



## Cloning and functional analysis of the ecdysteroid receptor complex in the opossum shrimp *Neomysis integer* (Leach, 1814)

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### ABSTRACT

In this paper, the non-target effects of tebufenozide were evaluated on the estuarine crustacean, the opossum shrimp *Neomysis integer* (Leach, 1814). Tebufenozide is a synthetic non-steroidal ecdysone agonist insecticide and regarded as potential endocrine-disrupting chemical (EDC). *N. integer* is the most used crustacean in ecotoxicological research in parallel to *Daphnia* sp. and has been proposed for the regulatory testing of potential EDCs in the US, Europe and Japan.

Major results were: (i) cDNAs encoding the ecdysteroid receptor (EcR) and the retinoid-X-receptor (RXR), were cloned and sequenced, and subsequent molecular phylogenetic analysis (maximum likelihood and neighbor-joining) revealed that the amino acid sequence of the ligand binding domain (LBD) of *N. integer* EcR (NiEcR) clusters as an outgroup of the Crustacea, while NiRXR-LBD clusters in the Malacostracan clade (bootstrap percentage = 75%). (ii) 3D-modeling of ligand binding to NiEcR-LBD demonstrated an incompatibility of the insecticide tebufenozide to fit into the NiEcR-ligand binding pocket. This was in great contrast to ponasterone A (PonA) that is the natural molting hormone in Crustacea and for which efficient docking was demonstrated. In addition, the heterodimerization of NiEcR-LBD with the common shrimp *Crangon crangon* (Linnaeus, 1758) RXR-LBD (CrcRXR-LBD) was also modeled *in silico*. (iii) With use of insect Hi5 cells, chimeric constructs of NiEcR-LBD and CrcRXR-LBD fused to either the yeast Gal4-DNA binding domain (DBD) or Gal4-activation domain (AD) were cloned into expression plasmids and co-transfected with a Gal4 reporter to quantify the protein–protein interactions of NiEcR-LBD with CrcRXR-LBD. Investigation of the ligand effect of PonA and tebufenozide revealed that only the presence of PonA could induce dimerization of this heterologous receptor complex. (iv) Finally, in an *in vivo* toxicity assay, *N. integer* juveniles were exposed to tebufenozide at a concentration of 100 µg/L, and no effects against the molting process and nymphal development were scored.

In conclusion, the *in vitro* cell reporter assay, based on NiEcR-LBD/CrcRXR-LBD heterodimerization in Hi5 cells and validated with the natural ecdysteroid hormone PonA, represents a useful tool for the screening of putative EDCs. As a test example for non-steroidal ecdysone agonist insecticides, tebufenozide had no negative effects on NiEcR/RXR receptor dimerization *in vitro*, nor on the molting process and nymphal development of *N. integer* at the tested concentration (100 µg/L) *in vivo*.

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### 1. Introduction

It is realized that today in densely populated areas a wide variety of chemicals is reaching the aquatic environment and can have effects on the health of marine and freshwater animals (Sumpter, 2009). Effects of endocrine-disrupting chemicals (EDCs) include mimicking endogenous hormones, antagonizing

normal hormones, altering the natural pattern of hormone synthesis or metabolism, and/or modifying hormone receptor levels. EDCs thus have the potential to interfere with normal reproduction and development that are controlled by an array of hormonal signals (Colborn et al., 1993). Because EDCs have been found in freshwater, estuarine and marine ecosystems, the aquatic environment has been termed the ultimate sink for natural and man-made chemicals (Sumpter, 1998). Further research needs to clarify whether EDCs actually impact aquatic organisms and to what extent (Mills and Chichester, 2005).

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Although most reports have focused on the impact of environmental compounds on physiological and reproductive functions in vertebrates, a global effort has been made more recently to develop appropriate testing systems to evaluate potential EDC effects on invertebrates such as crustaceans (LeBlanc, 2007; Rodríguez et al., 2007; Verslycke et al., 2007; Tatarazako and Oda, 2007; Ford, 2012). Despite the considerable increase in efforts, however, further research is needed to develop additional test systems and to include additional possible contaminants to estimate their environmental impact. For example, insecticides with ecdysone agonist activity constitute a group of compounds with potential impact on aquatic crustaceans because of their possible interaction with the receptor of the arthropod molting hormone, the ecdysteroid receptor (EcR), in crustaceans, which is evolutionary related to the insect EcR. Although the general toxicities of ecdysone agonists are low, their possible interaction with the EcR of crustaceans could have profound effect on their development and reproduction. Because ecdysone agonists act through interaction with the endocrine system, they can therefore be regarded as potential endocrine disruptors or endocrine-disrupting chemicals (EDC) (Sonnenschein and Soto, 1998; Vos et al., 2000).

In the Arthropoda phylum, the ecdysteroid hormone receptor complex is of crucial importance and it consists of a heterodimer of two nuclear receptors: EcR and Ultraspiracle or retinoid-X-receptor (USP or RXR; Kato et al., 2007; Yao et al., 1992). Both EcR and USP-RXR are part of the large nuclear receptor superfamily which has a general modular structure, commonly divided in four or five domains: A/B, C, D, E, and in some receptors also an F-domain. The DNA-binding domain (DBD), also called the C-domain, and the ligand-binding domain (LBD) or E-domain are the most conserved across all taxa for both receptors. A ligand-binding pocket, which has been characterized by X-ray crystallography (Billas et al., 2003; Carmichael et al., 2005), is formed within the E-domain of EcR heterodimerized to USP-RXR. The D-domain, also referred to as the hinge region, laying between the C- and E-domains, can have an influence on ligand binding as seen in some insect EcRs (Graham et al., 2007).

The natural ligands of EcR/USP-RXR are ecdysteroids. These are small lipophilic molecules, controlling a variety of physiological processes in the Arthropoda phylum containing insects and crustaceans (as Ecdysozoa), especially during growth and differentiation (Hopkins, 2009). Due to their high membrane permeability, ecdysteroids are able to directly interact with the EcR/USP-RXR complex in the nucleus, regulating its binding to ecdysteroid responsive elements (EcRE) in the promoter regions of the ecdysteroid responsive genes (EcRG). The two most active molting hormones in decapod crustaceans (and arthropods in general) are the two hydroxylated ecdysone molecules, namely 20-hydroxyecdysone (20E) and 25-deoxy-20-hydroxyecdysone or also called ponasterone A (PonA), which are known to bind in the ligand-binding pocket of the EcR.

Because the regulation of the molting and metamorphosis process by ecdysteroids is unique to arthropods, this system has been targeted for the development of arthropod pest-specific insecticides. To date, four synthetic non-steroidal ecdysone agonists with a dibenzoylhydrazine-based structure, are on the market as a new class of insecticides, also called molting-accelerating compounds. Tebufenozide (RH-5992), methoxyfenozide (RH-2485) and chromafenozide (ANS-118) are used to control lepidopteran insects (Dhadialla et al., 2005; Yanagi et al., 2006), while halofenozide (RH-0345) is used to control coleopteran (scarabid larvae) and lepidopteran insects in turf and ornamentals (Dhadialla et al., 2005). These molecules are highly effective against various lepidopteran species, while having a benign profile toward non-target species such as mammals, birds, fish and bees (Dhadialla et al., 2005; Mommaerts et al., 2006), making them safe insecticides.

However, data from Tomlin (2003) suggested that tebufenozide could potentially contaminate ground and/or surface waters. Even though the estimated maximum solubility in water is rather low (0.83 mg/L), corresponding to a –theoretical– maximum concentration of 2.35  $\mu$ M, the (soil) adsorption coefficient ( $K_{oc}$ ) is also low (605). This implies that most of the tebufenozide molecules will remain dissolved in the water column, rather than adsorbed to the soil particles. Since the compound is also quite resistant to photolytic and hydrolytic degradation (Sundaram, 1994), residual tebufenozide is expected to persist in the aquatic environment for extended periods after its application as insecticide (Kreutzweiser and Thomas, 1995; Kreutzweiser et al., 1998; Sundaram, 1995). Because its target site, i.e. the ligand-binding pocket of the EcR, is conserved among arthropods, environmental tebufenozide could act as an EDC in arthropods, mainly insects, but possibly also crustaceans.

For crustaceans, the mysid or opossum shrimp, *Neomysis integer* is the most used crustacean in ecotoxicological research in parallel to *Daphnia* sp. and has been proposed for the regulatory testing of potential EDCs in the US, Europe and Japan (deFur et al., 1999; Verslycke et al., 2007). Mysids are relatively small crustaceans, on average 0.5–2.5 cm in total length, that are characterized by the presence of a marsupium – or brood pouch – in females. They are widespread over all continents and occur in various aquatic environments, including freshwater, groundwater, brackish, estuarine, coastal, and oceanic habitats. Mysids are omnivores, feeding on phytoplankton, zooplankton, detritus and carrion (Fockedeey and Mees, 1999; Lesutiene et al., 2007). They form a vital link in the food webs of aquatic ecosystems, making them interesting study objects for ecotoxicological studies. Of these, *Neomysis* sp. are widely distributed in the coastal zone of the temperate seas of the northern Hemisphere and South America and are found in fresh and brackish waters.

Here we report on the molecular cloning and analysis of full length EcR and nearly full length RXR of the mysid *N. integer*. Subsequently, we developed a Gal4 cell reporter system with insect cells to evaluate the heterodimerization of EcR/USP-RXR. In this *in vitro* Gal4 two-hybrid system, we investigated the impact of the natural ligand PonA and the non-steroidal agonist tebufenozide on heterodimer formation of crustacean EcR and USP-RXR. Tebufenozide, one of the most widely-used members of the class of commercial molting-accelerating insecticides and a potential EDC, was used as a model test substance. Many studies have been carried out on the activity of tebufenozide against insects of different orders (Soin et al., 2010; Swevers et al., 2004; Zotti et al., 2012) and it is of interest to investigate whether it can potentially affect endocrinological processes in other arthropods, such as crustaceans. In parallel, we modeled the binding of PonA and tebufenozide in the ligand-binding pocket of the NiEcR and also evaluated potential interference with the heterodimerization EcR/USP-RXR by hormone and hormone agonist *in silico*. Finally, we exposed *N. integer* juveniles to environmentally relevant concentrations of tebufenozide, in order to evaluate potential effects on the molting process *in vivo*. The developed Gal4 reporter system in combination with the modeling/docking software package represents a valuable fast screening tool to predict whether candidate EDC substances, such as tebufenozide, can disrupt molting and growth of *N. integer* *in vivo*.

## 2. Materials and methods

### 2.1. Cloning of NiEcR and NiRXR and molecular phylogenetic analysis

Crustacean *N. integer* mysids were collected in the Braakman, a brackish water near the Scheldt estuary in Hoek (the Netherlands)

and in Galgenweel, a brackish water near the river Scheldt in Antwerp (Belgium). Species identity was confirmed with PCR using universal primers for a fragment of the 16S rRNA gene and degenerate primers for a partial sequence of cytochrome b. Positive fragments were purified, ligated in the pGEM<sup>®</sup>-T vector (Promega, Madison, WI) and cloned. After colony PCR, plasmids were extracted and then sequenced by LGC Genomics (Berlin, Germany).

Total RNA was isolated from pooled *N. integer* using Tri-Reagent<sup>®</sup> (Sigma–Aldrich, Bornem, Belgium). cDNA was synthesized with SuperScript<sup>®</sup> II Reverse transcriptase (Invitrogen, Merelbeke, Belgium), according to the manufacturer's protocol. All gene-specific primers were designed with Primer3 (Rozen and Skaletsky, 2000), unless stated otherwise.

Partial sequences encoding EcR and RXR were initially obtained by RT-PCR using degenerate primers directed to the highly conserved DBD for each nuclear receptor. Based on the thus obtained sequences, gene specific primers were designed to bridge the zone from DBD to the LBD, while both fragment termini were amplified using Rapid Amplification of cDNA Ends (RACE). 5'-ends were amplified with the SMART<sup>®</sup> RACE kit (Clontech, Mountain View, CA) and 3'-ends using the FirstChoice<sup>®</sup> RLM-RACE kit (Ambion, Austin, TX). Unfortunately, we did not obtain the complete 3'-end of RXR because the oligodT-adaptor from the FirstChoice<sup>®</sup> RLM-RACE kit annealed to a polyA rich region in the open reading frame (ORF).

The LBD amino acid sequences were aligned and analyzed using MUSCLE (in the default settings; Edgar, 2004) and COBALT (Papadopoulos and Agarwala, 2007). The evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model (Jones et al., 1992). The analysis involved 47 (EcR-LBD) and 37 (RXR-LBD) amino acid sequences. All evolutionary analyses were conducted with MEGA5 (www.megasoftware.net, Tamura et al., 2011).

## 2.2. Construction of the reporter system

Restriction primers were designed for insertion of the ORF corresponding to domains D to F of NiEcR (NiEcR-DEF) and *C. crangon* RXR-DEF ORF into the BamHI-site of the pEA-Myc-Gal4-DBD or pEA-Myc-Gal4-AD plasmid polylinker. pEA-Myc-Gal4-DBD and pEA-Myc-Gal4-AD are two modified pEA-NMyc lepidopteran expression vectors (Douris et al., 2006) into which the respective Gal4-DBD or AD domains, derived from yeast two-hybrid vectors pGBT9 and pGAD424 (Clontech), were cloned as BglII-BamHI fragments. In the expression plasmids, the polylinker derived from pGBT9 or pGAD424 (including BamHI) is available for generation of fusion constructs with Gal4-DBD or -AD, respectively. NiEcR-DEF and CcrRXR-DEF were cloned as BamHI-fragments into the corresponding site of pEA-Myc-Gal4-DBD/AD.

## 2.3. Heterodimerization assays in transfected Hi5 cells

*Trichoplusia ni* HighFive<sup>™</sup> (Hi5) cells (Life Technologies, LT, Belgium), were cultured in IPL41 medium (Gibco<sup>®</sup>, LT, Belgium), supplemented with 10% fetal bovine serum (LT). Hi5 cells were seeded in a 6-wells plate at  $5 \times 10^5$  cells per mL for transfections. For one well or  $1 \times 10^6$  cells, 5  $\mu$ L Escort IV<sup>™</sup> (Sigma–Aldrich) was incubated together with 295  $\mu$ L of basal IPL41 medium for 15 min on ice and then 15 min together with 1.3  $\mu$ g total plasmid DNA (in 286  $\mu$ L basal IPL41 medium) on ice. Plasmid DNA consisted of 100 ng pCMV-CLuc 2 Control Plasmid (New England Biolabs, NEB, Ipswich, MA), 400 ng pNEBR-X1GLuc Gal4 Reporter Control Plasmid (NEB) and twice 400 ng of a vector expressing either NiEcR or CcrRXR fused to Gal4-DBD or -AD (or the corresponding empty constructs). The cells were incubated for 6 h with the transfection medium at 27 °C in the dark. Then 24 h after transfection, cells were

seeded at a density of 50,000 cells in 100  $\mu$ L and incubated for 24 h with ponasterone A (PonA, >95% pure; LT), tebufenozide (technical grade, >95% pure; kind gift by Rohm and Haas Co, Spring House, PA) or ethanol. The BioLux<sup>™</sup> *Gaussia* (Gluc) or *Cypridina* (Cluc) luciferase assay kits (NEB) were used to measure the luciferase expression 48 h post-transfection in an Infinite M200 luminometer (Tecan, Switzerland). Specific activation/heterodimerization of the NiEcR-LBD/CcrRXR-LBD complex was determined by the amount of luminescence by *Gaussia* luciferase normalized to the amount of luminescence by *Cypridina* luciferase. The effect of each treatment was tested for significance using one-way ANOVA, followed by a *post hoc* test (all pairwise multiple comparison procedures; Holm–Sidak method) with a significance level of  $p = 0.05$  in SigmaPlot v11 (Systat Software, London, UK).

## 2.4. In silico modeling of NiEcR-LBD docked with ponasterone A and tebufenozide, and of NiEcR-LBD/CcrRXR-LBD heterodimerization

Homology modeling of NiEcR-LBD and CcrRXR-LBD was performed with the YASARA Structure program (Krieger et al., 2002) running on a 2.53 GHz Intel core duo Macintosh computer. Different models of NiEcR-LBD were built from the X-ray coordinates of the ecdysone receptor of the Lepidoptera *Heliothis virescens* (Fabricius, 1777) in complex with a synthetic dibenzoylhydrazine-based ecdysone agonist ligand (PDB code 3IXP), the USP-RXR receptor of the Coleoptera *Tribolium castaneum* (Herbst, 1797) bound to PonA (PDB Code 2NXX; Iwema et al., 2007), the EcR-LBD of the Hemiptera *Bemisia tabaci* (Gennadius, 1889) complexed to PonA (PDB code 1Z5X; Carmichael et al., 2005), the EcR-USP of *H. virescens* in complex with 20E (PDB code 2R40; Browning et al., 2007), and the human RXR $\alpha$  (PDB code 3FC6; Washburn et al., 2009) used as templates, respectively. Finally, a hybrid model was built up from the five previous models. Similarly, four CcrRXR-LBD models were built from the X-ray coordinates of human RXR $\alpha$ -ligand-binding domain complexed to rhein (PDB code R2A; Zhang et al., 2011a), human RXR $\alpha$  complexed with bigelovin (PDB code OZJ; Zhang et al., 2011b), human RXR $\alpha$  in complex with 9-*cis*-retinoic acid (PDB code 3OAP; Xia et al., 2010), human PPAR $\gamma$ -RXR $\alpha$  nuclear receptor complexed to DNA (PDB code 3E00; Chandra et al., 2008). Finally, a hybrid model was built up from the four previous models. PROCHECK (Laskowski et al., 1993) was used to assess the geometric quality of the three-dimensional (3D) model. In this respect, all of the residues of NiEcR-LBD were correctly assigned in the allowed regions of the Ramachandran plot. Using ANOLEA (Melo and Feytmans, 1998) to evaluate the models, only two residues of NiEcR-LBD over 234 exhibited an energy over the threshold value. Both residues are located in a loop region connecting  $\alpha$ -helices. Docking of PonA, tebufenozide and retinoic acid to EcR and RXR domains, respectively, was performed with the YASARA structure program. In order to define the dimerization interface between NiEcR-LBD and CcrRXR-LBD, both domains were modeled and superimposed to the structure of the heterodimer of the tobacco budworm *H. virescens* in complex with PonA (PDB code 1R1K), used as a template (Iwema et al., 2007). Molecular cartoons were drawn with YASARA and Chimera (Pettersen et al., 2004).

## 2.5. Chronic toxicity assay with *N. integer* juveniles in vivo with tebufenozide

Standardized chronic toxicity tests were done as described previously by Ghekiere et al. (2006). Gravid *N. integer* females were collected in the Braakman, a brackish water near the Scheldt estuary in Hoek (the Netherlands), and individually transferred to aquaria. The aquaria were examined each 12 h for birth of



juveniles. The juveniles from one female were placed individually and at random in 80 mL glass recipients containing 50 mL of  $100 \mu\text{g L}^{-1}$ , corresponding to 248 nM, tebufenozide (Mimic®, formulated tebufenozide) at a salinity of  $5 \text{ g L}^{-1}$ , a temperature of  $15^\circ\text{C}$  and a photoperiod of 16L:8D. Exposure lasted 4 molts (70–100 h per molt, see Fig. 5), and juveniles were examined at 12 h-intervals to assess disturbance of molting and toxicity. The criteria for death were scored by eye when there was no movement and/or no response to stimuli with a pair of tweezers. Exposure solutions were renewed every 48 h. The juveniles were fed daily with newly hatched (<24 h old) *Artemia nauplii ad libitum*. At least 12 replicates were used per concentration, based on optimization experiments as published by Ghekiere et al. (2006). The effect of the treatment was tested for significance using non-parametric Mann–Whitney or Kruskal–Wallis tests in SPSS v15 (IBM, Armonk, NY).

### 3. Results

#### 3.1. Cloning and molecular phylogeny of the EcR and RXR of *N. integer*

The full length nucleotide sequence of NiEcR (1713 bp, EU912574) was obtained from *N. integer*. The full amino acid sequence revealed low identities when compared to other known EcRs; the highest BLAST identities found were 53% with *Blattella germanica* (Linnaeus, 1767) EcR, 52% with *Pediculus humanus corporis* (Linnaeus, 1758) EcR, and 50% with *Uca pugilator* (Bosc, 1802) EcR. As expected, the identity with the mysid *Americamysis bahia* (Molenock, 1969) EcR was the highest: 73% for the full-length sequence, and 77% for the LBD (Fig. 1A).

In the COBALT alignment, the *T. castaneum* EcR sequence was used as template for the location of the secondary structures. A phylogenetic tree representing the evolutionary history of the EcR in the Arthropoda phylum was constructed with all available crustacean EcR-LBD sequences and a large selection of insect EcR-LBD sequences, representing all major orders. A vertebrate analog of EcR, i.e. *Homo sapiens* LXR $\alpha$ , was used as an outgroup to root the tree (Fig. 1B). NiEcR-LBD forms a distinct mysid clade (BP=99%), not grouping together with the other crustaceans that form separate clades, namely Branchiopoda (BP=99%) and Decapoda (BP=64%). The EcR-LBDs from the class of the Insecta are also distinct from all others (BP=66%) with the Lepidoptera grouping clearly together (BP=100%).

The (almost) full NiRXR sequence revealed 76% similarity with *C. crangon* RXR (Fig. 1A), 74% with *Marsupenaeus japonicus* (Spence Bate, 1888) RXR and 72% with *U. pugilator* RXR.

A phylogenetic tree containing all available crustacean RXR-LBDs, in addition to a large selection of insect and chordate RXR-LBD sequences, as an outgroup with *Tripedalia cystophora* RXR-LBD (Cnidaria, similar as in Bonneton et al., 2003) to root the tree, was constructed in MEGA 5 (Fig. 2B). Similar to the EcR-LBD phylogenetic tree, the *Daphnia* RXRs formed their own clade: namely the Branchiopoda, which are branching off separately from the rest of the Crustacea (BP=100%). The other crustaceans grouped together as the Malacostraca (BP=75%) which was again divided into two subclades: namely one containing the Decapoda (BP=95%) and another comprising the mysid group (BP=100%).

#### 3.2. Functional assays in Hi5 cells for heterodimerization between NiEcR-LBD and CrcRXR-LBD

Co-transfection of NiEcR-LBD fused to Gal4-AD (GAD-NiEcR-LBD) and CrcRXR-LBD fused to Gal4-DBD (GDBD-CrcRXR-LBD), revealed a concentration-dependent increase of GAD-NiEcR-LBD/GDBD-CrcRXR-LBD dimerization in the presence of PonA

(Fig. 3A). The interaction between NiEcR-LBD and CrcRXR-LBD was specific, since no activation was observed with the other –supposedly inactive– combinations: GDBD/GAD, GDBD-CrcRXR-LBD/GAD and GDBD/GAD-NiEcR-LBD (Fig. 3A). With tebufenozide, there was no concentration-dependent increase in heterodimer interaction at 100 and 1000 nM (Fig. 3B).

#### 3.3. In silico modeling of NiEcR-LBD docked with ponasterone A and tebufenozide

The NiEcR-LBD domain exhibits the typical canonical 3D-conformation, built up from 12  $\alpha$ -helices (numbered H1–H12) associated to a short hairpin of two  $\beta$  strands ( $\beta$ 1 and  $\beta$ 2), and delineating an ecdysteroid-binding pocket (Fig. 4A).

The ligand-binding pocket of NiEcR-LBD readily accommodates the steroidal hormone PonA (Fig. 4B) through a network of hydrogen bonds, associated to hydrophobic and stacking interactions with hydrophobic and surrounding aromatic residues, respectively (Fig. 4C). However, docking of the dibenzoylhydrazine-based tebufenozide to the ligand-binding pocket of NiEcR-LBD creates a rather severe clash with the ethyl-group at the phenyl ring that prevents tebufenozide to become correctly bound to the ligand-binding pocket of NiEcR-LBD (Fig. 4D). Both the size and shape of the ligand-binding pocket are responsible for the lack of interaction of NiEcR-LBD with the non-steroidal ecdysone agonist tebufenozide.

#### 3.4. In silico modeling of NiEcR-LBD/CrcRXR-LBD heterodimerization

In order to define the dimerization interface between NiEcR-LBD and CrcRXR-LBD, both domains were modeled from the structure of the heterodimer of *H. virescens* in complex with PonA (Iwema et al., 2007). As shown from the structural analysis of interfaces in *H. virescens* EcR-RXR, *B. tabaci* EcR/USP-RXR and *T. castaneum* EcR-USP, two types of heterodimerization interface between EcR and USP-RXR have been identified (Iwema et al., 2009). They correspond to the ancestral Mecopterida EcR-RXR interface (*Heliothis*) and the non-Mecopterida EcR-RXR interface (*Bemisia*, *Tribolium*), respectively. The structural analysis of the NiEcR-LBD/CrcRXR-LBD interface revealed a non-Mecopterida interface pattern, characterized by a limited number of interactions between both domains (Fig. 4E). In this respect, only two hydrogen bonds occur between residues Asp318 of the loop L8–9 and Glu329 of helix H9 of CrcRXR-LBD, and residues Lys471 and Arg474 of helix H7 of NiEcR-LBD, respectively (Fig. 4F). In addition, the extended loop L1–3 which is responsible for the displacement of helix H3 toward helix H12 in the Mecopterida *Heliothis* RXR structure, becomes readily shorter in the CrcRXR-LBD domain like in other non-Mecopterida RXR domains. Both NiEcR-LBD and CrcRXR-LBD in the dimer, readily accommodate PonA and 9-*cis*-retinoic acid, respectively (Fig. 4G). Because docking studies indicated that tebufenozide cannot efficiently bind to NiEcR-LBD, it is expected that heterodimer formation is not affected by this compound. Thus, the presence of tebufenozide alone will not stimulate heterodimerization, while it is also not expected to prevent the ligand-induced heterodimerization by PonA.

#### 3.5. Chronic toxicity test with ecdysone agonists

When *N. integer* juveniles were exposed for 96 h to a concentration of  $100 \mu\text{g/L}$  (corresponding to 284 nM) tebufenozide, mortality was lower than 10% and not significantly different from the control. No clear sublethal effects on molting, as assayed on the duration of the intermolt periods (IMP), were observed (Fig. 5).

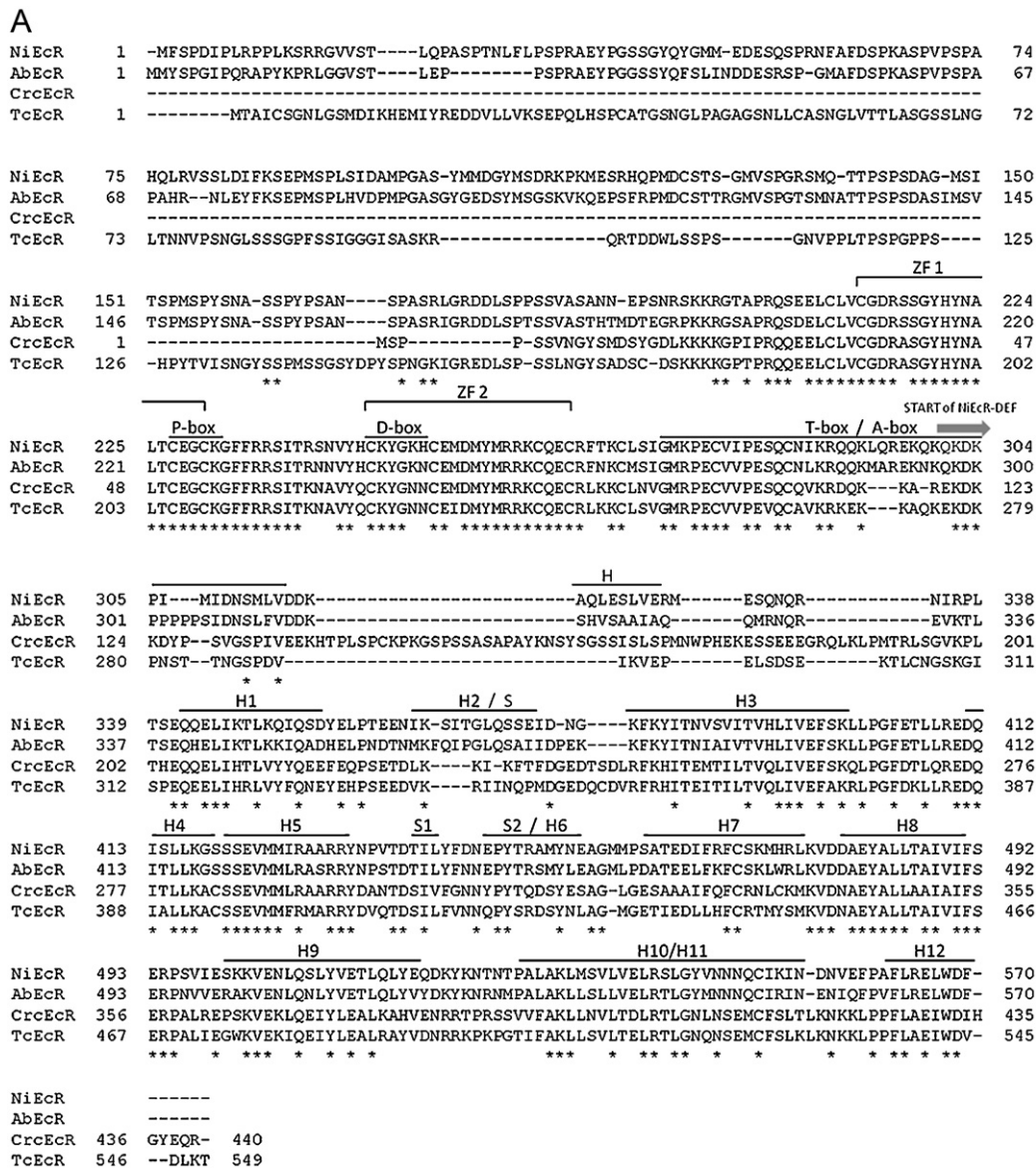
#### 4. Discussion

In this study, the two nuclear receptors that form the ecdysone hormone receptor heterodimer, EcR and USP-RXR, were cloned from the mysid *N. integer*, an important crustacean sentinel species that is used as a marker for environmental pollution in estuaria and coastal waters. Since the NiRXR sequence was incomplete at the C-terminal end of the ORF, it was chosen to carry out all functional and modeling studies using RXR of another crustacean, *C. crangon* (Verhaegen et al., 2010). CrRXR is the most closely related RXR to NiRXR with 76% amino acid sequence identity, and they cluster together in the Malacostracan clade (BP=75%). Since the common shrimp RXR is the most similar to the opossum shrimp RXR sequence, it is believed that it is the most valid candidate to replace the opossum shrimp RXR. However, it is realized that its use could be a source of non-specific

effects and that it would be preferable to use NiRXR in future assays.

In models of NiEcR-LBD, PonA could be docked with similar efficiency as in insect receptors (Fig. 3A–C, Billas et al., 2003; Carmichael et al., 2005; Soin et al., 2009, 2010). In contrast, for tebufenozide, a steric clash was observed which prevented binding, indicating that it is not likely that tebufenozide could act as a ligand for NiEcR. Similarly, Zotti et al. (2012) found that the apparent insensitivity of the Neuroptera species *Chrysoperla carnea* (Stevens, 1836) to tebufenozide is most likely due to the lack of space available in the ecdysone-binding pocket of CcEcR, making it unable to support the anchoring of the ethyl-phenyl ring of tebufenozide.

Our modeling results were confirmed by the *in vitro* assays since it was observed that addition of PonA enhanced dimerization of the NiEcR-DEF/CrcRXR-DEF complex, while addition of tebufenozide had no effect. The effect of PonA was strongest at a concentration



**Fig. 1.** Amino acid sequence alignment and phylogenetic analysis of NiEcR. (A) Sequence alignment of EcR using COBALT. Alignment of EcR sequences of the crustaceans *N. integer* (NiEcR, ACJ68423), *A. bahia* (AbEcR), *C. crangon* (isoform 1, ACO44665), and the red flour beetle *T. castaneum* (CAL25730). Conserved  $\alpha$ -helices (H1–H12),  $\beta$ -sheets (S1–S2), Zinc fingers (ZF1 and ZF2) and other functional domains are indicated above the sequence. (B) Phylogenetic analysis of 47 EcR-LBD amino acid sequences by maximum likelihood method. The tree of EcR-LBDs was constructed with 46 sequences from several Insecta and Crustacea, with *Homo sapiens sapiens* LXRA-LBD to root the tree. Bootstrap values as percentage of 1000 replicates are indicated on the tree. The tree is drawn to scale, with branch lengths measured in the number of amino acid substitutions per site.

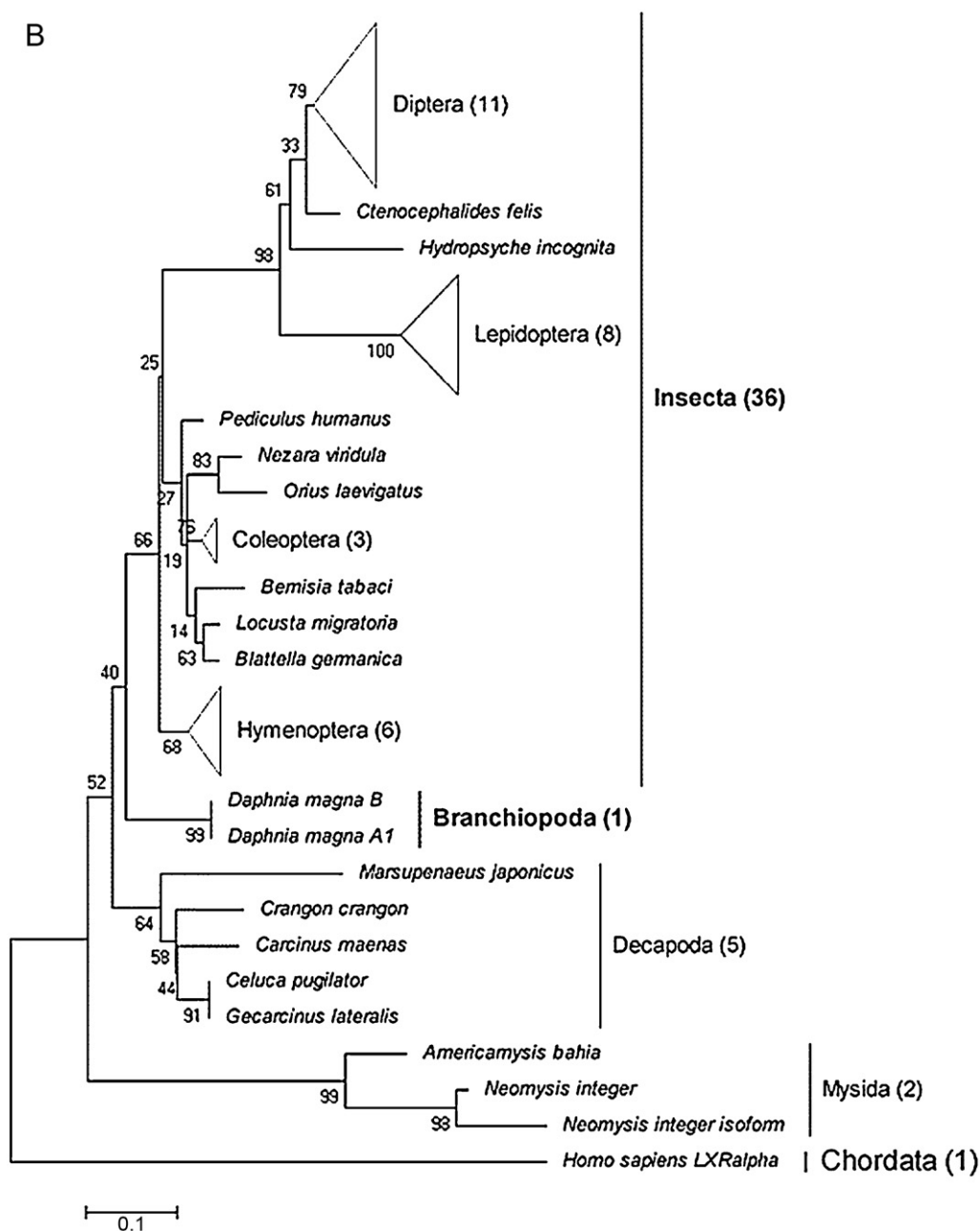


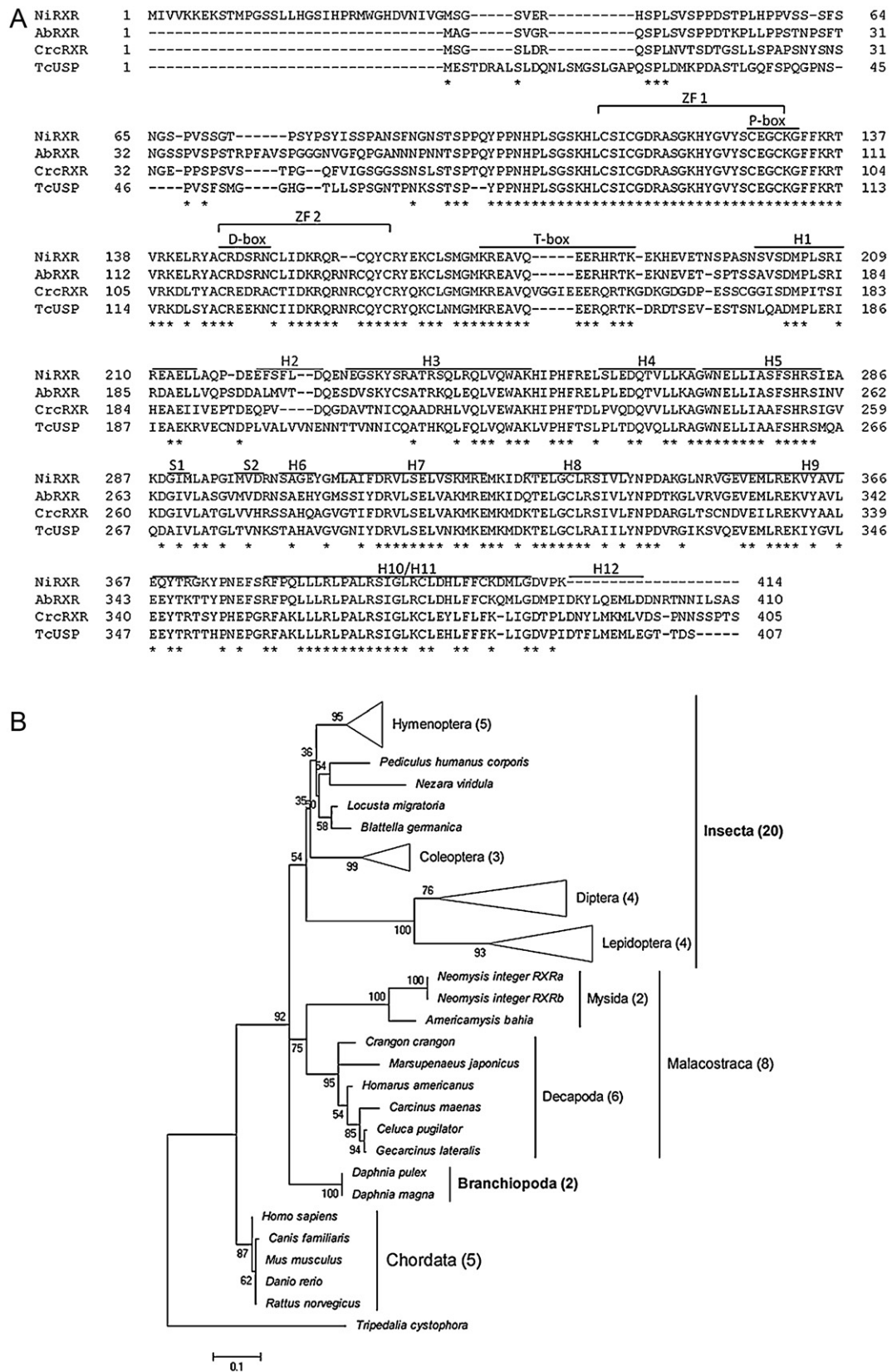
Fig. 1. (Continued).

of 1  $\mu$ M, but even at a lower concentration of 10 nM the reporter activation was still almost 3 times higher than the control. Previously, binding assays have been used to assess interaction of PonA with EcR/USP-RXR. For the American lobster (*Homarus americanus*; H. Milne-Edwards, 1837), Tarrant et al. (2011) reported a dissociation constant  $K_d$  of 2.5 nM for the PonA-HaEcR-DEF/HaRXR-DEF complex, and also Yokota et al. (2011) found a similar low value for *A. bahia* with a  $K_d$  of 2.14 nM for the PonA-AbEcR-DEF/AbUSP-DEF complex. However, it must be remarked that binding assays usually report much lower  $K_d$ -values than cell-based reporter assays. So far, no reports on an established crustacean cell line exist (Claydon and Owens, 2008; Lee et al., 2011), and reporter assays have been developed based on non-crustacean cell lines. Such reporter assays, as those developed by Verhaegen et al. (2010, 2011) who used EcR-deficient *Drosophila*-derived KcL57-3-11 cells transfected with CrcEcR/RXR expression plasmids, are less labor intensive than

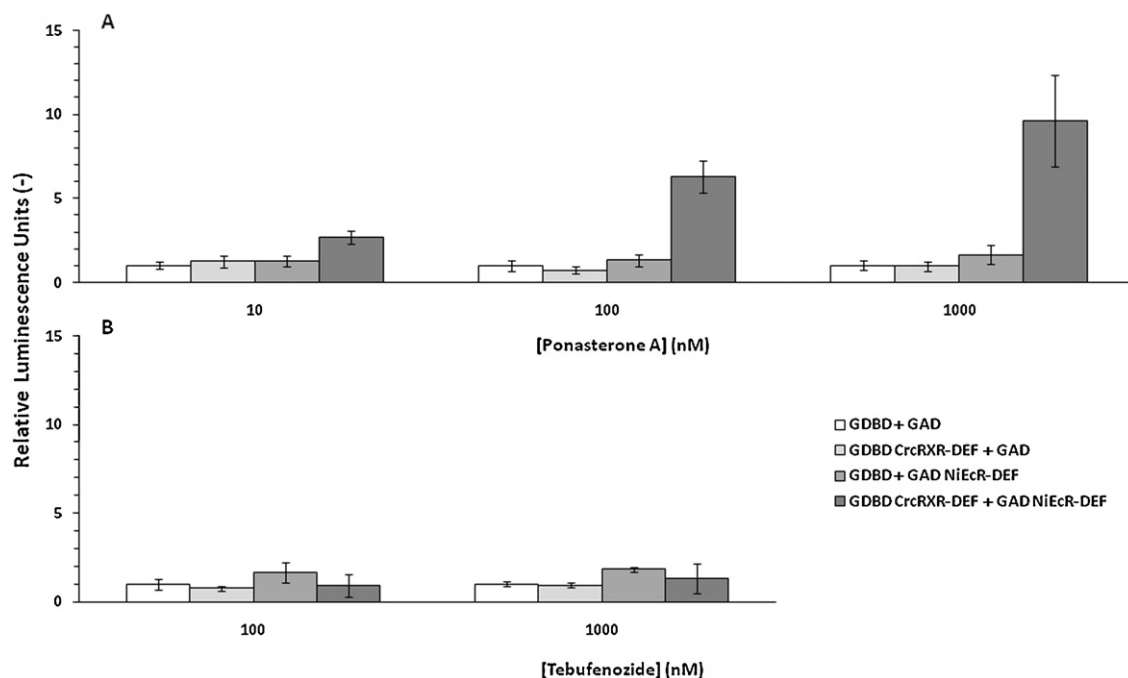
binding assays and clearly represent more useful tools for practical applications such as the prediction of endocrine-disrupting activities of exogenous compounds. Other research groups have developed reporter assays based on mammalian cells, which have the major advantage that endogenous EcR is naturally absent. For instance, Kato et al. (2007) have used a two-hybrid system with transfected chimeric genes, containing the LBDs of *Daphnia magna* (Straus, 1820) EcR and RXR in vertebrate CHO cells. In our study, a similar two-hybrid approach was used, but based on insect Hi5 cells which express endogenous EcR. However, from the set of experiments, it appears that by using crustacean EcR or RXR constructs as Gal4-fusions, no interference with endogenous receptors is observed, illustrating the feasibility of our approach.

On the other hand, it can also be noted that activation by PonA is less than 10-fold in our experiments. One possible explanation could be that the Gal4-AD functions as a rather weak activator in

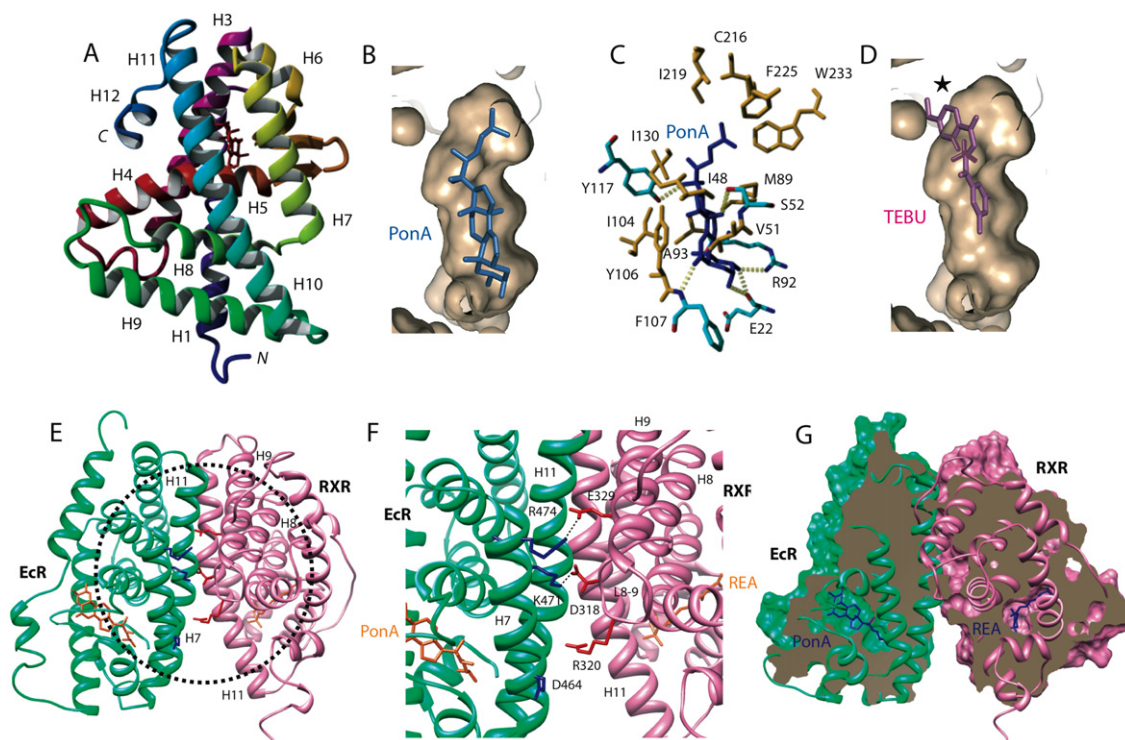




**Fig. 2.** Amino acid sequence alignment and phylogenetic analysis of NiRXR. (A) Sequence alignment of USP-RXR using COBALT. Alignment of the retinoid-X-receptor (RXR) or Ultraspiracle (USP) LBD sequences of the crustaceans *N. integer* (NiRXR), *A. bahia* (AbRXR) and *C. crangon* (isoform 1, AC044668), and the red flour beetle *T. castaneum* (EFA04649). Conserved  $\alpha$ -helices (H1–H12),  $\beta$ -sheets (S1–S2), zinc fingers (ZF1 and ZF2) and other functional domains are indicated above the sequence. Only the amino acids that differ between NiRXRa and NiRXRb are displayed. NiRXRa has an extra 34 extra amino acids at the NH<sub>2</sub>-terminal. Note that the carboxy-terminal of NiRXR is incomplete; helix H12 of the LBD and the F-domain are missing (see Material and methods (Section 2.4)). (B) Phylogenetic analysis of 37 USP-RXR-LBD amino acid sequences by maximum likelihood method. The tree of RXR-LBDs was constructed with 31 sequences from several Insecta and Crustacea. Chordata RXR-LBD sequences were used as an outgroup and *Tripedalia cystophora* RXR-LBD to root the tree. Bootstrap values as percentage of 1000 replicates are indicated on the tree. The tree is drawn to scale, with branch lengths measured in the number of amino acid substitutions per site.

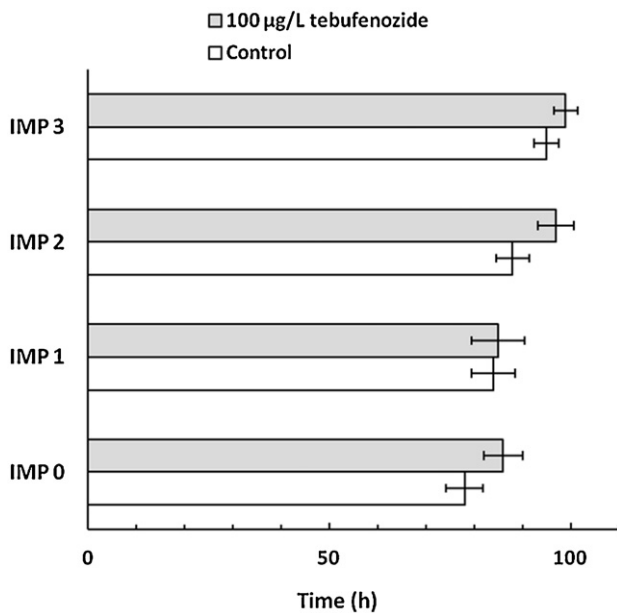


**Fig. 3.** Effect of different ponasterone A and tebufenozide concentrations on NiEcR-LBD/CrcRXR-DEF heterodimerization. Ponasterone A (A) or tebufenozide (B) was added 24 h after co-transfection of the Hi5 cells with the different constructs and the luminescence was measured 24 h later (48 h post-transfection). The overall significance level of the *post hoc* Holm–Sidak tests performed was exactly 0.05 ( $n=4$ ).



**Fig. 4.** Computer model of NiEcR-LBD docked with ponasterone A and tebufenozide and NiEcR-LBD/CrcRXR dimerization. (A) Ribbon diagram of NiEcR-LBD in complex with ponasterone A (PonA, red stick), showing the 3D-organization of the twelve  $\alpha$ -helices (H1–H12) arrayed along the polypeptide chain of the LBD. The two short strands of  $\beta$ -sheet forming the hairpin connecting  $\alpha$ -helix H6 to  $\alpha$ -helix H7 are labeled ( $\beta$ 1,  $\beta$ 2). (B) Clip views (dashed red line) of the ligand-binding pocket of NiEcR-LBD harboring PonA (blue stick). (C) Network of hydrogen bonds (dashed yellow lines) anchoring PonA (blue stick) to NiEcR-LBD. Residues interacting with the ligand by hydrophobic and stacking interactions are colored orange. Residues are labeled according to the 3D-models built for the NiEcR-LBD. (D) Clip views (dashed red line) of docking of tebufenozide (TEBU, purple stick) into the ligand-binding pocket of NiEcR-LBD. Steric clash of tebufenozide with LPBP is indicated by asterisk. (E) Ribbon diagrams showing the interface (dotted circle) between the NiEcR-LBD (EcR) in complex with PonA (orange stick) and CrcRXR-LBD (RXR) in complex with 9-*cis*-retinoic acid (REA, orange stick). The EcR and RXR domains are colored in green and pink, respectively. (F) Enlargement of the interface showing the interactions between residues (colored blue) of the  $\alpha$ -helix H7 of NiEcR-LBD domain and residues (colored red) of the loop L8–9 of CrcRXR-LBD domain. Hydrogen bonds are represented in black dotted lines. (G) Clip view of the NiEcR-LBD/CrcRXR-LBD dimer showing the ligand-binding pockets of both domains harboring PonA (blue stick) and REA (blue stick), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).





**Fig. 5.** Effect of the non-steroidal ecdysone agonist tebufenozide on the intermolt periods (IMP) of *Neomysis integer* juveniles. IMP0 is the time (h) between birth and the first molt. IMP1–3 represent the times of intermolt periods 1–3 (first to second molt; second to third molt, third molt to adult).

Hi5 cells. Recently, Kobayashi et al. (2011) found that activation domains derived from viral VP16 and mouse NF- $\kappa$ B are 17 and 107 times stronger, respectively, than Gal4-AD. In future experiments, it would therefore be prudent to include one of these domains.

In the assays using Gal4-AD and -DBD fusions, activation of the reporter gene is realized only after co-expression of Gal4-AD-NiEcR-DEF and Gal4-DBD-CcrRXR-DEF. This indicates that addition of PonA induces heterodimerization of crustacean EcR and RXR and that heterodimerization is weak in the absence of ligand. Indeed our modeling studies indicated the formation of a dimerization interface when the LBP of NiEcR-LBD is occupied by PonA and the LBP of CcrRXR occupied by retinoic acid. It indicates that heterodimerization is an important aspect for receptor functioning and that the reporter assay can be used to detect endocrine disruptors that interfere with the heterodimerization process. Since tebufenozide did not bind efficiently in the LBP of NiEcR-LBD, it is expected also not to influence heterodimerization significantly as was observed in the reporter assays.

To confirm our assumption that tebufenozide is unable to interfere with ecdysteroid signaling in *N. integer*, an *in vivo* toxicity assay was performed. Here, the non-steroidal ecdysone agonist tebufenozide did not significantly affect the intermolt period of *N. integer* juveniles at environmentally realistic concentrations of 100 µg/L (corresponding to 284 nM). So, we believe that in natural mysid populations, molting aberrations by tebufenozide are not expected, since our results indicated that (i) at the highest concentrations expected to occur in the environment, tebufenozide did not affect *Neomysis* development *in vivo*, and (ii) even at higher concentrations tested *in vitro*, it did not interact with EcR/USP-RXR heterodimerization. In literature, reports on effects of pesticides against (non-target) crustaceans are scarce. Song et al. (1997) tested tebufenozide against two saline arthropod species: *Artemia* sp. (Crustacea) and the mosquito *Aedes taeniorhynchus* (Wiedemann, 1821), as well as against two freshwater arthropods: the crustacean *D. magna* and the mosquito *Aedes aegypti* (Linnaeus in Hasselquist, 1762). In this study (Song et al., 1997), tebufenozide caused molting defects in both crustaceans and insects, but only mortality was observed in the two mosquito *Aedes* (insect) species. Also Hirano

et al. (2004) found no toxic effects in the cladoceran *D. magna* nor in the mysid *A. bahia* at concentrations of >10 mg/L tebufenozide. However, it should be remarked here that comparisons between our findings with *Neomysis* and the other studies in literature with species from other crustacean classes cannot be easily done because the sequence of NiEcR-LBD diverges considerably from that of any other known crustacean EcR-LBD.

In summary, we report in this project on (1) the cloning and characterization of *N. integer* EcR and RXR, (2) the 3D-reconstruction of the EcR-LBD and *in silico* docking of PonA and tebufenozide in this model, (3) the functionality of the NiEcR-DEF/CcrRXR-DEF complex in the insect Hi5 cell line through a Gal4-responsive luciferase reporter construct, and (4) the exposure of *N. integer* juveniles to environmentally realistic concentrations of tebufenozide. The results demonstrated that treated opossum shrimp juveniles showed no aberrations throughout the molting process and this agrees with the observations that tebufenozide did not activate the NiEcR-DEF/CcrRXR-DEF complex, which was under the control of the natural ligand PonA. We believe that such an *in vitro* reporter assay could prove a useful tool for screening of endocrine-disrupting effects of chemicals on this non-target crustacean species.

## Contributions

RDW wrote the manuscript, did the phylogenetic analysis and performed the transfections. TS did the cloning, the chronic toxicity tests and wrote part of the manuscript. RDW and LS made the plasmid constructs. OC helped with the phylogenetic analysis. PR and GS did the *in silico* analysis. LS and GS wrote the manuscript. All authors read and approved the final manuscript.

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