAR/RXRalpha

    heterodimer *Molecular Cell, 16*(6), 919-928.

CHAPTER FOUR

CONCLUSION

This study is a partial characterization of the NR1L group that was initially identified in *Daphnia pulex*. The study proved the existence of HR97g, HR97a, and HR97b in *Daphnia magna*, and verified that the three receptors do form a distinct group (NR1L) in the NR1 subfamily of nuclear receptors. This group was first found in the crustacean *Daphnia pulex*, however our study showed that *Ixodes scapularis* also have a receptor (IsHR97) that can be phylogenetically positioned in this group. The NR1L group is related to the NR1J group that contains HR96 (Thomson et. al., 2009), which in turn is related to the NR1I group that contains CAR, PXR, and VDR (Lin, Kozaki, & Scott, 2010). In searching for the activators for the three receptors, we had set up two objectives:

Objective 1: Cloning, sequencing, and characterization of magnaHR97g.

We successfully cloned and sequenced magnaHR97g. We analyzed the gene structure of HR97g in terms of position, length, and phase of each intron. Also, we aligned the sequence of magnaHR97g to similar receptors and conducted phylogenetic studies to study the events during evolution of HR97g and related receptors. To search for the activators for magnaHR97g, we constructed the chimeric plasmid GAL4-97gDEF. We found that HR97g functions as a repressor when in the absence of activators, but in the presence of pyriproxyfen or methyl farnesoate it induces transcription.

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Objective 2: Cloning, sequencing, and characterization of magnaHR97a and HR97b.

We cloned and sequenced magnaHR97a and HR97b. We analyzed their gene structures and compared them to magnaHR97g. Both HR97a and HR97b only have 3 exons, however, within the first exon of HR97a, we found a possible intronic site that obeys the GT-AG rule, which could make HR97a more similar to HR97g that has 4 exons. We aligned the five domains of magnaHR97a to similar receptors and conducted phylogenetic studies to study the events during evolution of the HR97s and related receptors. To search for the activators for magnaHR97a and b, we constructed the chimeric plasmids GAL4-97a/bDEF. We found that HR97a, same as HR97g, functions as a repressor when in the absence of activators, but HR97b constitutively activates transcription. Both magnaHR97a and HR97b are activated by pyriproxyfen, but the effect is not consistently strong.

Pyriproxyfen is a juvenile hormone analog that has been shown to induce male broods with very high potency (Olmstead & Leblanc, 2002; Tatarazako, Oda, Watanabe, Morita, & Iguchi, 2003). MF, the unepoxidated form of insect JH III, also induces the production of male broods in *Daphnia magna* (Olmstead & Leblanc, 2002). Since we discovered that pyriproxyfen activates all three HR97 receptors and that MF activates HR97g, it is interesting to further investigate whether the HR97s are involved in *Daphnia* male production. According to my labmate Gautam Ginjupalli's data, magnaHR97g is primarily expressed in the mandibular organs, ovaries, and gut, and is expressed more in

reproductively active adults. This further supports our hypothesis that HR97g is involved in *Daphnia* responding to environmental signals including food (gut) and altering to sexual reproduction (ovaries) under the regulation of MF (produced by the mandibular organ).

Switching from parthenogenic cloning of females to the production of males for sexual reproduction is a protective mechanism for *Daphnia* in response to many different environmental signals such as short day length, food shortage, and high population density (Tatarazako & Oda, 2007), as the ephippial eggs from sexual reproduction can survive through harsh conditions. Juvenile hormones are believed to be involved in this process as they induce the production of males at nanomolar concentrations (Olmstead & Leblanc, 2002). We speculate that instead of one single JH receptor, *Daphnia* might have multiple JH receptors with different functions. Similarly there are multiple retinoic acid binding receptors in mammals (Chambon, 1996).

We hypothesize that one or more of the HR97s are involved in the JH regulated male production. But it is possible that similar to PXR, which interacts with itself and RXR to recruit co-activators such as SRC-1 (Carnahan & Redinbo, 2005; Noble et al., 2006), HR97s may interact with RXR or other receptors that allow them to recruit co-activators and achieve greater activation. Future work should investigate the receptors' interactions with RXR and co-activators. For example, transactivations can be performed in the presence of RXR and SRC-1. Considering the repressive and constitutive functions of

HR97a and HR97b, future work should also investigate whether these receptors interact with each other.

Since food-shortage could cause male production in *Daphnia* (Tatarazako&Oda, 2007), it is interesting to see the effect of dietary fatty acids on the HR97s. Our preliminary data showed that the long chain polyunsaturated fatty acid (PUFA) arachidonic acid represses the activity of all HR97s (data not shown). If one or more of the HR97s is a JH receptor, the repression of HR97g by arachidonic acid in live daphnids could prevent the production of males when food is abundant. It has been shown that absence of long chain PUFAs limits egg production of *Daphnia magna* (Martin-Creuzburg & Elert, 2009), suggesting that PUFAs have some role in development.

MagnaHR97g and magnaHR97a repress transcription when in the absence of activators while HR97b constitutively activates transcription. Opposing regulatory functions between closely related receptors in the absence of activators have been observed for CAR and PXR, both NR1I members. PXR represses gene expression in the absence of ligands (Hernandez, Mota, & Baldwin, 2009; Takeshita et. al., 2002) by binding the co-repressors N-CoR or SMRT. CAR is constitutively active, but held in the cytosol so it does not have significant activity *in vivo*. Activation causes the loss of CAR cytosolic retention protein (CCRP) and allows for translocation to the nucleus (Kobayashi et. al., 2003). However, because CCRP and other cytosolic retention proteins are not expressed in most immortal cell lines, CAR is constitutively active in HepG2 cells (Baldwin &

Roling, 2009; Kawamoto et al., 1999; Kobayashi et al., 2003). It is possible that during

evolution HR97a has retained the structure of its precursor HR97g, which facilitates the

recruitment of co-repressors while HR97b, through subtle tinkering, adopted a structure

that is more like CAR that allows constitutive activity. It would be worthwhile to perform

parallel studies using another cell line such as an insect cell line.

Another subsequent study for the HR97s should be aimed at their possible interactions

with each other. We have cloned the full-length magnaHR97a, magnaHR97b, and

magnaHR97g into the mammalian expression plasmid pACT (Promega CheckMate

Mammalian Two-hybrid system, Promega, Madison, WI). In our next set of

transactivation assays we will co-transfect HepG2 cells with pBIND-GAL4-97a/b/gDEF

plasmids, or pBIND-GAL4-RXRpaired with the pACT plasmids expressing the full-

length magnaHR97a/b/g to see if there is any interaction between the receptors that

affects transcriptional regulation in the presence of activators. This assay is a modified

mammalian two-hybrid assay called a co-activator or co-partner dependent receptor

ligand assay (CARLA). Preliminary studies using dual pBIND plasmids suggest that

HR97a and HR97b work together, but the assay also allowed for GAL4 competition at

the response element. Using the pACT system will eliminate this obstacle.

We had hypothesized that HR97s are xenobiotic receptors that are activated by multiple

toxicants because it is found in the same nuclear receptor clade as HR96, CAR, and PXR,

all of which are xenosensors. However in our transactivation assays only juvenile

hormone analogs activated them. In addition, ClustalW alignments showed little similarity between HR97s and Fin-magnaHR96, DmDHR96, CeDAF12 or HsCAR. So we conclude that HR97s might not be xenobiotic sensors like the NR1I and NR1J receptors, and we hypothesize that they are receptors involved in development.

To further prove that HR97g is a JH receptor, we could try to purify the receptor from *Daphnia* and determine the native ligand bound to the receptor (provided that there is one). A binding assay could also be performed using radio labeled putative ligands.

It would greatly help the characterization of the HR97s if RNAi technology is available. Although RNAi has only been used in developing *Daphnia* embryos (Kato et al., 2011), it is beyond doubt worthwhile to try this technology in reproductive adults. Knocking down the HR97s would conveniently reveal the physiological functions related to the receptors, though it will require other studies to differentiate the direct functions from indirection functions. With the sequences of the three HR97 receptors available and putative activators found, it would also be interesting to see what genes are regulated by the HR97s. A ChIP assay can be conducted to find the response elements these receptors bind to; hence the genes directly regulated by them can be recognized.

This study alone is not going to solve the puzzle of environmental sex determination. However, it contributes to the sea of studies on this question. If our subsequent studies prove that HR97g is a JHA receptor, the Gal4-97gDEF plasmid can be used as a

detection system to screen potential male inducers from other environmental chemicals and help protect our ecosystems. But for now, this study at least adds to our understanding of a new branch in the enormous tree of nuclear receptors that was only a seedling in 1985.

References

Baldwin, W. S., & Roling, J. A. (2009). A concentration addition model for the activation of the constitutive androstane receptor by xenobiotic mixtures. *Toxicological Sciences, 107*(1), 93-105.

Carnahan, V. E., & Redinbo, M. R. (2005). Structure and function of the human nuclear xenobiotic receptor PXR *Current Drug Metabolism, 6*(4), 357-367.

Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology, 10*(9), 940-954.

Hernandez, J. P., Mota, L. C., & Baldwin, W. S. (2009). Activation of CAR and PXR by dietary, environmental and occupational chemicals alters drug metabolism, intermediary metabolism, and cell proliferation. *Current Pharmacogenomics and Personalized Medicine, 7*(2), 81-105.

Kawamoto, T., Sueyoshi, T., Zelko, I., Moore, R., Washburn, K., & Negishi, M. (1999). Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene *Molecular and Cellular Biology, 19*(9), 6318-6322.

Kato, Y., Kobayashi, K., Watanabe, H., & Iguchi, T. (2011). Environmental sex determination in the branchiopod crustacean *Daphnia magna*: Deep conservation of a doublesex gene in the sex-determining pathway. *PLoS Genetics, 7*(3), e1001345.

Kobayashi, K., Sueyoshi, T., Inoue, K., Moore, R., & Negishi, M. (2003). Cytoplasmic accumulation of the nuclear receptor CAR by a tetratricopeptide repeat protein in HepG2 cells. *Molecular Pharmacology, 64*(5), 1069-1075.

Lin, G. G., Kozaki, T., & Scott, J. G. (2010). Hormone receptor-like in 96 and broad-complex modulate phenobarbital induced transcription of cytochrome P450 CYP6D1 in drosophila S2 cells. *Insect Molecular Biology.*

Martin-Creuzburg, D., & Elert, E. (2009). Good food versus bad food: The role of sterols and polyunsaturated fatty acids in determining growth and reproduction of *Daphnia magna*. *Aquatic Ecology, 43*(4), 943-950.

Noble, S. M., Carnahan, V. E., Moore, L. B., Luntz, T., Wang, H., Ittoop, O. R., et al. (2006). Human PXR forms a tryptophan zipper-mediated homodimer *Biochemistry, 45*(28), 8579-8589.

Olmstead, A. W., & Leblanc, G. A. (2002). Juvenoid hormone methyl farnesoate is a sex

    determinant in the crustacean *Daphnia magna. The Journal of Experimental*

    Zoology, 293(7), 736-739.

Takeshita, A., Taguchi, M., Koibuchi, N., & Ozawa, Y. (2002). Putative role of the

    orphan nuclear receptor SXR (steroid and xenobiotic receptor) in the mechanism of

    CYP3A4 inhibition by xenobiotics. *The Journal of Biological Chemistry, 277*(36),

    32453-32458.

Tatarazako, N., & Oda, S. (2007). The water flea *Daphnia magna* (crustacea, cladocera)

    as a test species for screening and evaluation of chemicals with endocrine disrupting

    effects on crustaceans. *Ecotoxicology (London, England), 16*(1), 197-203.

Tatarazako, N., Oda, S., Watanabe, H., Morita, M., & Iguchi, T. (2003). Juvenile

    hormone agonists affect the occurrence of male *Daphnia. Chemosphere, 53*(8), 827-

    833.

Thomson, S. A., Baldwin, W. S., Wang, Y. H., Kwon, G., & Leblanc, G. A. (2009).

    Annotation, phylogenetics, and expression of the nuclear receptors in *Daphnia pulex.*

    BMC Genomics, 10, 500.

APPENDICES

Appendix A

Nuclear receptors used in phylogenetic analysis

[a]NC-IUPHAR	Receptor	Species (common name)	GenBank Accession No.
0A	DappuKNR-R1	*Daphnia pulex* (D. pulex)	EFX76945.1
0A	DappuKNR-R2	*Daphnia pulex* (D. pulex)	EFX76947.1
0A	KNI	*D. melanogaster* (fruitfly)	CAA31709
0A	KNRL	*D. melanogaster* (fruitfly)	AAF51627
0A	EGON	*D. melanogaster* (fruitfly)	CAA34626
1H	magnaEcR	*D. magna*	ABP48741
1I	VDR	*H. sapiens* (Human)	AAB95155
1I	PXR	*H. sapiens* (Human)	AAD05436
1I	PXR	*Danio rerio*	NP_001092087
1I	CAR	*H. sapiens* (Human)	AAY56401
1I	VDRL	*Ciona intestinalis*	NP_001071847
1J	DappuHR96	*Daphnia pulex* (D. pulex)	EFX89804.1
1J	DHR96	*D. melanogaster* (fruitfly)	AAC46928
1J	DAF-12	*Caenorhabditis elegans(C. elegans)*	AAD34462
1J	NHR-8	*Caenorhabditis elegans(C. elegans)*	AAP31437
1J	NHR-48	*Caenorhabditis elegans(C. elegans)*	CAD36502
1L	DappuHR97a	*Daphnia pulex* (D. pulex)	EFX79885.1
1L	DappuHR97b	*Daphnia pulex* (D. pulex)	442724[b]
1L	DappuHR97g	*Daphnia pulex* (D. pulex)	EFX77588.1
1L	magnaHR97g	*D. magna*	JF792806

[b] For DappuHR97b, the JGI genome portal version 1.1 protein identification number is provided.

Phylogenetic Comparison of NR1I/J/L/0A Nuclear Receptors Using Only Their DBDs
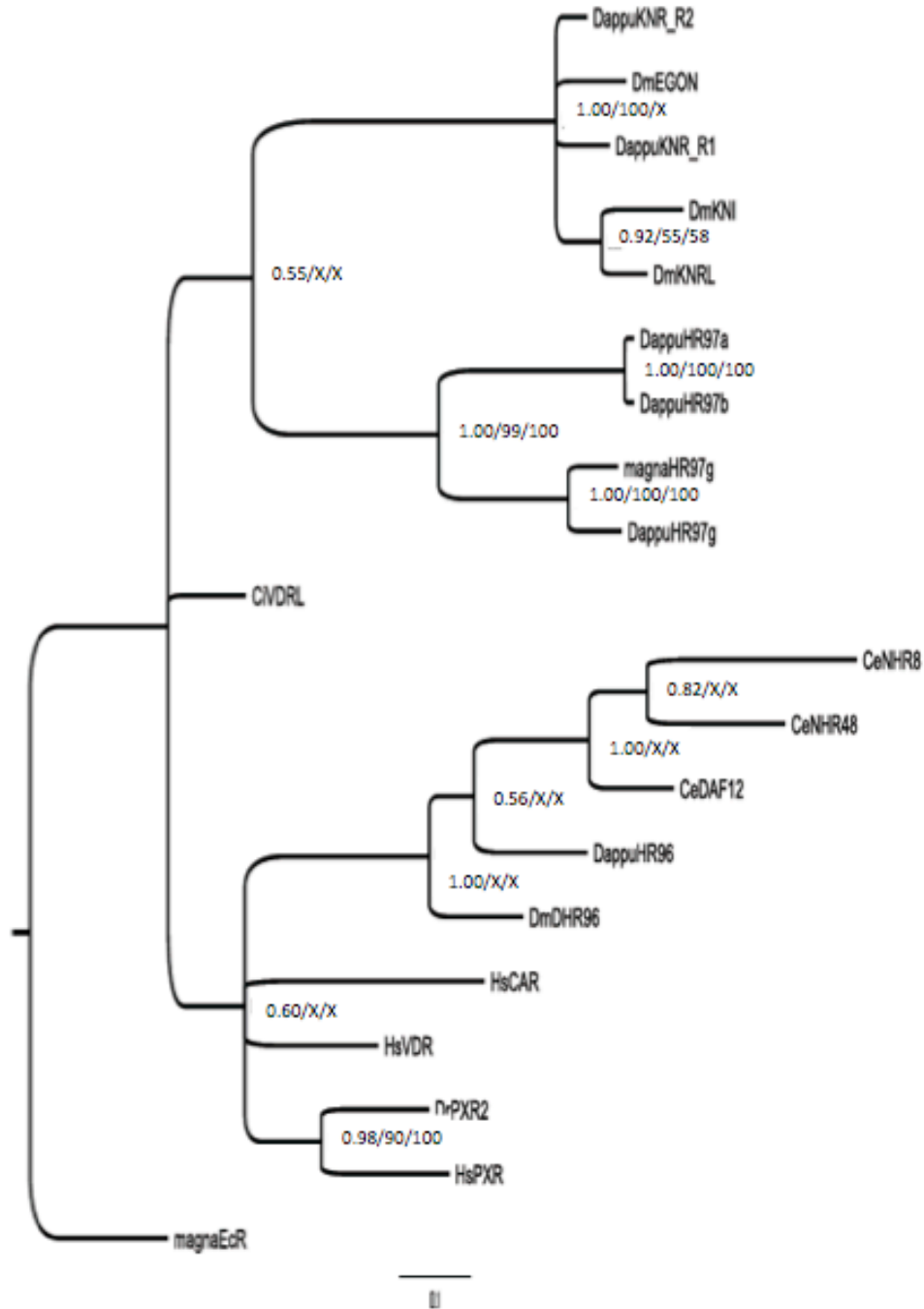
Probability values were provided from left to right as from Bayesian Inference, Neighbor-Joining and Maximum Parsimony respectively. An X indicates disagreement from the Bayesian tree.

Phylogenetic Comparison of NR1I/J/L Nuclear Receptors Using Only Their LBDs

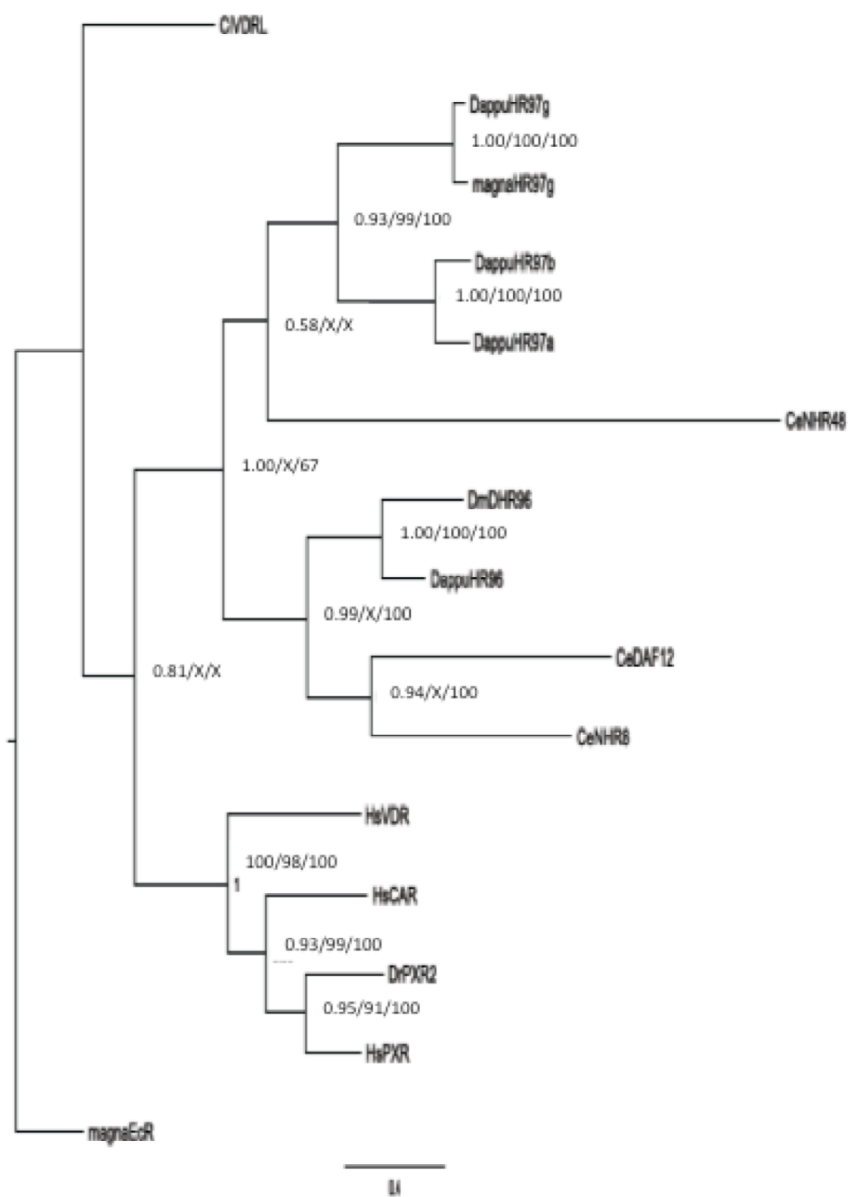Probability values were provided from left to right as from Bayesian Inference, Neighbor-Joining and Maximum Parsimony respectively. An X indicates disagreement from the Bayesian tree.
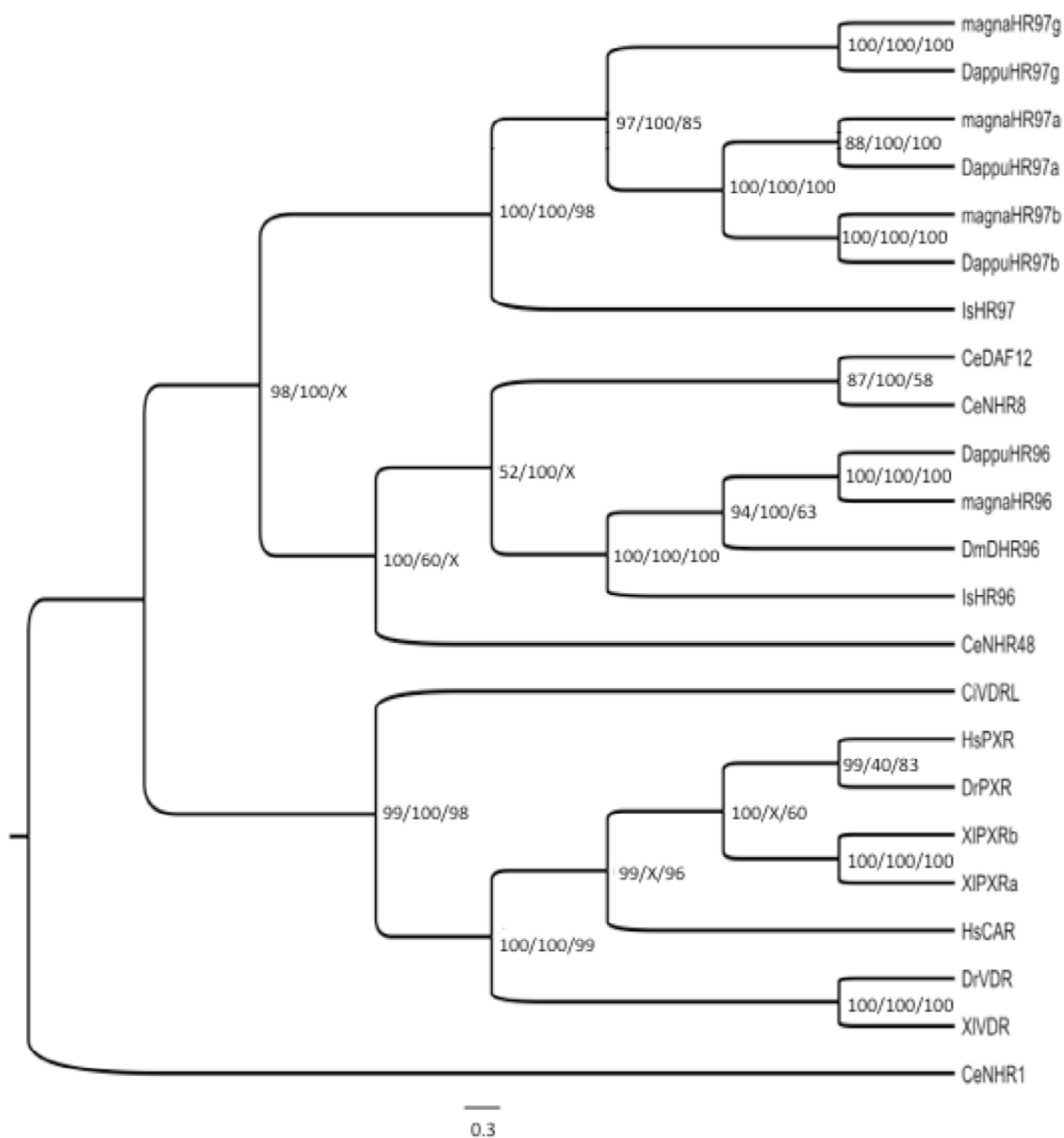
Appendix D

ClustalX Alignment File Comparing the Sequences of MagnaHR97g and DappuHR97g


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         ************** .****;.*;******.*;***************************;*******;;::.****************.***;******;*********** * **** :.    :  ..:. .::  :*.*;* **
pulex97g MDDSNSSLGSSNEEAVPENKSTNKICGVCGDKAKSYHFGGLCCESCKAFFRRAMQNDSFKAFFCVHGQSCIVNKENRRSCQKCRIQKCFSIGMEKGWLRTDEENVKMREAKLVN-KPAKQNVELDELGIRSSSPDSASQNTSTYQPRFE  149
magna97g MDDSNSSLGSSNEENVVPENRETKKICGVCSDRAKSYHFGGLCCESCKAFFRRAMQNDSFKSFFCVHGQTCVITKENRRSCQKCRIQKCFQIGMDKGWLRTEEENVKMREAKLGNSKPAKAQTTEMTMEENNTPIGATLLVSSVYEPSFE  150
         1.......10........20........30........40........50........60........70........80........90.......100.......110.......120.......130.......140.......150

         :* *** ********;*** ****;*;*****;*****************;****;********** .;*******.**************;;**********;*********;*.****************************;;*;********
pulex97g NILVYLMPEDVKFLEKIVDDYWKAYKQVPFGAEVTQNSTPRSGLQVISMFTTGIRRWASFARSLTEFSSLCRQDQATLLQAAVMQLSILRAVASFRMTDKEWRINTPVDEFTPSLSLEDVQRLLSPQLAEMHLTFISSVQKLKVDEPTIM  299
magna97g DINVYLSPEDVKFLENIVDAIWKAIRQVPFGTEVTQNSTPRSGLQVISMFTTGIRRWAAFARSLSEFSSLCRQDQANILQAAVMQLSILRAVASFRMSEKEWRINTPVNEFTPSLSLEDVQRLLSPQLAEMBLTFISSMHKFKVDEPTIM  300
         ........160.......170.......180.......190.......200.......210.......220.......230.......240.......250.......260.......270.......280.......290.......300

         ******;***;**********.*;**************;***** ***********************.****************************;;****************;********************************.;::.**
pulex97g LLSLITLFTPERADLVARESIVKAQEQYILLLERYCHWKYGPDDKGRTFAHLLAKMSDLRELSDVHSDHQVRFANQDLHKLYQELDAEPRPHVNMDLNNTSHFLSAMEFSILKTHHTLPKPEPVADPTYRELAQIEEMCAVLEGKEKETA  449
magna97g LLSLITMFTPDRADLVARESIAKTQEQYILLLERYCNWKYGPADKGRTFAHLLAKMSDLRELSDLHSDHQVRFANQDLHKLYQELDAEPRPHVNMDLNNTNHFLSAMEFSILKTHHTLPKPEPVADPTYRELAQIEEMCAVLEDQQNSTA  450
         .......310.......320.......330.......340.......350.......360.......370.......380.......390.......400.......410.......420.......430.......440.......450

         : *.*   ******
pulex97g NDENQINMEETDNPVKDETLSCTK  473
magna97g DTESQQ--METDNPV---------  464
         .......460.......470....
```

Appendix E

HR97a/b Phylogenetics Accession Numbers

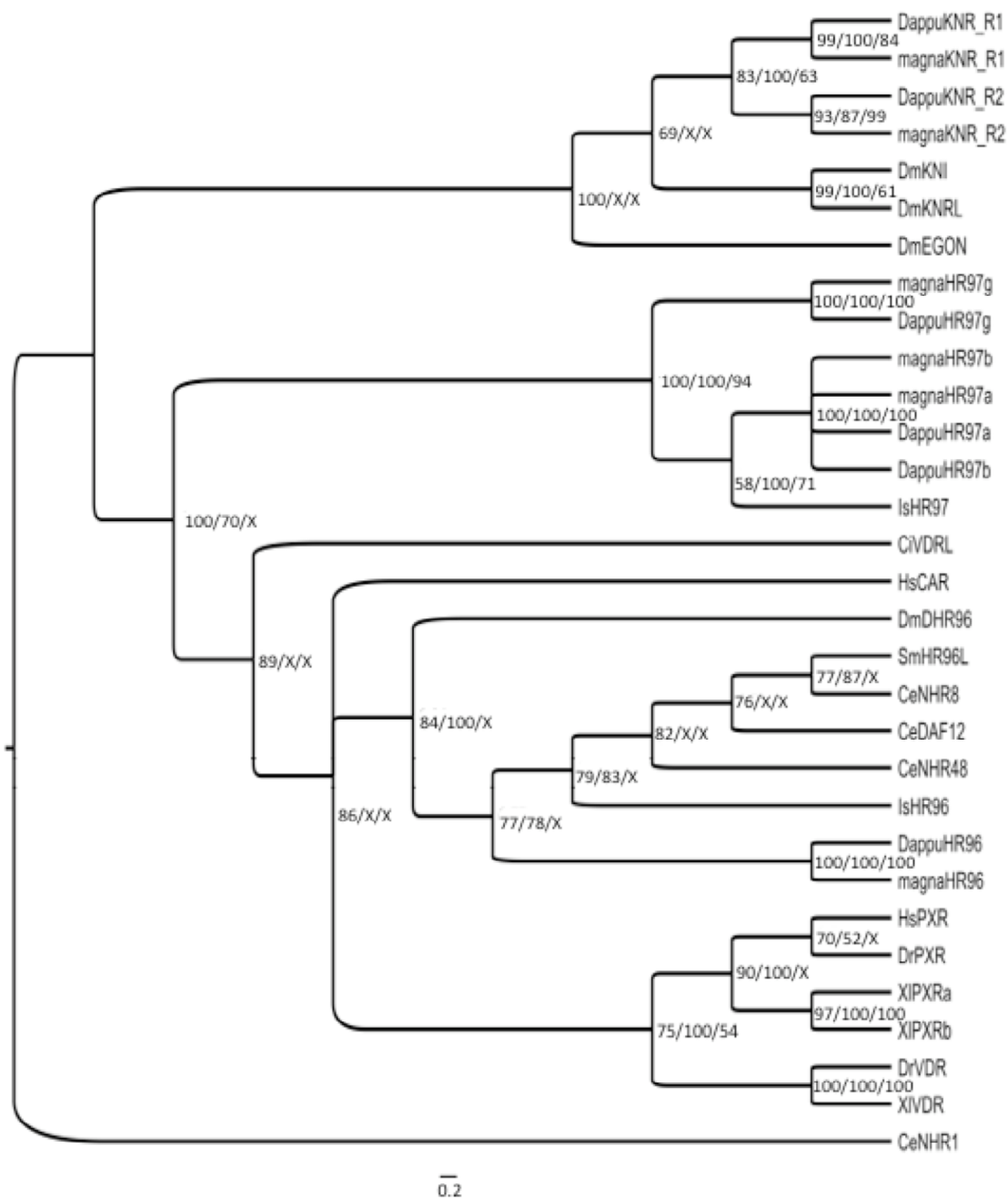| [a]NC-IUPHAR	Receptor	Species (common name)	Accession No.
1I	VDR	*Xenopus laevis*	NP_001079288
1I	VDR	*Danio rerio*	AAI62226
1I	VDR	*Danio rerio*	NP_001153457
1I	PXR	*H. sapiens* (Human)	AAD05436
1I	PXR	*Danio rerio*	NP_001092087
1I	PXR	*Xenopus laevis*	CAA53006
1I	PXR	*Xenopus laevis*	NP_001083606
1I	CAR	*H. sapiens* (Human)	AAY56401
1I	VDRL	*Ciona intestinalis*	NP_001071847
1J	HR96	*Daphnia pulex (D. pulex)*	EFX89804.1
1J	HR96	*Daphnia magna*	
1J	HR96	*Ixodes scapularis*	XP_002404556
1J	DHR96	*D. melanogaster* (fruitfly)	AAC46928
1J	DAF-12	*Caenorhabditis elegans (C. elegans)*	AAD34462
1J	NHR-8	*Caenorhabditis elegans (C. elegans)*	AAP31437
1J	NHR-48	*Caenorhabditis elegans (C. elegans)*	CAD36502
1B	SmHR96L	*Schistosoma mansoni*	XP_002575014
1K	NHR-1	*Caenorhabditis elegans (C. elegans)*	AAC48174
1L	HR97a	*Daphnia pulex (D. pulex)*	EFX79885.1
1L	HR97a	*Daphnia magna*	AUGep24b_p1s02190g158t1
1L	HR97b	*Daphnia pulex (D. pulex)*	442724[b]
1L	HR97b	*Daphnia magna*	AUGep24b_p1s02190g159t1
1L	HR97g	*Daphnia pulex (D. pulex)*	EFX77588.1
1L	HR97g	*Daphnia magna*	JF792806
1L	HR97	*Ixodes scapularis*	XP_002402961 EFX76945.1
0A	DpKNR-R1	*Daphnia pulex (D. pulex)*	
0A	KNR-R1	*Daphnia magna*	
0A	DpKNR-R2	*Daphnia pulex (D. pulex)*	EFX76947.1
0A	KNR-R2	*Daphnia magna*	
0A	KNI	*D. melanogaster (fruitfly)*	CAA31709
0A	KNRL	*D. melanogaster (fruitfly)*	AAF51627
0A	EGON	*D. melanogaster (fruitfly)*	CAA34626
2B	RXR	*Daphnia magna*	

HR97a/b Phylogenetic Comparison of NR1I/J/L Nuclear Receptors Using Only LBDs

Probability values were provided from left to right as from Bayesian Inference, Maximum Parsimony and Neighbor-Joining respectively. An X indicates disagreement from the Bayesian tree.

Appendix G

HR97a/b Phylogenetic Comparison of of NR1I/J/L/0A Nuclear Receptors Using DBDs

Probability values were provided from left to right as from Bayesian Inference, Maximum Parsimony and Neighbor-Joining respectively. An X indicates disagreement from the Bayesian tree.