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IMPACT OF ENDOCRINE DISRUPTING CHEMICALS IN NUCLEAR RECEPTOR SIGNALING IN MARINE ORGANISMS: INVERTEBRATE INSIGHTS

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Constam nesta tese os artigos já publicados, que a seguir se discriminam:

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Retinoid level dynamics during gonad recycling in the limpet *Patella vulgata*.
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Abstract

The retinoids are lipophilic molecules structural and functional related to vitamin A (or retinol), known to be essential for maintaining critical vertebrate biological processes, such as embryonic development, reproduction, and vision. To maintain such processes vertebrates have a complex mechanism to regulate the spatial and temporal distribution of retinoids that comprises a metabolic (including a synthesis, storage, transport and catabolic cascade) and a signaling pathway. Outside vertebrates, the presence of retinoid metabolic and signaling modules are still poorly understood. Yet, an increasing number of studies have hinted the presence of key molecular players of retinoid metabolic and signaling modules, benefiting from the increasing number of genome sequences from deuterostomes and protostome species. These findings suggest that retinoid pathways might have a more ancient evolutionary origin than previously thought. However, the presence of retinoid-related genes on protostomes, for instance, does not necessarily correlate with a comparable *in vivo* role as observed in vertebrates. Thus far, studies regarding the identification, isolation, and functional characterization of retinoid metabolic and signaling players have been conducted in a very limited number of protostome species.

In the present work we aimed to improve our knowledge regarding retinoid metabolism and signaling cascade evolution in lophotrochozoan protostomes. Therefore, here we describe the isolation and functional characterization of key molecular metabolic players in marine lophotrochozoan species (annelid and/or mollusks): the β-carotene cleaving oxygenase like enzyme (BCO), the diacylglycerol O-acyltransferase 1 (DGAT1) enzyme and the aldehyde dehydrogenase 1 (ALDH1) enzyme. We showed that the presence of metabolic vertebrate-like key players does not necessarily imply a conserved enzymatic function. 1) The BCO is an enzyme known to be involved in the oxidative cleavage of pro-vitamin A carotenoids into retinoic acid (RA) precursors, retinal (RAL) or β-apocarotenal. We have isolated a BCO-like enzyme from *Platynereis dumerilii* (annelid). In order to characterize the function of this enzyme, we used an *in vivo* color shift approach consisting in the expression of the *P. dumerilii* BCO protein in an *Escherichia coli* strain, able to produce and accumulating β-carotene. Our results indicate that this enzyme is able to cleave the carotenoids into retinoid precursors. 2) In vertebrates, the DGAT1 enzyme has a role in retinol (ROL) esterification and storage. Here, we isolated a DGAT1 gene orthologue in *P. dumerilii* and *Patella depressa* (mollusk) and demonstrated that key amino acid residues involved in vertebrate DGAT1 enzymatic function are fully conserved. In addition, we have determined the *Dgat1* gene expression in gonads and digestive gland upon ROL *in vivo* injection in *P. depressa*. No changes were observed in *Dgat1* gene expression compared to non injected control animals, suggesting that its

major role might not be ROL storage. 3) The ALDH1s are enzymes that irreversible metabolizes RAL into RA. For *P. dumerilii* and *P. depressa* we isolated two ALDH1s related enzymes. Through an *in vitro* protein expression and cell extracts-based biochemical assays we demonstrated that the enzymes are not able to oxidized RAL into RA like their vertebrate's counterpart. The lack of ability to use RAL as a substrate is in accordance to the key amino acid residues of the substrate entry channel that do not resembles vertebrate's ALDH1s but other family related proteins.

In parallel, with the isolation of retinoid metabolic modules we also carried out isolation and/or functional characterization of retinoid signaling modules, the nuclear receptors (NR), retinoid X receptors (RXRs) and retinoic acid receptor (RARs) orthologues. The RAR and RXR are transcription factors that mediate the effects of RA isomers by gene transcription regulation. Using an *in vitro* reporter gene transactivation assay we demonstrated that RXR in *P. dumerilii* (annelid) and *Crassostrea gigas*, *N. lapillus* and *Patella vulgata* (mollusks) conserve the ability to respond to the putative *bone fide* ligand 9-*cis*-RA, in accordance with the conserved amino acid residues of the NR ligand binding domain (LBD). In addition we demonstrated that mollusks RARs are not functionally similar to chordate RAR, since they are not responsive to retinoids. This feature appears conserved within the mollusk phylum. However, the mollusk RAR conserved the ability to form heterodimer with RXR. In this case, mollusks RAR/RXR heterodimer responds in the presence of RAR and RXR ligands by repressing target gene transcription, an action that seems to be ancestral and conserved with mammals.

Overall, our findings suggest that in lophotrochozoans it remains uncertain the exact role of retinoids and their metabolic and signaling modules. Yet, a retinoid metabolic cascade must be present in lophotrochozoans, at least in mollusks, since evidences revealed the presence of several endogenous retinoid precursors and active retinoids. In contrast, the retinoid signaling pathways appear to be conserved throughout evolution among vertebrates and lophotrochozoans *taxa*, although the functionality of such modules might not be fully conserved.

Although the repertoire of retinoid metabolic and signaling modules are not fully known and characterized in protostomes, evidences supports the conservation of some RA functions between vertebrates and lophotrochozoans. In this work, we also addressed the involvement of the retinoid system in gonad development in the gastropod *Patella vulgata*. The results are suggestive of an involvement of retinoids in gonad maturation, similar to the role demonstrated in vertebrates.

Environmental endocrine disrupting chemicals (EDCs) are able to interfere with the retinoid pathways. From an ecotoxicological standpoint, most studies have focused on the effects of EDCs mostly on vertebrates with invertebrates receiving less attention. The only

exception is the phenomenon of abnormal development of male reproductive structure in female gastropods, called imposex, that has been associated with organotin compounds (OTs) tributyltin (TBT) and triphenyltin (TPT) exposure. These OTs are known to bind to RXR and modulate their signaling pathways. Few studies have established a link between the retinoid system modulations and reported endocrine negative effects in invertebrate *taxa*. This aspect is strongly related with the poor knowledge regarding retinoid modules repertoire on invertebrates which consequently hampers our understanding on the wider biological processes and phylogenetic impact of EDCs. Since recent evidences indicate a more ancestral origin of the retinoid system we hypothesize that many more species can also be a target of EDCs. One of our main hypotheses explored in this thesis is related with RXR signaling pathway modulation by TBT and TPT in metazoans. Using an *in vitro* luciferase reporter assay, we showed that the RXR receptor from the *P. dumerilii* is also a prime target of TBT and TPT. We also provide evidences that support the hypothesis that in mollusks imposex is mediated by a RXR-dependent signaling cascade. We demonstrated *in vitro* that TBT, 9-*cis*-RA, HX630 and methoprene acid, compounds previously described to promote imposex in female *N. lapillus*, are able to induce target gene transcription activation regulated by RXR. Additionally we have demonstrated that although TPT induce very mild levels of imposex in female *N. lapillus* at environmentally relevant concentrations, is also a potential endocrine disruptor of RXR signaling pathways in gastropods: It binds to the NR and is able to activate gene transcription, though to a lesser extent than TBT which might explain the different sensitivity among gastropod species.

In parallel we also evaluated if target genes transcription regulated by RAR-dependent signaling pathway would also be a target of modulation by TBT in mollusks. Using an *in vitro* reported gene transactivation assay we demonstrate that RAR from both mollusks and human, as a monomer and in heterodimer with RXR in the presence of TBT display a gene transcription repression, more significant in the heterodimeric complex. One possible explanation for this response could be related with the TBT ability to bind to RXR and interact with RAR leading to repression in the heterodimer and RAR as monomer. The biological significance of this response remains to be investigated. It might be biological important given that for vertebrates RAR/RXR is implicated in the regulation of numerous physiological processes, and our data indicates that the heterodimeric complex might also be a target of modulation by TBT. Additionally, in the present thesis we also demonstrate that mollusks RAR, as a monomer, loss the ability to response to retinoids and that feature also seems to make then unresponsive to common environmental pollutants (i.e. organochlorine pesticides) that are known to interact with mammalian RARs.

Overall, the present thesis provides novel insights into the evolutionary origin of the retinoid metabolic and signaling pathways as well as into its modulation by EDCs in marine lophotrochozoans.

Resumo

Os retinoides são moléculas lipófilicas, estrutural e funcionalmente relacionados com a vitamina A (ou retinol); reconhecidos por serem essenciais para a manutenção de importantes processos biológicos em vertebrados, tais como o desenvolvimento embrionário, a reprodução e a visão. Para manter esses processos, os vertebrados possuem um mecanismo complexo para a regulação da distribuição espácio-temporal de retinoides que engloba uma via metabólica (incluindo uma cascata de síntese, armazenamento, transporte e catabolismo) e uma via de sinalização. Fora do grupo dos vertebrados, a presença de módulos das vias de metabolismo e de sinalização de retinoides ainda são pouco conhecidas. No entanto, vários estudos têm sugerido a presença de módulos chave do metabolismo e de sinalização de retinoides, beneficiando do crescente número de genomas sequenciados em espécie de deuterostómios e protostómios. Estas evidências sugerem que as vias de sinalização dos retinoides poderão ter uma origem evolutiva mais ancestral do que anteriormente sugerida. No entanto, a presença de genes relacionados com as vias de retinoides em protostómios, por exemplo, não implica necessariamente que tenha uma mesma função *in vivo* que a reportada em vertebrados. Até à presente data, têm surgido vários estudos focados na identificação, isolamento e caracterização funcional de componentes das vias metabólicas e de sinalização de retinoides mas num número bastante limitado de espécies de invertebrados.

O presente trabalho visa melhorar o nosso conhecimento sobre as vias do metabolismo e da sinalização de retinoides em lofotrocozoários protostómios. No presente trabalho descreve-se o isolamento e a caracterização funcional de alguns dos principais componentes moleculares do metabolismo em espécies lofotrocozoárias marinhas (anelídeos e/ou moluscos): Enzima de clivagem oxidativa de β-caroteno (BCO), a enzima diacilglicerol O-aciltransferase 1 (DGAT1) e enzimas aldeído desidrogenases 1 (ALDH1). Demostrámos que a presença de componentes chave do metabolismo idênticos (ortólogos) a vertebrados não significa necessariamente que apresentem uma função enzimática conservada. 1) A BCO é uma enzima que se encontra envolvida na clivagem oxidativa de pró-vitamina A carotenoides em precursores de ácido retinoico (RA), retinal (RAL) ou β-apocarotenal. Em *Platynereis dumerilii* (anelídeo), isolamos uma enzima ortóloga da BCO dos vertebrados. De modo a caracterizar esta enzima

funcionalmente, utilizamos uma abordagem *in vivo* de observação de mudança de cor que consiste na expressão da proteína de BCO de *P. dumerilii* numa estirpe de *Escherichia coli*, que apresenta a capacidade de produzir e acumular β-caroteno. Os nosso resultados apontam para que a enzima tenha a capacidade de clivar oxidativamente os carotenoides em percussores de retinoides. 2) Nos vertebrados, a enzima DGAT1 desempenha um papel na esterificação e armazenamento de retinol (ROL). Aqui, isolamos um gene ortólogo de DGAT1 em *P. dumerilii* e em *Patella depressa* (molusco). Mostramos que os aminoácidos chave envolvido na função enzimática de DGAT1 em vertebrados estão conservados. Adicionalmente, determinamos a expressão génica de *Dgat1* nas gonadas e glândula digestiva de *P. depressa* 48 h após injeção *in vivo* de ROL. Não foram observadas diferenças na expressão do gene em comparação com animais controlo não injetados, o que sugere que a enzima possa não apresentar um papel a nível da esterificação e armazenamento de ROL. 3) As ALDH1s são enzimas que metabolizam irreversivelmente RAL em RA. Para *P. dumerilii* e *P. depressa* isolamos duas enzimas relacionadas com as ALDH1s. Através de um ensaio baseado na expressão *in vitro* das proteínas em células e ensaios bioquímicos de extração de extratos celulares demonstramos que as enzimas são incapazes de usar RAL como substrato na síntese de RA como o seu homólogo em vertebrados. A incapacidade de usar RAL como substrato está de acordo com a alteração de aminoácidos chave no canal de entrada do substrato que não são idênticos aos das ALDH1s em vertebrados, mas de outras proteínas relacionadas da família.

Em paralelo com o isolamento de módulos metabólicos dos retinoides, procedemos ao isolamento e/ou á caracterização de módulos das vias de sinalização de retinoides, os recetores nucleares (NR): recetores X do ácido retinoico (RXRs) e recetores do ácido retinoico (RARs). Os RARs e RXRs são fatores de transcrição que medeiam os efeitos dos isómeros de RA ao regularem a transcrição de genes. Usando um ensaio *in vitro* de transativação do gene repórter da luciferase demonstramos que RXR em *P. dumerilii, Nucella lapillus, Crassostrea gigas* e *P. vulgata* conservaram a capacidade de responder ao suposto ligando natural, o 9-*cis*-RA, de acordo com a conservação de aminoácidos chave no domínio de ligação ao ligando (LBD) do recetor. Adicionalmente demonstramos que os RARs em moluscos não são funcionalmente idênticos aos RARs de cordados, uma vez que não respondem de modo similar à presença de retinoides, e que esta característica parece estar conservada ao longo de todo o filo. Por outro lado, o RAR em moluscos conservou a capacidade de formar heterodímero com o RXR. Neste caso, o heterodímero RAR/RXR em moluscos responde à presença de ligandos de RAR e RXR reprimindo a transcrição de genes alvo, uma ação que em parte parece ser ancestral e conservada com os mamíferos.

De um modo geral os nossos dados sugerem que em lofotrocozoários permanece incerto o papel exato dos retinoides e dos seus módulos metabólicos e de sinalização. Contudo, uma cascata metabólica deverá estar presente em lofotrocozoários, pelo menos em moluscos, uma vez que evidências revelam a presença de vários retinoides endógenos precursores e ativos. Em contraste, as vias de sinalização de retinoides parecem estar conservadas ao longo da evolução entre *taxa* de vertebrados e lofotrocozoários, embora a funcionalidade desses módulos possa não estar inteiramente conservada.

Embora o repertório dos componentes metabólicos e de sinalização de retinoides não seja totalmente conhecido nem tenha sido inteiramente caracterizado em protostómios, evidências suportam a conservação de algumas funções de RA entre vertebrados e lofotrocozoários. Neste trabalho também foi abordado o envolvimento das vias de sinalização de retinoides no desenvolvimento da gonada no gastrópode *Patella vulgata*. Os resultados sugerem o envolvimento dos retinoides na maturação da gonada, idêntica à verificada em vertebrados.

Os disruptores endócrinos químicos ambientais (EDCs) possuem a capacidade de interferir com as vias de retinoides. Do ponto de vista ecotoxicológico, muitos estudos têm-se focado no efeito de EDCs sobretudo em vertebrados, recebendo os invertebrados menos atenção. A única exceção é o fenómeno do desenvolvimento anormal de estruturas reprodutoras masculinas em fêmeas de gastrópodes, designado de *imposex*. Este fenómeno tem sido associado com a exposição a compostos organoestanhos (OTs): o tributilestanho (TBT) e o trifenilestanho (TPT). Estes OTs têm a capacidade de se ligar e de modular as vias de sinalização dependentes de RXR. Um número reduzido de estudos tem estabelecido uma ligação entre a modulação da cascata de retinoides e efeitos endócrinos reportados em *taxa* de invertebrados. Este facto está em grande parte relacionado com a limitação do conhecimento sobre o repertório de componentes chave das vias de retinoides em invertebrados que consequentemente condicionam a nossa compreensão do impacto de EDCs em processos biológicos. Uma vez que evidências recentes apontam para uma origem evolutiva mais ancestral das vias de síntese e sinalização de retinoides, nós colocamos a hipótese que muitas mais espécies poderão também ser alvo por um mesmo EDCs. Uma das principais hipóteses analisadas nesta tese está relacionada com a modelação do RXR por TBT e TPT em metazoários. Usando um ensaio *in vitro* de transativação do gene repórter da luciferase, demonstrámos que o RXR de *P. dumerilii* é também alvo de modulação por TBT e TPT. Apresentámos também evidências que suportam a hipótese de que em moluscos o *imposex* é mediado por uma cascata de sinalização dependente de RXR. Demonstrando *in vitro* que o TBT, 9-*cis*-RA, HX630 e ácido *methoprene*, compostos anteriormente descritos por apresentarem a

capacidade de promover o desenvolvimento de *imposex* em fêmeas de *N. lapillus*, são capazes de induzir a ativação da transcrição de genes alvo mediada por RXR. Adicionalmente também demonstra-mos que embora o TPT não induza o desenvolvimento de *imposex* em fêmeas de *N. lapillus* a concentrações ambientalmente relevantes, é também um potencial EDCs das vias de sinalização de RXR em gastrópodes: liga-se e tem a capacidade de ativar a transcrição de genes alvo mas a níveis mais baixos que o TBT, explicando a diferente sensibilidade a este composto entre espécies de gastrópodes.

Em paralelo também avaliámos se a transcrição de genes alvo regulada por via de sinalização dependente de RAR seriam também alvo de modelação por TBT em moluscos. Utilizando o ensaio *in vitro* de transactivação da transcrição do gene repórter da luciferase mostramos que RAR de moluscos e de humano, tanto em monómero como em heterodímero com RXR na presença de TBT produz uma resposta de repressão da transcrição, sendo esta mais significativa no caso do complexo heterodimérico. Uma possível explicação para esta resposta pode estar relacionada com a capacidade do TBT para se ligar a RXR e interagir com RAR levando a repressão no heterodímero em RAR como monómero. O significado biológico desta resposta permanece por investigar. Esta resposta será biologicamente bastante relevante, uma vez que por exemplo, pelo menos para os vertebrados, RAR/RXR está implicada na regulação de inúmeros processos fisiológicos e os nossos dados prevêm que o complexo heterodimérico possa ser também um alvo da modulação por TBT. Adicionalmente, na presente tese também mostrámos *in vitro* que RAR de moluscos, enquanto monómero perdeu a capacidade de resposta a retinoides e que essa mesma característica parece também ter levado à perda de resposta a poluentes ambientais comuns (ex. pesticidas organoclorados) que são conhecidos por interagir com RARs em mamíferos.

No geral, a presente tese fornece novos conhecimentos sobre a origem evolutiva das vias metabólicas e de sinalização de retinoides, bem como da sua modulação por EDCs em lofotrocozoários marinhos.

Abbreviations List

ACAT	Acyl-CoA: cholesterol acyltransferase
ADHs	Alcohol dehydrogenases
AF	Activation function
ALDHs	Aldehyde dehydrogenases
AP	Anterio - posterior
ARAT	Acyl-CoA: retinol acyltransferase
βc	β-carotene
BCO	β-carotene oxygenase
BCO-I	β-carotene 15,15'-monooxygenase
BCO-II	β-carotene 9',10'-dioxygenase
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CD2665	4- [6-[(2-Methoxyethoxy) methoxy] -7-tricyclo [3.3.1.13, 7] dec-1-yl-2-naphthalenyl) benzoic acid
CRABP	Cellular RA binding proteins
CRALBP	Cellular retinaldehyde-binding protein
CRBP-I	Cellular retinol-binding protein type I
CRBP-II	Cellular retinol-binding protein type II
CNS	Central nervous system
CYP26	Cytochrome P450 family 26
DBD	DNA binding domain
DGAT1	Diacylglycerol O-acyltransferase 1
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulfoxide
DR	Direct repeat
DTT	DL-Dithiothreitol
EcR	Ecdysone receptor
EDCs	Endocrine disrupting chemicals
EDTA	Ethylenediaminetetraacetic acid
EF-1α	elongation factor alpha gene
GSP	Gene specific primers
HAT	Histone acetyltransferase
HDAC	Histone deacetylase activity
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HNF4	Hepatocyte nuclear factor 4 receptor
HPLC	High-performance liquid chromatography
HPLC-MS	High-performance liquid chromatography - mass spectrometry
HSCs	Hepatic stellate cells
HX630	4- (7, 8, 9, 10 -Tetrahydro-7, 7, 10, 10-tetramethylbenzo[b] naphtha [2, 3- f] [1, 4]) thiazepin-12-yl-benzoic acid
Ip	Isoelectric point
kDa	Kilodaltons
LBD	Ligand binding domain
LBP	Ligand-binding pocket
LC/MS/MS	High-performance liquid chromatography -tandem mass spectrometry
LE 135	4- (7, 8, 9, 10-Tetrahydro-5, 7, 7, 10, 10 -pentamethyl- 5H-benzo [e]naphtha [2, 3-b] [1, 4]diazepin-13-yl) benzoic acid
LRAT	Lecithin: retinol acyltransferase
LXR	Liver X receptor
MA	Methoprene acid
MBOAT	Membrane-bound O-acyltransferases
MDRs	Medium-chain dehydrogenase/reductases family
Mw	Molecular weight
NAD+	Nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NR	Nuclear receptor
NR1	Nuclear receptor 1
NR2	Nuclear receptor 2
ORF	Open reading frame
OTs	Organotin compunds
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator-activated receptor
PPARγ	Peroxisome proliferator-activated receptor gamma
p,p′, DDE	1, 1-dichloro-2, 2-bis(p-chlorophenyl)ethane
RA	Retinoic acid
RACE	Rapid amplification of cDNA ends
RAL	Retinaldehyde or retinal
RALDH	Retinal dehydrogenase
RAR	Retinoic acid receptor

RARE	Retinoic acid responsive elements
RBP	Retinol binding protein
RDH	Retinol dehydrogenase
RE	Retinyl ester
REH	Retinyl ester hydrolase
ROL	Retinol
RP	Retinyl palmitate
RT-PCR	Real time - polymerase chain reaction
RXR	Retinoic X receptor
SDR	Short-chain dehydrogenase/reductase
SEC	Substrate entry channel
STRA6	Stimulated by retinoic acid protein 6 receptor
TBT	Tributyltin
TCDD	2, 3, 7, 8-tetrachlorodibenzo-p-dioxin
TFA	Trifluoroacetic acid
ThR	Thyroid hormone receptor
TPT	Triphenyltin
TTNPB	4- [(E)-2- (5, 6, 7, 8-Tetrahydro-5, 5, 8, 8-tetramethyl- 2-naphthalenyl) - 1-propenyl] benzoic acid
TTR	Transthyretin
USP	Ultraspiracle
VDR	Vitamin D receptor

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CHAPTER 1

1 General Introduction

1.1 Retinoids

The retinoids are a family of chemical compounds that encompasses molecules structurally and/or functionally related to retinol (ROL) or vitamin A (Blomhoff and Blomhoff, 2006). Structurally, all retinoids encompass a polar end group and a polyunsaturated polyene hydrophobic side chain attached to a cyclic six-carbon ring (Kam et al., 2012). The major natural retinoids occurring, i.e., vitamin A derivatives, are retinyl esters (REs), retinaldehyde (retinal or RAL) and retinoic acid (RA) (Fig. 1.1) (Blomhoff and Blomhoff, 2006). Retinol and REs are the alcohol and esters retinoid forms, respectively. In vertebrates, the first is the predominant retinoid in circulation during the fasting state, serving as a precursor for RAL and RA synthesis; while the former is the main storage form in tissue, also found in the circulation incorporated in lipoprotein particles such as chylomicrons and their remnants (Theodosiou et al., 2010; O'Byrne and Blaner, 2013). Retinyl esters has no biological activity, but it can be converted to active retinoids when needed to satisfy the vitamin A requirements, also serving as substrate for the formation of the visual chromophore 11-*cis*-retinal (Theodosiou et al., 2010; O'Byrne and Blaner, 2013). Retinal is the aldehyde form; it is a precursor for RA synthesis, but has also a crucial role in vertebrate's vision, mainly in the metabolism of the pigment rhodopsin (Blomhoff and Blomhoff, 2006; D'Ambrosio et al., 2011). It acts as a hormone controlling the expression of many genes, RA is vitamin A major biologically active form (Blomhoff and Blomhoff, 2006).

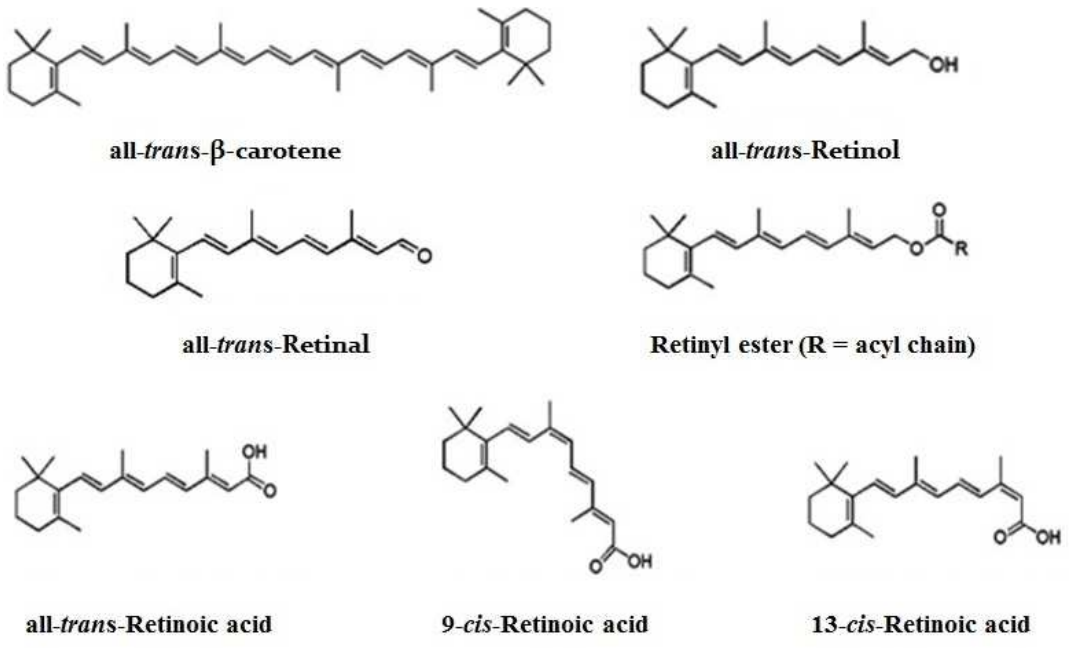


Figure 1.1: Chemical structural formulas of naturally occurring retinoids and β -carotene (adapted from O'Byrne and Blaner, 2013).

In vertebrates, several physiological processes are regulated by retinoids: cell differentiation and apoptosis, embryonic development, growth, reproduction and vision (Clagett-Dame and DeLuca, 2002; Blomhoff and Blomhoff, 2006; D'Ambrosio et al., 2011). Retinoid's biological importance is well known, being recognized that both the excess and deprivation can have several deleterious effects on vertebrate's health (Maden et al., 1998; Zile, 1998, 2001; Blomhoff and Blomhoff, 2006). During embryonic development, retinoid imbalance produces congenital malformations, affecting the heart, ocular tissues and several major organ systems: the circulatory, urogenital and respiratory systems as well as the central nervous system (CNS) (Maden et al., 1998; Zile, 1998, 2001). Therefore, vertebrates require an accurate homeostatic control of retinoid levels. This is achieved through a complex network of metabolic and signaling pathways, with ROL playing a pivotal role linking both routes (Cañestro et al., 2006; Theodosiou et al., 2010) (Fig. 1.2). Depending on the homeostatic requirements, ROL can either enter a two-step oxidation cascade, sequentially producing RAL and RA, the biological active metabolite, or undergo esterification in the form of REs to promote retinoid storage; an additional oxidation inactivates RA (Theodosiou et al., 2010). In general, animals are not able to endogenously produce *de novo* vitamin A, relying exclusively upon ROL, REs, and/or pro-vitamin A (mainly β-carotene) dietary supply (Blomhoff and Blomhoff, 2006).

1.2 Overview of vertebrate's retinoid metabolic and signaling pathways

1.2.1 Retinoid uptake, storage and mobilization pathway

In vertebrates, retinoid levels are maintained at a homeostatic state through an effective mechanism that provides an adequate vitamin A supply regardless of daily nutritional fluctuation involving a ROL storage and mobilization cascade system (Fig. 1.2) (Blomhoff and Blomhoff, 2006; Theodosiou et al., 2010; Schreiber et al., 2012). Metabolism of vitamin A begins in the intestinal lumen where dietary performed REs are directly hydrolyzed to ROL by the action of retinyl ester hydrolases (REHs) (Schreiber et al., 2012). The resulting ROL is taken up by enterocytes where it is bound to cellular retinol binding protein type II (CRBP-II), re-esterified by lecithin: retinol acyltransferase (LRAT), and incorporated into chylomicrons, along with other dietary lipids and secreted to the general circulation via the lymphatic system (Noy et al., 2000; Blomhoff and Blomhoff, 2006; D'Ambrosio et al., 2011). Circulating REs are transported to storage tissues, such as the liver, white adipose tissue, intestine, lung, kidney and others (D'Ambrosio et al., 2011; O'Byrne and Blaner, 2013). The liver is the main storage organ for ROL. In the hepatocytes, REs are hydrolyzed back to ROL, bound to cellular retinol-binding protein type I (CRBP-I) and delivered to hepatic stellate cells (HSCs) for LRAT-catalyzed esterification and storage (D'Ambrosio et al., 2011; Schreiber et al., 2012). Whereas LRAT is the sole enzyme that esterifies hepatic ROL, in other tissues diacylglycerol O-acyltransferase 1 (DGAT1) and/or other yet unidentified enzymes with acyl-CoA: retinol acyltransferase (ARAT) activity can contribute to ROL esterification (D'Ambrosio et al., 2011). In times of insufficient dietary intake of vitamin A, REs stores are mobilized by REHs into ROL and released into the circulation to supply peripheral tissues (Theodosiou et al., 2010; Schreiber et al., 2012). Before entering circulation, ROL binds to retinol binding protein (RBP) (Blomhoff and Blomhoff, 2006; D'Ambrosio et al., 2011). Generally, ROL-RBP circulates in the blood as a 1:1 molar complex associated with another serum protein, the transthyretin (TTR) to avoid excretion by the kidney (Blomhoff and Blomhoff, 2006; D'Ambrosio et al., 2011).

1.2.2 Cellular uptake and active retinoid synthesis pathways: The canonical route

Once in target tissues, a membrane receptor, stimulated by retinoic acid protein 6 receptor (STRA6), mediates ROL cellular uptake (Theodosiou et al., 2010; D'Ambrosio et al., 2011). After delivery to target cells, ROL can be metabolized into RA by two consecutive enzymatic reactions: first ROL is reversibly oxidized producing RAL which is then irreversibly oxidized into RA (Parés et al., 2008; Fig. 1.2). Two enzyme families have been suggested to mediate the first conversion: the cytosolic alcohol dehydrogenases (ADHs), belonging to the medium-chain dehydrogenase/reductases family (MDRs), and the microsomal retinol dehydrogenases (RDHs) included in the short-chain dehydrogenases/reductases family (SDRs) (Duester et al., 2003; Sandell et al., 2007, 2012). Vertebrates present multiple ADH isoforms, but only three are suggested to have a major implication on ROL oxidation process: ADH1, ADH3 and ADH4 (Parés et al., 2008; Theodosiou et al., 2010). In addition to ADHs, RDHs also have a role in ROL oxidation to RAL, mainly the RDH10 (Sandell et al., 2007; Farjo et al., 2011; Sandell et al., 2012). The second step in RA synthesis involves the RAL irreversible oxidation into RA (Fig. 1.2). This reaction is carried out by ALDHs. There are generally three ALDHs class in vertebrates, which are named as ALDH1a1, ALDH1a2 and ALDH1a3 (Parés et al., 2008). For long, the specificity of RA synthesis was thought to reside exclusively at the level of the second reaction step with ALDH1a being considered the sole rate limiting enzyme; However, RDH10-mediated oxidation of ROL also plays an important role in the control and regulation of RA production as does the subsequent ALDH1a-mediated reaction establishing a novel nodal point in RA feedback regulation (Sandell et al., 2007; Farjo et al., 2011; Sandell et al., 2012).

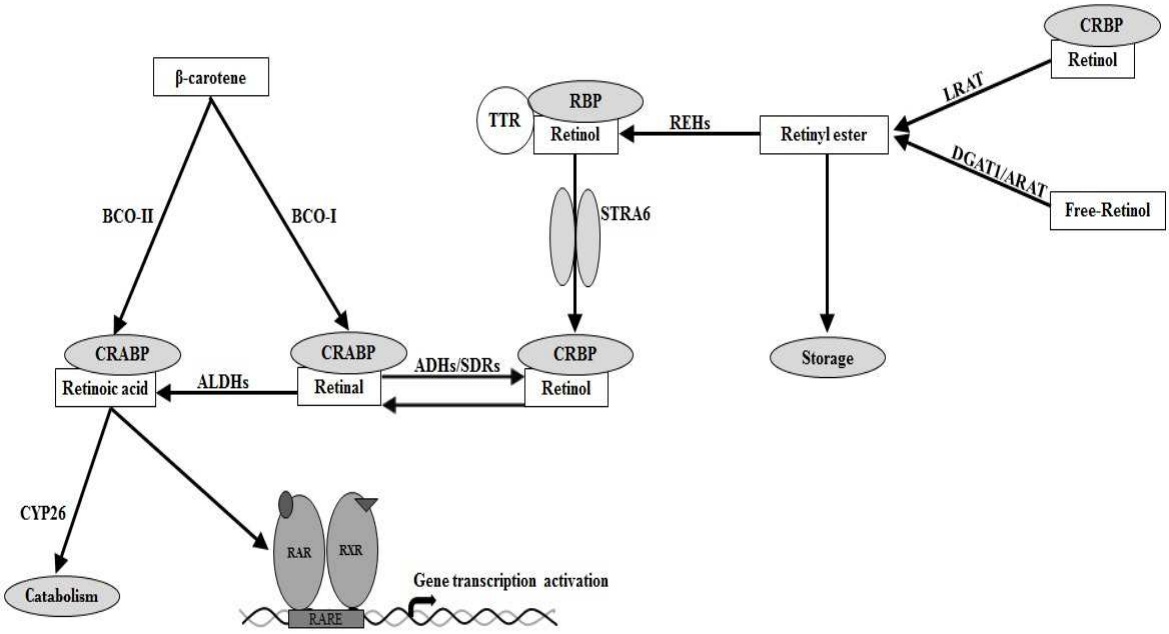


Figure 1.2: Main components of retinoid metabolic and signaling cascade in vertebrates (adapted from André et al., 2014).

1.2.3 RA alternative synthesis pathway: The non-canonical route

Some animals are also able to satisfy their own vitamin A requirement by cleaving pro-vitamin A carotenoids derived from fruits and vegetables diet source (Kiefer et al., 2001; Simões-Costa et al., 2008; Amengual et al., 2011). The β-carotene (βc) is the most abundant carotenoid present in the diet and tissues, and is one of the main retinoids synthesis precursors (von Lintig, 2010). In order to generate retinoids, βc must be cleaved. Two major enzymes, the β-carotene 15, 15'- monooxygenase (BCO-I) and β-carotene 9', 10'-dioxygenase (BCO-II), controls vitamin A synthesis from dietary βc and other carotenoids (von Lintig and Vogt, 2000; Kiefer et al., 2001). The BCO-I oxidative cleaves βc symmetrically at the 15, 15' carbon double bond yielding two molecules of RAL, which can then be either oxidized to RA or/and reduced to ROL by the enzymes from the canonical route (von Lintig, 2010). In contrast, BCO-II cleaved βc asymmetrically generating a molecule of β-ionone ring and one of β-apocarotenal (Kiefer et al., 2001). The β-apocarotenal is then converted to β-apocarotenoic acid that is stepwise oxidized to RA in a process involving enzymes that have yet to be identified (Kiefer et al., 2001).

1.2.4 Retinoids signaling pathway

The resulting RA from βc asymmetric cleavage and ALDH1as activity can have two possible fates: being inactivated by degradation or used for biological functions regulation. Thus, cytosol RA levels, like ROL, are regulated by specific cellular binding proteins, the cellular retinoic acid binding proteins (CRABPs) type I and II. CRABP-I delivers RA for degradation, whereas CRABP-II transports RA to the cell nucleus, mediating their binding to nuclear receptor (NR) superfamily members (Blomhoff and Blomhoff, 2006). Retinoic acid serves as ligand to the retinoic acid receptors (RARs) and/or retinoid X receptors (RXRs), regulating the transcription of several genes (Szanto et al., 2004; Blomhoff and Blomhoff, 2006). For each retinoid receptor, vertebrates have three distinct isoforms: RXRα (NR2B1), RXRβ (NR2B2), RXRγ (NR2B3), RARα (NR1B1), RARβ (NR1B2), and RARγ (NR1B3) (Theodosiou et al., 2010). Both all-*trans*-RA and 9-*cis*-RA isomers serve as ligands for all RARs, whereas for RXRs only 9-*cis*-RA specifically bind with high affinity (Mangelsdorf et al., 1992; Allenby et al., 1993; Germain et al., 2006a, b).

Like other members of NRs superfamily, RARs and RXRs are organized in five to six modular regions: A/B, C, D, E and/or F (Aranda and Pascual, 2001; Germain et al., 2006a) (Fig. 1.3). The A/B region is a poorly conserved N-terminal domain that contains a constitutively active transactivation function 1 (AF-1) to which the coactivators bind (Germain et al., 2003). The C region is highly conserved, and it corresponds to the DNA-binding domain (DBD); its small motifs, the P-, D- and T-box are important for conferring sequence-specific DNA recognition and ligation for the response elements in the promotor region of a target gene and/or dimerization (Germain et al., 2003; Bastien and Rochette-Egly, 2004; Germain et al., 2006a). The C region is connected to the E region via a flexible hinge, the so-called D domain (Bastien and Rochette-Egly, 2004; Germain et al., 2006a, b). The D domain is poorly conserved, and it allows C and E domains to adopt different conformations (Bastien and Rochette-Egly, 2004; Germain et al., 2006). The E region is also known as the ligand binding domain (LBD) and mediates transcription activation of target genes (Aranda and Pascual, 2001; Germain et al., 2006a). The LBD harbors four structurally surfaces that has distinct function: 1) a dimerization surface, which interacts with LBDs from other NRs; 2) a surface that recognize and interacts with ligands, termed as ligand-binding pocket (LBP); 3) a surface that interacts and binds to cofactors (coactivators or corepressors) to regulate target gene transcription and 4) an activation function, so-called AF-2, which mediates ligand-dependent transactivation (Germain et al., 2003; Germain et al., 2006a). RARs also has an F domain, which is absent in RXRs, the function still remains unknown (Aranda and Pascual, 2001; Bastien and Rochette-Egly, 2004; Dawson and Xia, 2012).

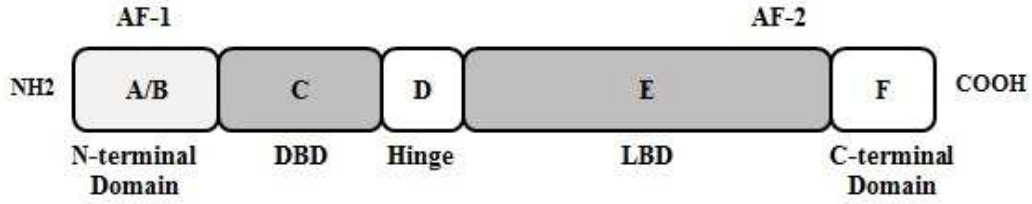


Figure 1.3: Schematic modular structure of nuclear receptors. A/B domain, with an AF-1 activation function located in the N-terminal. A highly conserved DNA-binding domain, the C domain. D domain, a highly flexible hinge region. E domain responsible for ligand-binding and converting NRs to active forms that bind to DNA, which holds a AF-2 activation domain. A F domain with an unknown function (adapted from Aranda and Pascual, 2001).

To modulate RAR-dependent gene transcription, RXRs serve as an obligate heterodimeric partners to RARs. The RXR is required for efficient binding of RAR/RXR heterodimer to the retinoic acid response elements (RAREs) in the regulatory regions of target genes (Fig. 1.4) (Blomhoff and Blomhoff, 2006; Germain et al., 2006b). The heterodimer RAR/RXR RAREs consists in two direct repeats of the nucleotide sequence, (A/G)G(G/T)TCA separated by a variable number of nucleotides (usually, one, two or five) (Bastien and Rochette-Egly, 2004; Szanto et al., 2004; McGrane, 2007). In the absence of RA, RAR/RXR heterodimer is bounded to RAREs and to a corepressor complex including a histone deacetylase activity (HDAC)/SIN3 complex, recruited via corepressors NCoR or SMRT, resulting in chromatin condensation and gene transcription silencing (Fig. 1.4) (Bastien and Rochette-Egly, 2004). When RA enters the cell nucleus, it binds to RARs and/or RXR, then a conformational change occurs. The corepressors dissociate and a coactivator complex possessing histone acetyltransferase (HAT), methyltransferase, kinase or ATP-dependent remodeling activities (SWI/SNF). These activities lead to chromatin decondensation and core transcription factors recruitment; facilitating the positioning of the transcriptional machinery at the promoter region, that ultimately leads to gene transcription initiation (Fig. 1.4) (Bastien and Rochette-Egly, 2004).

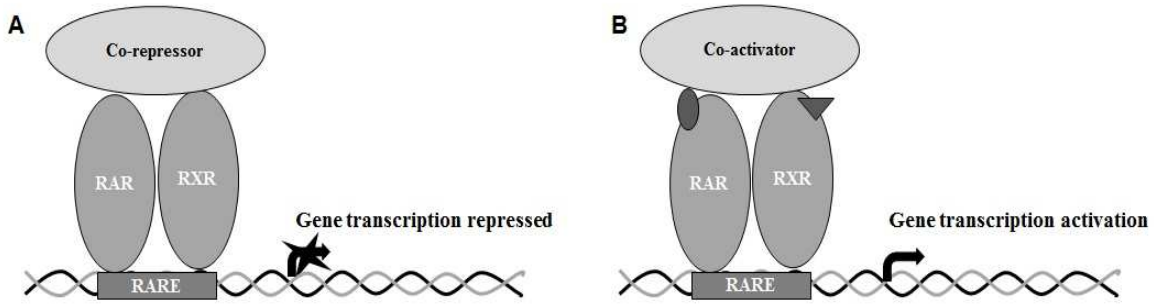


Figure 1.4: Mechanism of target gene regulation by RAR/RXR heterodimers. A) In the absence of ligand, the heterodimer RAR/RXR is associated with RARE recruiting corepressors complexes having an enzymatic activity of deacetylation of histones, which results in chromatin condensation and transcription repression. B) After RA binding to RAR, the heterodimer suffers a conformational change, releasing the corepressor complex and recruiting a coactivator complex with an enzymatic transacetilase activity of histones, resulting in chromatin descondensation and thus allowing the activation of target genes transcription (adapted from Marlétaz et al., 2006).

Apart from forming a heterodimer complex with RARs, RXRs also have the ability to functions as a dimer with itself (homodimer - RXR/RXR) or act in heterodimeric complexes with other NRs members, such as peroxisome proliferator-activated receptors (PPAR), thyroid hormone receptor (ThR), liver X receptor (LXR) (Szanto et al., 2004; Germain et al., 2006b). Therefore, RXRs are implicated in many signaling pathways apart from those involved in retinoid signaling, regulating transcription of several genes, and concomitantly being involved in a wide range of physiological functions, such as homeostasis, development and metabolism (Szanto et al., 2004). In the case of RXRs transcription regulation as a homodimer complex (RXR/RXR), the signaling pathways are believed to be 9-*cis*-RA binding dependent, but its exact function remains uncertain (Mangelsdorf et al., 1992; Allenby et al., 1993; Germain et al., 2006b).

In addition to RA functions mediated through NRs signaling pathway, their action via non-genomic mechanisms has also been reported, such as activation of protein kinase cascades, which influence gene expression through phosphorylation processes (Blomhoff and Blomhoff, 2006; Al Tanoury et al., 2013).

1.2.5 Retinoids catabolism pathway

Given its robust role in gene transcription regulation, RA catabolic inactivation is required to maintain a proper homeostatic equilibrium. This oxidative step is mainly catalyzed by members of the cytochrome P450 family 26 (CYP26) (Thatcher et al., 2010). The CYP26 enzymes family receives RA that is believed to be bound to cellular retinoic acid binding proteins (CRABPs), promoting the catabolism of RA by metabolizing into polar, and less potent, metabolites, including 4-hydroxy retinoic acid, 4-oxo retinoic acid, 18-hydroxy, 5, 6-epoxy RA or 5, 8-epoxy RA, and others (Blomhoff and Blomhoff, 2006; Theodosiou et al., 2010). The biological relevance of these metabolites as signaling molecules is still unclear, but there are reports that might have a biological activity (Thatcher et al., 2010; Theodosiou et al., 2010).

1.3 A comparative analysis of retinoid metabolism in invertebrates: molecular and biochemical evidences

In comparison with vertebrates, the information regarding the presence of an elaborated retinoid system remains fragmented and incomplete for invertebrate lineages. Initially, most of the retinoid key molecular components were described outside chordates. Therefore, retinoid metabolic and signaling pathways were considered to be a chordate innovation, probably related to the evolutionary origin of their body plan organization (Fujiwara and Kawamura, 2003; Simões-Costa et al., 2008). However, evidences from massive genome sequencing effort, regarding phylogenetically informative species, allowed to identify retinoid-related genes, from the metabolic and signaling cascade, and question this traditional view (Fig. 1.5) (e.g. Cañestro et al., 2006; Albalat and Cañestro, 2009; Theodosiou et al., 2010). In addition, although conducted in a limited number of invertebrate *taxa*, the endogenous retinoid profile has also been investigated, again supporting the assumption of a retinoid cascade presence outside vertebrates (Table 1.1). Altogether, findings point to an early emergence in basal bilaterians (Cañestro et al., 2006; Albalat and Cañestro, 2009; Theodosiou et al., 2010). Yet, the origin and evolution of retinoid pathways is still poorly understood. Retinoid cascade is not yet entirely characterized in most invertebrate phyla. For instance, the existence of invertebrate genes orthologues does not necessarily correlate with *in vivo* vertebrate identical roles at the level of retinoid metabolism and/or signaling (Albalat, 2009). Thus, so far studies regarding the identification, isolation, and functional characterization of key molecular modules have been restricted to a few species (Albalat, 2009; Theodosiou et al., 2010; Gesto et al., 2012, 2013). Studies on the physiology of retinoids are also scarce, or even non-existing, for most invertebrate groups. This current lack of knowledge limits our basic understanding regarding the organizational, functional similarities and differences

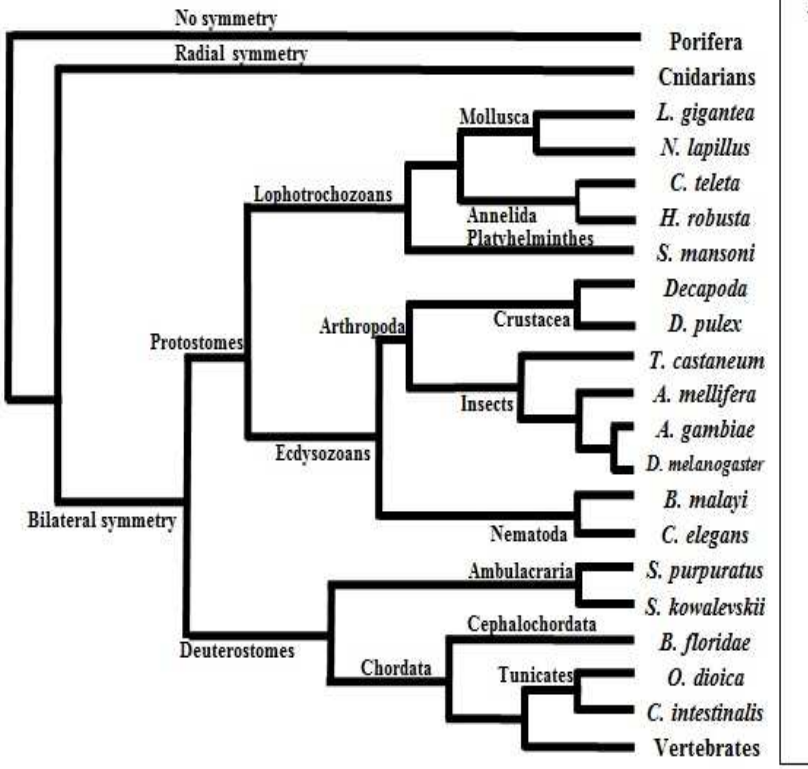
between invertebrate and vertebrate retinoid systems. Findings clearly point to an older ancestry of retinoid pathways than previously thought, despite some fundamental contrasts. The main focus has been drawn from urochordates, cephalochordates and mollusks. Most importantly, available reports suggest a mosaic pattern when compared to vertebrate's retinoid functions, with conserved aspects (e.g. REs storage in some mollusk species) and key differences (e.g. RAR presence and signaling in ecdysozoans and gastropods). In order to enhance our insights on retinoid biology and pathway evolution, future studies should focus on the isolation and functional characterization of the related molecular components and on the detection of endogenous retinoids.

In this section, the current knowledge on retinoid pathways in invertebrate phyla is presented.

Table 1.1: Overview of polar and non-polar retinoids as well as β-carotene presence in invertebrates. (+) Present in the species; (−) absent; (na) not analyzed. HPLC – high-performance liquid chromatography; HPLC–MS – high-performance liquid chromatography–mass spectrometry; GS/MS – gas chromatography–mass spectrometry.

Specie	Phylum; Class	Tissue	RAL	ROL	REs	all-*trans*-RA	9-*cis*-RA	13-*cis*-RA	βc	Analytic method	Ref.
Polyandrocarpa misakiensis	Chordate; Ascidiacea	Developing bud	+	-	na	+	na	-	na	HPLC	Kawamura et al., 1993
		Adults all body	+	-	na	+	na	-	na		
Halocynthia roretzi	Chordate; Ascidiacea	Eggs	+	+	+	na	na	na	na	HPLC	Irie et al., 2003, 2004
		Gonads	+	+	+	na	na	na	na		
		Hepatopancreas	+	+	+	na	na	na	na		
		Gill	+	+	+	na	na	na	na		
		Hemolymph cells	+	+	+	na	na	na	na		
		Hemolymph plasma	+	+	+	na	na	na	na		
		Body wall muscles	+	+	+	na	na	na	na		
Branchiostoma floridae	Chordate; Leptocardii	All body	+	+	na	+	+	+	na	HPLC	Dalfó et al., 2002
Locusta migratoria	Arthropoda; Insecta;	Embryos	na	na	na	+	+	na	na	HPLC-MS	Nowickyj et al., 2008
Uca pugilator	Arthropoda; Malacostraca	Blastema of regenerating limbs	+	na	na	+	+	-	na	HPLC and GS/MS	Hopkins, 2001; Hopkins et al., 2008
Litopenaeus vannamei	Arthropoda; Malacostraca	Ovary	+	+	na	na	na	na	+	Diode array spectrophotometer and HPLC	Paniagua-Michel and Liñan-Cabello, 2000; Liñán-Cabello et al., 2003
		Digestive gland	+	na	na	na	na	na	+		
Lymnaea stagnalis	Mollusca; Gastropoda	CNS	na	na	na	+	+	na	na	HPLC-MS	Dmetrichuk et al., 2008

Osilinus lineatus	Mollusca; Gastropoda	Gonads	na	+	+	+	+	+	na	HPLC and HPLC-MS	Gesto et al., 2012
		Digestive gland	na	+	+	+	+	+	na		
Patella depressa	Mollusca; Gastropoda	Gonads	na	+	+	+	+	+	+	Spectroscopically, HPLC and HPLC-MS	Goodwin, 1950; Gesto et al., 2013
		Digestive gland	na	+	+	+	+	+	na		
Nucella lapillus	Mollusca; Gastropoda	Gonads	na	-	-	+	+	+	na	HPLC and HPLC-MS	Gesto et al., 2013
		Digestive gland	na	-	-	+	+	+	na		
		Kidney	na	-	-	na	na	na	na		
		Gill	na	-	-	na	na	na	na		
		Prostate	na	-	-	na	na	na	na		
		CNS	na	-	-	+	+	+	na		
		Sperm-ingest gland	na	-	-	na	na	na	na		
		Albumen gland	na	-	-	na	na	na	na		
		Capsule gland	na	-	-	na	na	na	na		
Nassarius reticulatus	Mollusca; Gastropoda	Complex gonads/ digestive gland	na	-	-	+	+	+	na	HPLC and HPLC-MS	Gesto et al., 2013
Geodia cydonium	Porifera; Demospongiae	All body	na	na	+	+	-	+	+	HPLC	Biesalski et al., 1992



	Storage		Transport					Synthesis				Catabolism	Signaling	
	LRAT/DGAT1	REHs	RBP	STR6	TTR	CRBP	CRABP	BCO	ADHs	SDHs	ALDHs	CYP26	RAR	RXR
Porifera	unk/+	unk	-	-	-	-	-	+	unk	unk	+	unk	-	-
Cnidarians	unk/+	unk	-	-	-	-	-	+	+	+	+	-	-	a
L. gigantea	unk/+	unk	-	-	-	-	-	+	+	+	+	+	+	+
N. lapillus	unk/+	unk	unk	unk	unk	unk	unk	unk	+	unk	unk	unk	+	+
C. teleta	-/+	unk	-	-	unk	unk	unk	+	+	+	+	+	+	+
H. robusta	unk/+	unk	unk	unk	unk	unk	unk	unk	unk	unk	+	-	-	+
S. mansoni	unk/+	unk	unk	unk	unk	unk	unk	unk	unk	unk	+	-	-	+
Decapoda	unk/+	unk	unk	unk	unk	+	+	unk	unk	unk	unk	unk	unk	+
D. pulex	-/+	-	-	-	unk	-	unk	+	+	+	+	-	unk	+
T. castaneum	unk/+	unk	unk	unk	unk	unk	unk	unk	unk	unk	+	-	-	+
A. mellifera	unk/+	unk	unk	unk	unk	unk	unk	unk	unk	unk	+	-	-	+
A. gambiae	unk/+	unk	unk	unk	unk	unk	unk	unk	unk	unk	+	-	-	+
D. melanogaster	-/+	-	-	-	-	-	-	+	unk	+	+	-	-	+
B. malayi	unk/+	unk	unk	unk	unk	unk	unk	unk	unk	unk	-	-	-	+
C. elegans	unk/+	-	-	-	-	unk	unk	+	+	+	+	-	-	-
S. purpuratus	-/+	unk	-	-	-	+	-	+	+	+	+	+	+	+
S. kowalevskii	-/+	-	-	-	-	-	-	-	+	+	+	+	+	+
B. floridae	-/+	+	unk	unk	unk	+	unk	+	+	+	+	+	+	+
O. dioica	unk/+	unk	unk	unk	unk	unk	unk	unk	unk	unk	-	-	-	+
C. intestinalis	-/+	unk	-	-	-	-	unk	+	+	+	+	+	+	+
Vertebrates	+/+	+	+	+	+	+	+	+	+	+	+	+	+	+

Figure 1.5: Cladogram representing the current knowledge concerning the presence/absence of key molecular components in different metazoan groups. (+) indicates that molecular components are present in the phylum/species; (−) means that molecular components are absent from genome for analyzed species; (unk) absence of evidences (a) indicates that the molecular component is present in some species but absence in others from the same phylum. Hypothetic phylogenetic relationships according to Evans and Gundersen-Rindal (2003) and Theodosiou et al. (2010).

1.3.1 Tunicates

Tunicates are vertebrates' closest living relatives. However, the few available studies conducted so far suggest that retinoid spatiotemporal distribution is differently controlled in these two animal groups (Irie et al., 2003, 2004).

In *Ciona intestinalis* the presence of putative vertebrate-like components involved in RA synthesis, including ROL and RAL oxidation, NR-mediated signaling and RA catabolism were genome predicted, whereas a retinoid storage system seems to be lacking (Theodosiou et al., 2010). However, the majority of these genetic modules were not isolated or functionally characterized. Among the few cloned genes is a β-carotene oxygenase (BCO) orthologue, identified in the visual system of larvae and adult *C. intestinalis* (Nakashima et al., 2003; Takimoto et al., 2006). *Escherichia coli* purified BCO-like exhibited significant asymmetrical carotenoid cleavage activity, since no RAL was detected (Poliakov et al., 2012). A gene coding for a putative cellular retinaldehyde-binding protein like (CRALBP) expressing in the central nervous system (CNS) during embryonic development, was also identified (Nakashima et al., 2003).

In *Polyandrocarpa misakiensis* both RXR and RAR homologues were isolated (Hisata et al., 1998; Kamimura et al., 2000); whereas for *Botrylloides leachi* only a RAR homologue was identified (Rinkevich et al., 2007). RXR has also been cloned and its expression evaluated in *Halocynthia roretzi* (Maeng et al., 2012). Regarding these species, reports suggest a major role for RA as an endogenous signaling molecule, participating in the morphallactic bud development regulation and tissue regeneration (Kawamura et al., 1993; Kamimura et al., 2000; Rinkevich et al., 2007; Kaneko et al., 2010). RAR is, in fact, expressed during *B. leachi* whole body regeneration; RAR knockdown resulted in regeneration arrest and bud malformation. The same pattern was reported when RA synthesis was inhibited (Rinkevich et al., 2007). The presence of a RAR orthologue, as well as RA synthetic and catabolic enzymes ALDH1 (ALDH1a/b/c/d) and CYP26, has been also reported in C*. intestinalis* (Nagatomo and Fujiwara, 2003; Theodosiou et al., 2010; Sobreira et al., 2011). Interestingly, independent losses of the RA genetic machinery might have occurred amongst larvaceans, as suggested by the absence of ALDH1a, CYP26 and RAR orthologues in *Oikopleura dioica* genome (Cañestro et al., 2006). Consistently, treatment with RA, a classical morphogen, did not induce homeotic posteriorization of anterior structures; likewise, no developmental abnormalities were observed after treatment with an ALDH inhibitor (Cañestro and Postlethwait, 2007). Thus, *O. dioica* develops and maintains an RA-independent anterio–posterior (AP) axial patterning, typical of a chordate body plan (Cañestro and Postlethwait, 2007).

The available data on endogenous retinoid content highlights a major difference in the synthesis, storage and homeostatic mechanisms between ascidians and vertebrates. In contrast to the latter, RAL is seemingly the main storage and transport retinoid in tunicates, being hypothesized that it is rapidly metabolized to RA when required (Irie et al., 2004). In agreement, endogenous levels of all-*trans*-RA and RAL were detected in developing buds and adult *P. misakiensis* (Kawamura et al., 1993). Also, in *H. roretzi* eggs and adult tissues the most abundant retinoid was RAL and practically no ROL or REs could be detected (Irie et al., 2003, 2004). Recently, RA has been reported to participate in body patterning during embryo development in *C. intestinalis* (Pasini et al., 2012), supporting the biological relevance of active endogenous retinoids in most urochordates.

1.3.2 Cephalochordates

Comparatively, amphioxus is recognized to have a vertebrate-like response to RA, including the associated homeostatic control mechanisms and signaling pathways (Escriva et al., 2002; Marlétaz et al., 2006). Exhaustive genome analyses have identified various RA metabolic and signaling components in *Branchiostoma floridae* (Albalat et al., 2011; Poliakov et al., 2012). In some cases genes were isolated and their function assessed. For instance, an ADH3 orthologue was cloned and *in situ* hybridization analysis revealed restricted expression patterns: adult gut, embryos, late free-swimming and feeding larvae (Cañestro et al., 2000). No functional characterization of ADH3 was performed to elucidate its possible role in ROL oxidation. Two retinol dehydrogenase orthologues, RDH1 and RDH2, were identified and functional characterized. Both RDHs exhibited dehydrogenase activity catalyzing the reduction of all-*trans*-RAL into all-*trans*-ROL, using NADH as cofactor (Dalfó et al., 2007).

RAR and RXR orthologues were also isolated and shown to mediate RA-dependent transcription (Escriva et al., 2002; Tocchini-Valentini et al., 2009). The ability of RAR/RXR heterodimers to recognize retinoic acid responsive elements (RARE) was validated using electrophoretic mobility shift assays (Escriva et al., 2002). In the presence of RA the heterodimer activated transcription (Escriva et al., 2002). Using a cell-based assay, 9-*cis*-RA was reported to activate RXR, albeit at relative higher concentrations (Tocchini-Valentini et al., 2009). *B. floridae* putatively exhibits pathways for both β-carotene and ROL-derived RA synthesis and also RA catabolism, given that five BCO-like genes, ALDH1 isoforms (ALDH1a/b/c/d/e/f), SDRs and CYP26 orthologues were genome predicted (Albalat and Cañestro, 2009; Theodosiou et al., 2010; Albalat et al., 2011; Sobreira et al., 2011). Mechanisms for REs hydrolysis and ROL handling also seems to be present, since putative REHs and CRPB were genome predicted (Theodosiou et al., 2010). Regarding the counter esterification pathway, DGAT1 orthologues, but not LRAT,

can be retrieved in *B. floridae* genome (Albalat et al., 2011; Poliakov et al., 2012). *B. floridae* also seems to lack putative, STRA6, TTR and RBP orthologues, suggesting alternative transport and cellular uptake mechanisms (Theodosiou et al., 2010; Albalat et al., 2011).

In the adult *B. floridae*, endogenous levels of ROL, RAL and RA isomers were reported and appear to be involved in morphogenetic processes (Dalfó et al., 2002; Escriva et al., 2002; Schubert et al., 2004). The RA synthesis pathway is also active (Dalfó et al., 2002). In fact, ROL-exposed amphioxus developed morphological abnormalities as observed in RA-exposed animals (Dalfó et al., 2002). Further data suggests an important role for RA signaling in amphioxus nervous system AP patterning and development of epidermal sensory neurons. During embryonic development RAR is expressed in the CNS and strongly upregulated upon RA treatment; conversely, total down-regulation of RAR was reported in the presence of specific antagonists (Escriva et al., 2002). Regarding AP positioning of epidermal sensory neurons, treatments with RA and RAR antagonists affected the collinear expression of *Hox* genes, up- and down regulating expression, respectively (Schubert et al., 2004).

1.3.3 Mollusks

An increasing body of knowledge advocates for the presence of putative retinoid metabolic and signaling pathways in mollusks. However, studies have focused mainly in NR identification, cloning and their implication in a very limited number of biological functions. Although the detection of endogenous retinoids predicted that, in mollusks, retinoid pathways are active and present, the isolation of the associated genetic machinery and its functional characterization is still missing.

Genome searches have identified retinoid metabolic modules in the herbivore *Lottia gigantea* (Albalat and Cañestro, 2009; Theodosiou et al., 2010; Sobreira et al., 2011). This species hypothetically exhibits mechanisms for both β-carotene and ROL-derived RA synthesis and RA catabolism as BCO, ADHs, SDHs, three ALDH1a (ALDH1a/b/c) and CYP26 orthologues were genome predicted (Albalat and Cañestro, 2009; Theodosiou et al., 2010; Sobreira et al., 2011). Yet, *L. gigantea* appears to lack vertebrate-like retinoid storage, transport and mobilization system (Theodosiou et al., 2010). It remains to be investigated whether the referred enzymes are functionally similar to the vertebrate orthologues. For instance, in the dogwhelk *Nucella lapillus*, ADH3 was isolated and, unlike the ubiquitous vertebrate orthologue, has expression mostly in the digestive gland (Coelho et al., 2012).

The study of retinoid signaling pathways in mollusks emerged with the identification and cloning of RXR orthologues in diverse gastropod species, such as the

rock shell *Thais clavigera* (Nishikawa et al., 2004), the freshwater bloodfluke planorb *Biomphalaria glabrata* (Bouton et al., 2005), the dogwhelk *N. lapillus* (Castro et al., 2007), the mud snail *Ilyanassa obsoleta* (Sternberg et al., 2008), and the great pond snail *Lymnaea stagnalis* (Carter et al., 2010). *In vitro* binding assays demonstrated that 9-*cis*-RA binds, with relative high affinity, to the ligand-binding domain of *B. glabrata*, *T. clavigera* and *N. lapillus* RXR, highlighting a possible biological role of the molluskan RXR (Nishikawa et al., 2004; Castro et al., 2007). Furthermore, when transfected into mammalian cell lines *B. glabrata* RXR activated transcription in the presence of 9-*cis*-RA (Bouton et al., 2005). From a physiological standpoint, injection of the putative RXR ligand, 9-*cis*-RA, into females *T. clavigera* and *N. lapillus* induces the outgrowth of male reproductive structures (Nishikawa et al., 2004; Castro et al., 2007). RXR signaling was also hypothesized to regulate male and female seasonal reproductive development, since an increasing RXR expression pattern in *I. obsoleta* correlated with reproductive tract recrudescence in both sexes (Sternberg et al., 2008, 2010). This function might be evolutionary conserved since vertebrate retinoid signaling was also suggested to modulate reproductive differentiation and development (Kastner et al., 1996; Ogino et al., 2001; Bowles and Koopman, 2007). *L. stagnalis* RXR transcripts were also detected in developing embryos and in the adult CNS (Carter et al., 2010). Cloning of a RAR orthologue was reported in *N. lapillus* and *T. clavigera*; however, despite their ability to heterodimerize with RXR, no RA-mediated transcription activation was observed (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014a).

Gene expression and predictive data is consistent with the endogenous retinoid content. The first indication came from the scientific work of Dmetrichuk et al. (2008). Using analytical approaches they demonstrated the presence of 9-*cis*-RA and all-*trans*-RA in adult *L. stagnalis* CNS. The presence of RA isomers suggests that this species is capable of synthesizing RA, but the associated enzymes are yet to be identified. Evidences suggest the CNS as a potential physiological target for all-*trans*-RA and 9-*cis*-RA, modulating neuronal regeneration and outgrowth (Dmetrichuk et al., 2008). Additional studies are also indicative of a contribution of RA for the chemotropic response of growth cones in regenerating neurons (Carter et al., 2010). Vertebrate RA modulates several processes in the developing CNS, such as neural patterning, neural differentiation and neurite outgrowth (Maden, 2007) and the above mentioned results endorse a conservation of neuronal RA functions between mollusks and vertebrates (Maden, 2007; Campo-Paysaa et al., 2008; Dmetrichuk et al., 2008). Apart from the CNS, RA was also shown to take part in *L. stagnalis* embryonic development. RA-treated embryos exhibit eye and shell defects and are developmentally arrested at the trochophore stage. Similar

results were observed upon ROL and RAL treatment, further validating that both metabolites serve as RA precursor's *in vivo* (Créton et al., 1993).

ROL metabolism and signaling was also demonstrated in some mollusk species. RA, ROL and REs (mainly REs palmitate) were detected in the gastropod *Osilinus lineatus* and *Patella depressa*, mainly in male gonad-digestive gland complex (Gesto et al., 2012, 2013). The presence of β-carotene in *P. depressa* gonads was also described (Goodwin, 1950). In contrast, neither ROL nor REs trace levels were detected in *N. lapillus* nor *Nassarius reticulatus*, suggesting that esterified ROL storage might be absent (Gesto et al., 2013). When present, ROL and REs levels were higher in male gonads than in females, which could suggest a role in gonad maturation (Gesto et al., 2012, 2013). Together with the presented RXR gene expression data, these results support seasonal retinoid variations in the development of male reproductive tract in gastropod mollusks (Sternberg et al., 2008; Gesto et al., 2012, 2013). Active ROL esterification and RAL oxidative pathways were demonstrated using *in vivo* assays in *O. lineatus* (Gesto et al., 2012). This suggests that these mollusks have an elaborated, vertebrate-like, retinoid system able to control active retinoid levels through inactive REs storage. Consistently, a pharmacological *in vivo* assay, carried out with *N. lapillus*, demonstrated that, despite their ability to convert RAL into ROL or active RA isomers, they lack a metabolic esterification pathway (Gesto et al., 2013). Based on these evidence Gesto et al. (2013) proposed that retinoid homeostatic control through REs storage is not present in all gastropod clades.

1.3.4 Ecdysozoans

Little is known about the functional relevance of enzymes, binding proteins, receptors, as well as metabolites of the retinoid metabolism and signaling pathways in ecdysozoans. Once again studies have been conducted in a reduced number of species, most of the information was genome predicted and functional studies are scarce.

Genes displaying sequence similarity with vertebrate ALDH1a, responsible for RA synthesis, were predicted in several ecdysozoan genomes: *Drosophila melanogaster*, *Apis mellifera*, *Anopheles gambiae*, *Tribolium castaneum* and *Daphnia pulex*; yet, CYP26 homologues, which mediate RA oxidative clearance, are missing (Albalat and Cañestro, 2009; Baldwin et al., 2009; Theodosiou et al., 2010). Regarding *D. melanogaster* and *D. pulex*, the genetic machinery required for retinoid storage, transport and mobilization is also absent (Theodosiou et al., 2010). In *D. melanogaster* very few genes from the metabolic cascade were isolated *de facto*. One of such genes was a putative BCO homologue (von Lintig and Vogt, 2000). *E. coli* expression and subsequent biochemical analysis revealed that the encoded BCO enzyme was capable of catalyzing the symmetric oxidative cleavage of β-carotene to RAL (von Lintig and Vogt, 2000). A CRALBP-like

orthologue was also found in the *D. melanogaster* genome but its function has not been determined (Werner et al., 2000). Five short-chain dehydrogenases/reductases (SDRs) were identified and characterized, showing biochemical properties similar to the human retinol dehydrogenases 12 (RDH12): using as substrates both all-*trans*-RAL and all-*trans*-3-hydroxyRAL (Belyaeva et al., 2009). Apart from *D. melanogaster* and *D. pulex*, few studies have reported the isolation of retinoid metabolism-related genes within this group. Regarding transport, genes coding for ROL, RAL and RA binding proteins were identified in some arthropods (Mansfield et al., 1998; Gu et al., 2002; Wakakuwa et al., 2003). Vertebrate-like CRABPs, which have a ligand binding pocket capable of accommodating RA, were also described in the tobacco hornworm *Manduca sexta* (Mansfield et al., 1998). A member of retinoid/fatty acid binding-like protein family, that displayed characteristics of both CRABP and CRBP, was isolated from the crustacean *Metapenaeus ensis*. Further examination revealed its ability to bind RA and RAL, with similar affinity (Gu et al., 2002). A ROL-binding protein was also reported in the swallowtail butterfly, *Papilio xuthus*, yet it exclusively bound 3-hydroxyretinol and was suggested to take part in visual pigment turnover (Wakakuwa et al., 2003). Regarding NR-mediated pathways, genome database mining revealed that RAR signaling is probably absent in ecdysozoans, since it failed to identify RAR orthologues in the insects *D. melanogaster*, *A. mellifera*, *A. gambiae*, and *T. castaneum*, and in the crustacean *D. pulex* (King-Jones and Thummel, 2005; Albalat and Cañestro, 2009; Thomson et al., 2009; Theodosiou et al., 2010). RXR orthologues, on the other hand, have been described, and cloned, in several arthropod species including the water flea *Daphnia magna* and *D. pulex*, the scorpion *Liocheles australasiae*, the shrimp *Crangon crangon*, the centipede *Lithobius peregrinus*, the locust *Locusta migratoria*, the sand fiddler crab *Uca pugilator*, the fruit fly *D. melanogaster* and others; with respect to insects, RXR orthologues are known as ultraspiracle (USP) (Nakagawa et al., 2007; Wang et al., 2007; Hopkins et al., 2008; Nowickyj et al., 2008; Nakagawa and Henrich, 2009; Thomson et al., 2009; Bortolin et al., 2011).

Arthropod RXRs are known to serve as obligate heterodimeric partners of ecdysone receptors (EcR), a partnership responsive to ecdysteroid hormones (e.g. 20-hydroxy-ecdysone and ponasterona A) and involved in several physiological functions, such as development, reproduction, molting, metamorphosis and crab limb regeneration (Hall and Thummel, 1998; Nakagawa et al., 2007; Hopkins et al., 2008; Nowickyj et al., 2008; Lafont, 2009). In *D. melanogaster*, the EcR/USP heterodimer participates in head involution, dorsal closure and tracheal and midgut morphogenesis during embryo development (Chavoshi et al., 2010). RXR mRNA transcript levels during *D. magna* embryonic development also suggest a crucial role for this receptor, notably in stage 1

embryos when RXR is upregulated in comparison with the subsequent embryonic stages (Wang et al., 2007).

Heterodimerization with RXR/USP is, in fact, required to enhance EcR affinity towards ecdysteroids in *D. melanogaster* and in the fiddler crab, *U. pugilator* (Grebe et al., 2003; Hopkins et al., 2008). This mandatory partnership with the ecdysone-inducible EcR is often accompanied by a lack of high-affinity functional ligands for RXR orthologues. Yet, studies suggest that EcR-independent RXR signaling pathways might also exist (Nowickyj et al., 2008; Nakagawa and Henrich, 2009; Wang and LeBlanc, 2009). For instance, in the beetle *T. castaneum* neither 9-*cis*-RA nor crustacean hormones (methoprene acid and ponasterone A) were able to bind RXR *in vitro* (Iwema et al., 2007). *D. magma* RXR orthologue, on the other hand, is in fact able to bind 9-*cis*-RA and methyl farnesoate, a crustacean hormone; still no transactivation was observed (Wang and LeBlanc, 2009). In *L. migratoria* embryos, two RXR alternative splicing isoforms were isolated, both binding 9-*cis*-RA and all-*trans*-RA isomers with similar affinity. Furthermore, RXR transcripts were detected when EcR was absent: supporting an EcR-independent role for RA-mediated signal transduction during locust embryonic development (Nowickyj et al., 2008). More complex activation mechanisms were observed in the fiddler crab, *U. pugilator*, where two RXR splice variants, capable of binding 9-*cis*-RA, methyl farnesoate and farnesoic acid, were cloned (Hopkins et al., 2008). Interestingly, the RXR isoforms were shown to alter EcR sensitivity towards ponasterone A, but not ecdysone or 20-hydroxy ecdysone, suggesting a differential responsiveness of the EcR/RXR dimers (Hopkins et al., 2008).

In accordance with the molecular data, endogenous active retinoids were quantified in *U. pugilator* regenerating limb blastemas (all-*trans*-RAL, 13-*cis*-RAL, all-*trans*-RA and 9-*cis*-RA) and *L. migratoria* embryos (all-t*rans*- and 9-*cis*-RA) (Hopkins, 2001; Hopkins et al., 2008; Nowickyj et al., 2008). β-carotene, ROL and RAL were also reported in shrimp, *Litopenaeus vannamei*, developing oocytes whereas the digestive gland contained RAL and β-carotene (Paniagua-Michel and Liñán-Cabello, 2000; Liñán-Cabello et al., 2003) Interestingly, gonad retinoid content increased with oocyte maturation, stressing their during gonad maturation, as suggested for mollusks and tunicates (Paniagua-Michel and Liñán-Cabello, 2000; Liñán-Cabello et al., 2003; Irie et al., 2004; Gesto et al., 2012, 2013). This observation is also in agreement with previous reports by Alava et al. (1993) showing an increase in ovary development, in the shrimp *Penaeus japonicus*, upon administration of dietary retinyl palmitate.

1.3.5 Porifera

Porifera represents one of the oldest living metazoan phyla. An array of molecular and biochemical evidences predict the biological relevance of retinoids in this group.

The identification and functional validation of a BCO-I like enzyme from *Suberites domuncula* suggested that the β-carotene derived RA synthesis might be functional in sponges (Müller et al., 2011, 2012). Sponge RA synthesis was further supported by the isolation of a retinal dehydrogenase/reductase; yet no functional studies were conducted (Müller et al., 2011, 2012). Regarding the signaling pathways, no RXR and RAR orthologues exist (Bridgham et al., 2010). In the demosponge genome, *Amphimedon queenslandica*, only two NRs were identified: nuclear receptor 1 (NR1) and nuclear receptor 2 (NR2) (Bridgham et al., 2010). Phylogenetic studies by Bridgham et al. (2010) indicate that NR2 is an orthologue of the hepatocyte nuclear factor 4 receptor (HNF4) family and NR1 the unduplicated orthologue of all other NRs superfamily members, already present in the last common ancestor of Metazoans. However, their biological roles are unclear (Bridgham et al., 2010). Despite the absence of canonical RA-binding receptors, it has been reported that RA exerts a morphogenetic effect on developing gemmules in *S. domuncula* (Wiens et al., 2003).

Biesalski et al. (1992) demonstrated the presence of β-carotene, all-*trans* and 13-*cis*-RA, and two different REs forms (retinyl arachidate and retinyl stearate) in the sponge *Geodia cydonium*. In contrast to vertebrates, where retinyl palmitate is the most abundant storage form, retinyl arachidate is the predominant RE in *G. cydonium*. The presence of these endogenous retinoids suggests their ability to recruit β-carotene and REs for RA synthesis and maintain retinoid homeostasis through RE storage and mobilization.

Together, these results support a basal metazoan origin for the RA synthesis pathway through β-carotene cleavage. Regarding RA role in biological functions, it is still unclear whether it acts through NR-like signaling or via non-genomic pathways.

1.3.6 Other groups

Information regarding the remaining invertebrate phyla is particularly limited and exclusively based in genome predictions (Fig. 1.5). Basic Local Alignment Search Tool (BLAST) searches and phylogenic analysis in two deuterostome species, the echinoderm *Strongylocentrotus purpuratus* and the hemichordate *Saccoglossus kowalevskii*, suggest that BCO, ADH, SDH, ALDH, RAR and RXR orthologues are present but both species apparently lack the retinoid storage machinery (Cañestro et al., 2006; Theodosiou et al., 2010). In the nematodes, *Brugia malayi* and *Caenorhabditis elegans*, RAR and Cyp26 are absent; ALDH orthologues were also not predicted in the latter (Albalat and Cañestro, 2009; Theodosiou et al., 2010).

Regarding lophotrochozoans, most available studies focus on mollusks, as discussed above. In annelids and flatworms, gene isolation, transcription profiles, protein functional characterization and analytical retinoid detection is still missing. *Capitella teleta* and *Helobdella robusta* are, so far, the only available annelid genomes. BLAST search analysis propose vertebrate-like retinoid synthesis, degradation and signaling mechanisms in *C. teleta* (e.g. RAR, CYP26, ADH and ALDH1a); a typical retinoid storage and mobilization mechanism is missing (Albalat and Cañestro, 2009; Theodosiou et al., 2010). However, CYP26 and RAR orthologues seem to be absent from the annelid *H. robusta* and the platyhelminth *Schistosoma mansoni* (Albalat and Cañestro, 2009).

In the cnidarian *Tripedalia cystophora*, a RXR orthologue was isolated and shown to bind 9-*cis*-RA (Kostrouch et al., 1998). This is not a generalized feature in all cnidarian clades, since *Nematostella vectensis* lacks both RXR and RAR (Theodosiou et al., 2010). Yet, in *N. vectensis*, a BCO orthologue and enzymes from the ROL oxidative cascade were predicted (ADH, SDR, and ALDHs) (Theodosiou et al., 2010). Recently, a biological role has been suggested for RA in the cnidarian *Clava multicornis*, since it has been shown that RA treatment affected nervous system development in this species (Pennati et al., 2013). Similarly, 9-*cis*-RA was suggested to participate in neural differentiation in another cnidarian, *Renilla koellikeri* (Estephane and Anctil, 2010). More recently, in the *Aurelia aurita* transcriptome were identified two RDHs (RDH1 and RDH2) and a RXR gene homologues (Fuchs et al., 2014). In addition it was proposed that retinoid metabolic and signaling cascade might have a role during *A. aurita* metamorphosis (Fuchs et al., 2014). RXR and RDH2 were found to be upregulated express during the strobilation stage; and the exposure of *A. aurita* polyps to both ROL and 9-*cis*-RA leads to strobilation induction, which can be significantly delayed in the presence of a RXR antagonist and by an inhibitor of ALDH (Fuchs et al., 2014). These findings suggests that RXR seems to be crucial for the strobilation process whereas 9-*cis*-RA might serve as a signaling molecule, and also predicts the presence of enzymes able to convert ROL into RAL and other that subsequently convert RAL to 9-*cis*-RA (Fuchs et al., 2014). Yet, demonstration of endogenous retinoid levels is missing to support the findings as well as protein isolation and functional characterization (Fuchs et al., 2014).

1.4 Retinoid metabolism: mobilization and storage mechanism evolution

Organisms have to maintain most endogenous compounds at certain levels. In the case of essential nutrients like vitamins, there is an extra handicap for a proper control, since animals are not able to produce them endogenously. Regarding retinoids, vertebrates possess very effective strategies to maintain a homeostatic control of their levels such as esterification. Through this mechanism vertebrates are able to store the retinoid dietary excess as REs, which can be mobilized again when the retinoid intake is insufficient (Albalat, 2009). Hence, REs function as an endogenous and ready to-use retinoid source. An animal lacking a similar mechanism would be certainly more sensitive to strong variations in the dietary intake. This is especially important in the case of retinoids, since these compounds are known to be detrimental both in defect and in excess, markedly during embryo development (Marlétaz et al., 2006; Isken et al., 2007).

Despite early findings identifying REs in sponge tissues (Biesalski et al., 1992) and demonstrating shrimp's ability to recruit REs as retinoid precursors (Alava et al., 1993), it was widely believed that retinoid storage was vertebrate-specific (Albalat, 2009; Albalat et al., 2011). Non-vertebrates were considered to have alternative, and probably less strict, control mechanisms, for example by the accumulation of RAL (Irie et al., 2004). However, the recent discovery of endogenous REs in two gastropod species questions this view (Gesto et al., 2012, 2013) and suggests a more ancestral origin for ROL esterification. In contrast to vertebrates, where liver storage is predominant, gastropods store REs mainly in the gonads (D'Ambrosio et al., 2011; Gesto et al., 2012, 2013); hence, important physiological differences might exist.

Another interesting aspect is the relationship between feeding behaviors and ROL esterification (Gesto et al., 2013). Despite the limited number of studied gastropod species, an herbivore/carnivore dichotomy regarding esterified ROL content seems to exist: REs were detected in the grazing herbivores *O. lineatus* and *P. depressa* but not in the caenogastropoda *N. lapillus*, a carnivorous species that feeds mainly on mussels and barnacles (Gesto et al., 2012, 2013) or in *N. reticulatus*, also a carnivore. Unlike *N. lapillus*, whose diet might provide several RA precursors (Hertzberg et al., 1988; Hughes and Burrows, 1991; Simões-Costa et al., 2008; Gesto et al., 2013), *O. lineatus* and *P. depressa* retinoid intake is limited to carotenoids which serve as major precursors of both ROL and REs (Crothers, 2001; Wong and Wong, 2003; Gesto et al., 2012, 2013).

Despite the biochemical evidence, the clarification of the molecular mechanisms involved in ROL esterification and mobilization in invertebrates is yet to be concluded. Evidence points to the absence of several key components such as LRAT, RBP, TTR, STRA6, and REHs orthologues in invertebrate genomes, suggesting this acquisition as a vertebrate novelty (Albalat, 2009; Theodosiou et al., 2010; Albalat et al., 2011; Poliakov et

al., 2012). This raises a fundamental question: what is the enzymatic pathway involved in ROL esterification and REs storage in invertebrates? Studies highlighted that DGAT1, a well-known enzyme involved in the final step of triglyceride synthesis (Cases et al., 1998), also exhibited ARAT activity capable of ROL esterification in vertebrates (O'Byrne et al., 2005; Yen et al., 2005; Wongsiriroj et al., 2008; Shih et al., 2009). DGAT1 orthologues have been genome predicted in the referred invertebrate species, including *B. floridae*, *N. vectensis*, *A. mellifera* and *D. melanogaster* (Albalat et al., 2011; Turchetto-Zolet et al., 2011). Although the function of invertebrate DGAT1 remains to be evaluated, its distribution suggests that, in the absence of LRAT, it might be responsible for REs synthesis and storage. Yet, one cannot discard a possible role of other, unidentified, enzymes with ARAT activity. Although additional studies are required, this puts forward an ancestral role of DGAT1 in REs biosynthesis. If confirmed, the esterification pathway would not represent a chordate innovation but, instead, would precede the appearance of bilateral symmetry (Albalat et al., 2011). However, when compared to vertebrates, differences do exist, concerning not only the esterification mechanism but also the retinoid storage form (Biesalski et al., 1992; Kawamura et al., 1993; Irie et al., 2003, 2004; Albalat et al., 2011; Gesto et al., 2012, 2013).

1.5 Evolution of RA synthesis pathway

In contrast with the catabolism, storage, transport and signaling pathways, the retinoid biosynthesis routes appears to be one of the most conserved from an evolutionary point of view in metazoans (Fig. 1.5). Genome search in metazoan species predicts the presence of all main retinoid synthesis key players either from the canonical and non-canonical routes in different metazoan phyla, proposing that enzymes able of synthesizing retinoids have originated long before the protostome–deuterostome split, and might even have been present earlier in metazoan evolution (Albalat, 2009; Theodosiou et al., 2010). Yet, the function of these enzymes at the level of retinoids metabolism is still missing for most invertebrate lineages.

Some vertebrates are able to accumulate high contents of βc on their tissues, with the subsequent oxidative cleavage of this carotenoid representing an extra source of RA and an alternative mechanism that helps keeping a retinoid homeostasis balance independent of ROL mobilization stores (Napoli et al., 1988; Wang et al., 1996; Kiefer et al., 2001). Invertebrate genome studies revealed that they lack a vertebrate-like tool kit for retinoid storage as REs (Albalat, 2009; Theodosiou et al., 2010). Since BCO orthologues are present in invertebrates, βc oxidative cleavage might be an adaptive strategy mechanism by which they synthesize retinoids to keep a vitamin A homeostatic control in the absence of the ability to store and mobilize ROL (Napoli et al., 1988; Wang et al.,

1996; Kiefer et al., 2001; Simões-Costa et al., 2008). Yet, information regarding invertebrate lineages ability to accumulate βc, and BCO orthologues involvement in RA metabolism is still missing.

It has been proposed that the ADHs and ALDH1 enzymes initially had a role in cellular detoxification mechanisms, and only latter during evolution might have acquired in vertebrates a role on retinoid's biosynthesis cascade (Vasiliou and Nebert, 2005; Simões-Costa et al., 2008; Sobreira et al., 2011). For invertebrates lineages it is still unclear if a similar trend occurred. Yet, the role at retinoid metabolism is still not fully understood outside vertebrates. Given that the presence does not necessarily imply functional conservation; the hypothesis that ALDH1a and ADHs could have metabolic activities other than retinoid synthesis in invertebrates cannot be rejected. A functional characterization is missing.

1.6 Evolution of signaling pathway

An important feature of metazoan evolution was the development of an endocrine system that allows regulation of biological processes in response to environmental and physiological stimuli (Bertrand et al., 2004). Crucial components of the endocrine system are the NRs, a superfamily of ligand-depend and independent transcription factors that bind to specific DNA motifs and regulate target gene expression modulating several physiological processes (Thornton, 2003; Bertrand et al., 2004). Like other gene families, NRs superfamily resulted from a continuous evolutionary process of genomic diversification within and between species, achieving a great diversity already before bilateral symmetry appearance (Thornton, 2003; Bridgham et al., 2010).

Genome investigations in non-model invertebrates reveals the presence of NRs members once considered a chordate novelty such as RARs and RXRs orthologues (Thornton, 2003; Bridgham et al., 2010; Albalat, 2009). RXRs are the most highly conserved members of the NRs across metazoan lineages, have been found from diploblastic to triploblastic organisms (Fig. 1.5), being suggested that might be the oldest retinoid responsive pathway emerging long before the appearance of bilateral symmetry (Thornton, 2003; Bridgham et al., 2010; Gutierrez-Mazariegos et al., 2014b). RARs were traditionally thought to be a chordate-specific novelty because they are absent from model ecdysozoan *taxa*, and not initially identified in other invertebrate phyla. Thus, studies have genome predicted RARs orthologues in chordates, ambulacrarians, hemichordates and lophotrochozoans, but absent in cnidarians and sponges (Fig. 1.5) (Thornton, 2003; Bertrand et al., 2004; Albalat, 2009; Bridgham et al., 2010). These findings predict that RA signaling pathway mediated by RAR has a much more ancient evolutionary origin, possible already present in the last common ancestor of all bilaterians (Bertrand et al.,

2004; Albalat, 2009). Yet, presence of RAR and/or RXR orthologue in a species does not indicate a vertebrates-like RA-dependent response activity and similar gene expression modulation. Functional characterization is missing for most of the invertebrate phyla, making it hard to establish their evolutionary origin. The available findings predicts that retinoid signaling cascade has evolved differently along metazoan phyla and highlighting an evolutionary plasticity of the retinoid receptors LBD (Gutierrez-Mazariegos et al., 2014a, b). For instance, in ecdysozoa the ligand recognition and binding ability of RXR homologues suffered independently alteration in different *taxa* probably related with the organism physiological process requirement. In some ecdysozoan *taxa* RXR kept the ability to bind 9-*cis*-RA, whereas in other it recognizes and bind to other ligands (Gutierrez-Mazariegos et al., 2014b). In contrast in other protostomes lineages, the lophotrochozoans, it seems that RXR kept the ability to interact with 9-*cis*-RA and to mediate ligand-induced gene transcription activation (Bouton et al., 2005; Castro et al., 2007; Urushitani et al., 2011). Thus, the information available so far come from functional studies with mollusks and the existence of similar pathways in other lineages such as annelids is unknown. Regarding RARs, RARs gene from chordates invertebrates (cephalochordates and tunicates) seem to keep a vertebrate-like ability to bind and be activated by RA isomers, as well as to form heterodimer to RXR (Hisata et al., 1998; Escriva et al., 2002; Fujiwara and Kawamura, 2003; Rinkevich et al., 2007). In contrast, in the mollusks *T. clavigera* and *N. lapillus*, RARs is able to heterodimerize with RXR but are unresponsive to non- and active retinoids (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014a). Mutation in key amino acid residues, of mollusks RAR LBD might be responsible for the loss of its affinity for retinoids (Gutierrez-Mazariegos et al., 2014a).

1.7 Disruption of retinoid pathawys in Invertebrates

Over the years, mankind has witnessed an exponential increase in industry and technology development worldwide, and together with that a growth in chemical synthetic manufacturing also arose. These man-made chemicals include pharmaceutical, agriculture and industrial compounds that became a part of everyday life. Even though they became essential in human life and provide certain benefits in numerous areas, a growing body of evidence suggests that once released in the environment they may represent a serious threat (Hotchkiss et al., 2008; Diamanti-Kandarakis et al., 2009, Castro and Santos, 2014; Trasande et al., 2015; Santos et al., 2016). Indeed, in the past decades, the protection of the environment from contamination, not only by anthropogenic compounds but also by natural occurring, has become a matter of worldwide growing concern (WHO, 2002; Matthiessen, 2003; Hotchkiss et al., 2008). A wide range of environmental contaminants, such as pesticides, detergent residues, organotin

compounds, pharmaceutical products, natural and synthetic hormones, etc., have been identified to be able to impact the environment and cause adversely health effects in humans and wildlife (Colborn et al., 1993; McLachlan, 2001; Matthiessen, 2003; Trasande et al., 2015). Evidence from laboratory and field studies reveals that some of these environmental contaminants are able to interfere with numerous endocrine-related mechanisms and, consequently, affect diverse biological processes (Colborn et al., 1993; McLachlan, 2001; WHO, 2002; Matthiessen, 2003; Hotchkiss et al., 2008; EEA report, 2012; Trasande et al., 2015). These compounds are commonly referred to as endocrine disrupting chemicals (EDCs). They consist on exogenous substance and/or mixtures that causes adverse health effects in an intact organism or its progeny or (sub)populations, subsequent to changes in endocrine function (WHO, 2002). EDCs are able to interfere with endocrine system via acting through receptor-mediated and non-receptor-mediated cascades (WHO, 2002). Through receptor-mediated cascades, mimicking the action of natural hormones and thereby setting off identical chemical reactions (agonistic) or by blocking the receptors (hormone and NRs) in the cells that normally receive the hormones, thereby preventing their action (antagonistic); through non-receptor mediated mechanism, where EDCs can interact with the endocrine system by affecting the synthesis, transport, metabolism and excretion of hormones and other natural molecules (WHO, 2002; Noppe, 2006). A large range of impacts resulting from EDCs exposure have been described and documented. This includes effects on development, reproduction, energy metabolism disorder, increased cancer incidence, nervous and immune system disorders of all major vertebrate groups and some invertebrate phyla, including local population decline and extinction (Colborn et al., 1993; WHO, 2002; Matthiessen, 2003; Grün et al., 2006; Castro et al., 2007; EEA report, 2012; Trasande et al., 2015).

The disruption of the endocrine system in metazoans from terrestrial and aquatic ecosystems has becoming a matter of great concern. However, much attention has been given to aquatic species (Sumpter, 2005). The focus on aquatic organism is due to the fact that they are exposed to more contaminants than terrestrial ones (Sumpter, 2005). Aquatic environments receive most of the chemicals released from anthropogenic activities via direct input from discharge form sewage treatment plants, agricultural and industrial runoff, accidental releases of chemicals spills and atmospheric deposition (Langston et al., 2005; Sumpter, 2005).

Initially, most studies focused on estrogen-mimicking compounds and estrogenic pathways (Colborn et al., 1993; Matthiessen, 2003; Sumpter, 2005). In addition, it is known that EDCs are also capable of interfering and modulating other metabolic and signaling pathways (Janošek et al., 2006; Novák et al., 2008; Hotchkiss et al., 2008; EEA report, 2012; Defo et al., 2014). One of such targets is the retinoid system (Novák et al.,

2008; Inoue et al., 2010). Many studies have focused on the disruption of retinoid pathways in chordates, with other lineages receiving far less attention (Novák et al., 2008; Inoue et al., 2010; Defo et al., 2014), mostly because of the lack of knowledge regarding their metabolic and signaling modules. However, the recent discovery that, other metazoan lineages are able to maintain a retinoid homeostatic control, support the notion of an evolutionary conserved biological role of retinoid metabolism. Simultaneously, the presence of these pathways across metazoan species, even though probably operating under distinct genetic modules, implies that more metazoan species are potential targets for EDCs known to affect retinoid pathways.

Indeed, one of the best-documented examples of endocrine disruption in wildlife is the masculinization of female gastropods by organotin compounds, tributyltin (TBT) and triphenyltin (TPT), a phenomenon termed imposex (Smith, 1971; Bryan et al., 1986; Santos et al., 2000). Imposex in gastropods consists in the superimposition of male secondary sexual characteristics (penis or/and vas deferens) onto females (Bettin et al., 1996). It appears to be an irreversible condition and, in advanced stages, could induce female sterilization, leading to population declines and even local extinctions (Bryan et al., 1986; Schulte-Oehlmann et al., 2000; Sternberg et al., 2010). A growing number of studies have causally linked organotin-induced imposex with an inappropriate modulation of RXR (e.g. Nishikawa et al., 2004; Castro et al., 2007; Horiguchi et al., 2010; Sternberg et al., 2010; Lima et al., 2011; Stange et al., 2012; Abidli et al., 2013). Nishikawa et al. (2004) demonstrated that both TBT and TPT efficiently bind human and *T. clavigera* RXR with high affinity. They also showed that the natural ligand 9-*cis*-RA promotes imposex in gastropod females. In this context, organotins would mimic an endogenous ligand of RXR and thus activate the signaling cascades, which apparently are RA dependent. This hypothesis was confirmed with *N. lapillus*, where injections of 9-*cis*-RA and a selective RXR agonist, methoprene acid, induced imposex (Castro et al., 2007), thus reinforcing the hypothesis of an RXR-mediated induction of imposex by TBT. Stange et al. (2012) further supported these finding reporting that TBT and the RXR agonist HX630 induced imposex in female *N. lapillus*. RXR gene transcription is also altered after TBT and TPT-exposure in a tissue and sex-specific manner (Horiguchi et al., 2010; Lima et al., 2011; Abidli et al., 2013). At advanced stages of imposex RXR is highly expressed in female penis, which displayed transcription patterns identical to those of males, therefore pointing to a functional role of RXR in penis development of both genders (Lima et al., 2011; Abidli et al., 2013). Although the exact cascade of events that leads to imposex induction is not fully understood, the available evidences suggest that retinoids could have a major role not only locally in imposex/male penis development, but also at the CNS level (Lima et al., 2011).

Recent findings also indicate that organotin compounds are potential obesogens (e.g. Inadera and Shimomura, 2005; Kanayama et al., 2005; Grün et al., 2006; Janer et al., 2007; Santos et al., 2012; Lyssimachou et al., 2015). Apart from RXR, TBT compounds also serve as high affinity ligands to peroxisome proliferator-activated receptor gamma (PPARγ), activating the PPARγ/RXR pathway (Kanayama et al., 2005). The PPARγ/RXR heterodimer is a ‘‘master regulator’’ of adipocyte differentiation, lipid storage and lipid metabolism in vertebrates (Santos et al., 2012). Several studies have demonstrated that TBT induces adipocyte differentiation and lipid accumulation in 3T3-L1 cells, and increases lipid accumulation in newborn rats exposed to TBT, through modulation of the PPARγ/RXR signaling pathway (Inadera and Shimomura, 2005; Kanayama et al., 2005; Grün et al., 2006). In invertebrates, TBT also seems to disturb fatty acid homeostasis and enhance lipid accumulation, but the underlying mechanisms are still unknown (Janer et al., 2007; Jordão et al., 2015).

TBT-sensitive RXR signaling pathways have also been reported in other invertebrate groups. For instance, in crustaceans, TBT seems to interact with RXR and negatively affect EcR/RXR-dependent gene expression. In *D. magna*, RXR is activated by TBT whereas the EcR/RXR heterodimer is synergistically activated only when exposed to combinations of TBT and 20-hydroxyecdysone, both *in vitro* and *in vivo* (Wang and LeBlanc, 2009; Wang et al., 2011). Exposure to TBT and high concentrations of 20-hydroxyecdysone led to incomplete molting and increased mortality; aggravating the effect of either ligand alone (Wang et al., 2011). In the shrimp *C. crangon*, *in vivo* acute exposure to TBT prompted EcR/RXR transcript downregulation, notably in the ovaries. Also, *in vitro* EcR/RXR transactivation was reduced upon TBT exposure (Verhaegen et al., 2011). These effects were suggested to derive from TBT binding to the ligand binding pocket of crustacean RXR, as supported by *in silico* analysis (Verhaegen et al., 2011).

In addition to receptor-mediated disruption, vertebrate retinoid metabolism can also serve as target of EDCs (Novák et al., 2008; Berghe et al., 2013). For instance, the 2,3,7,8-Tetrachlorodibenzo *p*-dioxin (TCDD) seems to affect retinoid metabolism by diminishing REs tissue levels through modulation of LRAT-dependent ROL esterification (Nilsson et al., 1996, 2000). Adult frog *Rana temporaria* exposed to 1,1-dichloro-2,2-bis(p-chloro-phenyl)ethene (*p,p*-DDE) exhibited a reduction of hepatic CYP26 mRNA expression in a dose-specific manner. This led to a reduction of CYP26 enzymatic activity and hepatic ROL metabolism imbalance (Leiva-Presa et al., 2006).

Still, it is presently unclear if invertebrate retinoid homeostasis is also a target of EDCs, as seen for their vertebrate counterparts. A very limited number of studies addressed this issue. ADH3 expression, in the ascidian *C. intestinalis*, has been shown to be down regulated by TBT (Azumi et al., 2004). However, no similar effects were

observed in the gastropod *N. lapillus* (Coelho et al., 2012). Interestingly, retinoid storage mechanisms, in the form of REs, were suggested to be absent in imposex-susceptible gastropods (*N. lapillus* and *N. reticulatus*). This raises a fundamental question linking RE-related retinoid homeostasis and differential sensitivities towards retinoid signaling disruptors, such as TBT (Gesto et al., 2013). Clearly, the lack of knowledge on the endocrine role of key metabolic and signaling components is a limitation to our understanding of the threat EDCs pose on invertebrates.

Overall, from an ecotoxicological standpoint, studies with invertebrates (mostly mollusks) have just focused on the effects of EDCs, mostly organotin compounds, in the retinoid signaling pathway. The effects of EDCs in retinoid metabolic pathways have not been addressed and further studies should focus on such aspects. Once retinoid metabolic and signaling pathways are established our understanding of the adverse effects of EDCs on invertebrates will be significantly improved.

1.8 Model species

Lophotrochozoans have retained several key molecular features of the retinoic metabolic and signaling cascade (Fig. 1.5) including those though to be chordate specific innovations (Albalat, 2009; Theodosiou et al., 2010). Therefore, lophotrochozoans are good model organisms to study the evolutionary origin of retinoid pathway and to address the issue of their modulation by EDCs. We selected for the present study lophotrochozoan species representatives of two different phyla, mollusks and annelids (Fig. 1.6), which are present in intertidal rocky shores along the Portuguese coast.

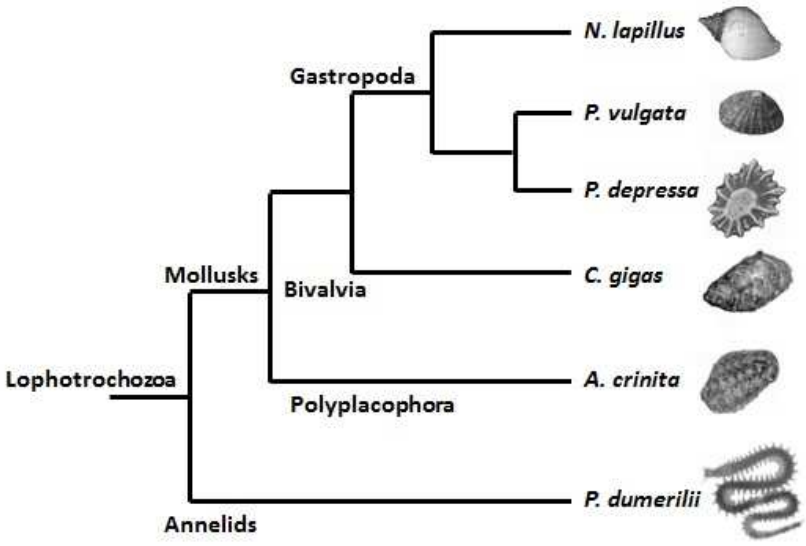


Figure 1.6: Phylogenetic relationship of the selected Lophotrochozoan species (based on Kocot et al., 2011). Mollusks picture were retrieved and modified from www.aphomarine.com, whereas the annelid picture was retrieve and modified from Barford, 2013 (Doi:10.1038/nature.2013.13833).

1.8.1 *Nucella lapillus*

The dogwhelk *N. lapillus* (Linnaeus, 1758) (class gastropoda, family Muricidae) is a common gastropod of the rocky shores (intertidal) that can be found throughout the North Atlantic, from northern Russia to Portugal, Iceland and Greenland, and from southern Newfoundland to the New York region (Gibbs, 1999). It is a carnivore species that feed almost exclusively on barnacles and mussels, but also on other mollusks by drilling the shell, using combined radula scraping and enzymatic-chemical dissolution (Feare, 1970; Crothers 1974, 1985). Reproductions in this gastropod species occur throughout much of the year, with their breeding season being accompanied by an aggregative behavior (Gibbs, 1999). The sexes are separated and the fertilization is internal. It's easy to determine the gender in the living snails without removing it from the shell; being the males recognize by the presence of a penis in the head behind the right tentacle, and the females by the absence of this structure.

N. lapillus is an excellent candidate model species to study the impact of EDCs in retinoid signaling for the following reasons: 1) its wide distribution along the northern Portuguese coast and easy maintenance under laboratory conditions; 2) previous molecular and biochemical studies revealed the presence of the retinoid cascade in this gastropod by isolating important retinoid metabolic (ADH3 and CYP26) and signaling molecular components (RAR and RXR) as well as detection of endogenous retinoids; and additionally 3) it is very sensitive to low levels of the RXR agonist TBT, developing imposex at the low ng/L range (Castro et al., 2007; Lima et al., 2011; Coelho et al., 2012; Gesto et al., 2013).

1.8.2 *Patella vulgata* and *Patella depressa*

The limpet *P. vulgata* (Linnaeus, 1758) and *P. depressa* (Pennat, 1777) (class gastropoda, family Patellidae) are grazing mollusks, whose geographical distribution extends from North Africa, along the Atlantic coasts of Europe to southwest England and Wales (Orton and Southward 1961; Guerra and Gaudêncio 1986). In Portugal they can be founded along the entire coast distributed over all the intertidal rocky shores. Like other limpet species, they play a fundamental ecological role in controlling algae communities on the shore through their grazing feeding activity (Guerra and Gaudêncio, 1986; dos Santos, 1994; Boaventura et al., 2002a, b; Silva et al., 2003).

In these two species the sexes are separated and the fertilization is external, with the reproductive cycle being largely influenced by environmental conditions (e.g. wave action and weather) (Guerra and Gaudêncio 1986; Brazão et al., 2003). *P. depressa* displayed almost continual gonad maturation, with two main spawning periods, September/January and March/June, and minor spawning events dispersed throughout

the year (Ribeiro et al., 2009). In contrast, *P. vulgata* has a highly synchronized reproductive cycle, with well defined breeding and resting periods with gonad development, along the Portuguese continental coast, starting in September and spawning taking place from November/December to March (Ribeiro et al., 2009). Limpets spawn resulting in the release of gametes (eggs and sperm) directly into the water. After a period in the plankton, the short-lived free-swimming planktotrophic veliger larvae, settle lower on the shore or in damp crevices. As they grow, they move up shore inhabiting different shore levels (Guerra and Gaudêncio 1986). The choice of these species for the isolation and functional characterization of retinoid metabolic and signaling modules is due to the recent report that both species display the ability to maintain a retinoid homeostatic control through REs storage and the genome prediction of another limp, *L. gigantea*, of several retinoid components (Albalat, 2009; Gesto et al., 2013).

1.8.3 *Crassostrea gigas*

The pacific oyster *C. gigas* (Thunberg, 1793) is a mollusk species that belongs to the class bivalve (order: Ostreoida and Family: Ostreidae). This oyster species is native to Japan, but has been successfully introduced in many parts of the world mostly for aquaculture purposes (FAO 2012). *C. gigas* is a lamellibranch filter feeder consuming planktonic organisms and detritus from the surrounding water (Harris, 2008; Troost, 2010). The oyster is a marine and estuarine species which occurs in water of 0 to 40 m depth; it lives attached on both hard and soft substrates along exposed shores and form reef structures on tidal flats (Harris, 2008; Troost, 2010; FAO 2012). *C. gigas* are oviparous protandrous hermaphrodite species; initially develop as males and after their first year they can mature as either male or female and can spawn annually in warm months (Harris, 2008).

We selected this species due to its position in the Mollusca phylum and the available genome indicates the presence of a nuclear receptor conserved repertoire (Vogeler et al., 2014). We chose the selected species to study if RAR in bivalves' class might have conserved a functional role on gene regulation similar to that described in vertebrate.

1.8.4 *Acanthochitona crinita*

Acanthochitona crinita (Pennant, 1777) is a mollusk that belongs to the polyplacophora (order Neoloricata family and Acanthochitonidae). This mollusks species have is body only protected by a shell composed of eight interlocked transverse, coarsely keeled plates or valves (Ruppert et al., 2004; Bonfitto et al., 2011). The polyplacophora are likely the most basal group and closely related to the molluscan ancestor (Ruppert et

al., 2004; Kocot et al., 2011). This species can be found in North American Atlantic coast and the eastern Atlantic along the European coast from Norway to Azores, Madeira, Canary and Cape Verde Islands (Bonfitto et al., 2011). In Portugal can be found in the south and northern coast (dos Santos, 1994). The species is very common in the lower shore or shallow subtidal areas but can occur to a depth of 50 to 175 m (Bonfitto et al., 2011). Lives on the underside of rocks and boulders usually lightly embedded in sand or gravel (Gowlett-Holmes 1998; Bonfitto et al., 2011). It may be associated with the barnacle zone or with several kinds of algae (Mather and Bennett, 1993; Gladfelter 1988; Gowlette-Holmes 2001; Bonfitto et al., 2011). It feeds by grazing material from the rock surface using its radula (Ruppert et al., 2004). Like almost other all polyplacophorans chitons are dioecious (separate sexes), it reproduces through external fertilization and has indirect development passing through larva stages (Kaas et al., 1998; Ruppert et al., 2004).

We selected this species due to its key position on the mollusk evolution closed to the last common ancestral of all mollusks.

1.8.5 *Platynereis dumerilii*

P. dumerilii (Audouin and Milne Edwards, 1834) (class Polychaeta, family Nereididae) is a marine annelid widely distributed all over the world from temperate to tropical seas (Hay et al., 1988; Fischer and Dorresteijn, 2004). In Portugal this species is widely distributed along the northern coastal areas (dos Santos, 1994). This worm is quite common in the intertidal zone of rocky substrate, lying mainly in the lower eulittoral and littoral fringe, living in mucous tubes attached to algae, especially laminaria species (Hay et al. 1988; dos Santos 1994; Giangrande et al., 2002).

The life-cycle of *P. dumerilii* comprises three major larval developmental stages, the trochophore, the metatrochophore and the nectochaete; and two adult stages: the atkom, sexually immature young adult, and epitoke, sexually mature adult (Dorresteijn, 1990). The life-cycle ends in a brief sexual maturation stage and reproductive event (Fischer and Dorresteijn, 2004).

P. dumerilii is an emerging model organism, widely used in various fields of research such as molecular development, evolution, neurobiology, ecology and ecotoxicology. (e.g. Hutchinson et al., 1995; 1998; Hagger, et al., 2002; Raible et al., 2005; Denes et al., 2007; Tessmar-Raible, 2007; Kerner et al., 2009; Fischer et al., 2010). It is an excellent model for ecotoxicological studies because it displays a short life-cycle, an indirect development; their embryos and larvae are transparent; additionally they are easily maintained in the laboratory (Dorresteijn, 1990; Hutchinson et al., 1995; Hagger et al., 2002; Dean, 2008; Fischer et al., 2010). It is a relevant model organism to study

ancestral characteristics in comparison with bilateral deuterostomes, since they seem to have retained many ancestral characteristics in contrast with ecdyzoan model species (Tessmar-Raible and Arendt, 2004; Raible et al., 2005; Denes et al., 2007; Tessmar-Raible, 2007; Fischer et al., 2010).

1.9 Objectives

Given that there is still a current lack of knowledge of invertebrate's endocrinology in regard to the retinoid system, one major aim of this thesis is to elucidate about the presence and functional characterization of key molecular components of retinoid in an evolutionary perspective. To study retinoid signaling pathway's modulation by EDCs in marine lophotrochozoans is also a major goal of this PhD thesis. To achieve the main goals of the present work the following specific objectives were established:

1. To isolate and functional characterize the orthologues of vertebrate-like components of the retinoid metabolic route, i.e., the BCO, DGAT1 and ALDHs, in selected lophotrochozoans.

2. To provide evidences that the retinoid system is present in mollusks and it regulates key physiological functions. We address the involvement of retinoids in the gonad maturation regulation process in the marine gastropod *Patella vulgata*.

3. Address if the loss of vertebrate-like function of RAR orthologues in mollusks is conserved along clades, and get insights on the mode of action of RAR/RXR heterodimer. To evaluate the modulation of mollusks RAR and RAR/RXR heterodimer by EDCs.

4. To isolate a RXR orthologue in the annelid *Platynereis dumerilii* and address the possible interference of organotin compounds, TBT and TPT, on RXR-dependent signaling pathway. We aimed to provide evidences to support the hypothesis that RXR modulation by certain EDCs might be conserved across metazoans.

5. Strengthen the previous assumption that the imposex induction by organotin compounds in gastropods is associated with the modulation of RXR-dependent signaling cascade.

The objectives of this thesis are explored through the following chapters. Some of the chapters are already published in International Journals and others are being prepared for publication, as indicated below:

Chapter 1: General introduction
Retinoid metabolism in invertebrates: When evolution meets endocrine disruption. André, A., Ruivo, R., Gesto, M., Castro, F.L.C., Santos, M.M., 2014. Gen. Comp. Endocrinol., 208, 134-145. DOI: 10.1016/j.ygcen.2014.08.005 (adapted from).

Chapter 2: Isolation and preliminary functional characterization of a BCO-like gene orthologue in the marine annelid *Platynereis dumerilii*. André, A., Castro, F.L.C., Santos, M.M. (in preparation).

Chapter 3: Diacylglycerol O-acyltransferase 1 (DGAT1) in lophotrochozoans: insights into the mechanisms of retinol esterification and storage. André, A., Santos, M.M., Castro, F.L.C. (in preparation).

Chapter 4: Aldehyde dehydrogenase type 1 enzyme in lophotrochozoans and their implication in retinoic acid synthesis: an evolutionary perspective. André, A., Ruivo, R., Castro, F.L.C., Santos, M.M. (in preparation).

Chapter 5: Retinoid level dynamics during gonad recycling in the limpet *Patella vulgata*. Gesto, M., Ruivo, R., Páscoa, I., André, A., Castro, F.L.C., Santos M.M., 2016. Gen. Comp. Endocrinol., 225, 142-148. DOI: 10.1016/j.ygcen.2015.10.017 (adapted from).

Chapter 6: The Retinoic acid receptor (RAR) in mollusks: function, evolution and endocrine disruption insights. André, A., Ruivo, R., Fonseca, E., Castro, F.L.C., Santos M.M. (in preparation).

Chapter 7: Cloning and functional characterization of a retinoid X receptor orthologue in *Platynereis dumerilii*: insights on evolution and toxicology. André, A., Ruivo, R., Páscoa, I., Castro, F.L.C., Santos M.M. (submitted).

Chapter 8: *Nucella lapillus* retinoid X receptors isoforms activate transcription of reporter genes in response to imposex-induction compounds. André, A., Ruivo, R., Castro, F.L.C., Santos M.M. (in preparation).

Chapter 9: General discussion, Conclusion and future perspectives

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CHAPTER 2

2 Isolation and preliminary functional characterization of a BCO-like gene orthologue in the marine annelid *Platynereis dumerilii*

2.1 Abstract

Here we report the isolation and functional characterization of a cDNA encoding β-carotene cleavage enzyme like from the BCO family in the marine lophotrochozoan Polychaeta *Platynereis dumerilii*. We provided a phylogenetic analysis that revealed that the isolated BCO protein from *P. dumerilii* holds the key amino acid for enzymatic function characteristic of BCO family members. Also we performed a partial functional characterization that demonstrates the ability of the enzyme to use and cleave β-carotene into active retinoid synthesis precursors. Even though we have been not able to determine the metabolization products of this enzyme, the preliminary data presented here indicates that in annelids a pathway for retinoid synthesis through carotenoids cleavage is present and active. Altogether, the data reinforces the hypothesis of the existence of alternative ancestral retinoid biosynthesis pathways with a basal origin that is conserved throughout metazoans, including lophotrochozoans.

2.2 Introduction

Vitamin A (or retinol) and their natural occurring derivatives, the so-called retinoids, are fat-soluble organic components essential to maintain physiological functions in vertebrates, such as vision, immune responses, reproduction and embryonic development (Blomhoff and Blomhoff, 2006). The diverse functions of retinoids are carried out by the active metabolite retinoic acid (RA) that generally act as a ligand for nuclear retinoic acid receptors (RARs) and/or retinoid X receptors (RXRs) (Theodosiou et al., 2010; André et al., 2014). Inadequate levels (excess or lack) of retinoids, is well known to cause several deleterious effects in humans and animals, suggesting that a careful balance of retinoids levels is require (Blomhoff and Blomhoff, 2006). Despite nutritional importance and biological significance, in general animals lack the ability of endogenously synthesized retinoids *de novo* (Novák et al., 2008). The only source of retinoids is diet-derived. Thus, vitamin A requirements can be satisfied directly from diet with retinol (ROL) or retinyl ester (REs) (mainly retinyl palmitate) forms; or by ingestion of pro-vitamin A carotenoids rich vegetables and fruits (Krinsky and Johnson, 2005; Blomhoff and Blomhoff, 2006).

Regarding carotenoids, the β-carotene (βc) is the most abundant in the diet, blood, and tissues and the main precursor for retinoid synthesis in vertebrates (von Lintig, 2010). Once absorbed into the body βc can be oxidative cleaved by means of two protein members of non-heme iron–containing oxygenases family protein (Fig. 2.1): the β-carotene 15, 15′-monooxygenase (BCO-I or BCMO1) and β-carotene 9′, 10′-dioxygenase

(BCO-II or BCDO2) (Kiefer et al., 2001; Lindqvist and Andersson, 2002; von Lintig, 2010). The BCO-I is a cytosolic enzyme that symmetrically cleaves βc at the 15, 15′ carbon double bond given rise to two molecules of retinaldehyde (retinal or RAL), which is further reversibly reduced to ROL or irreversibly oxidized to RA (Lindqvist and Andersson, 2002; Duester et al., 2003). In contrast, the enzyme BCO-II is localized in the mitochondria and asymmetrical cleaves βc at the 9', 10' carbon double bond to yield β-apocarotenals and β-ionone (Fig. 2.1). Then, through a stepwise process involving enzymes whose identity remains unknown, β-apocarotenal is converted to β-apocarotenoic acid that is further oxidized to RA (Kiefer et al., 2001; Amengual et al., 2011).

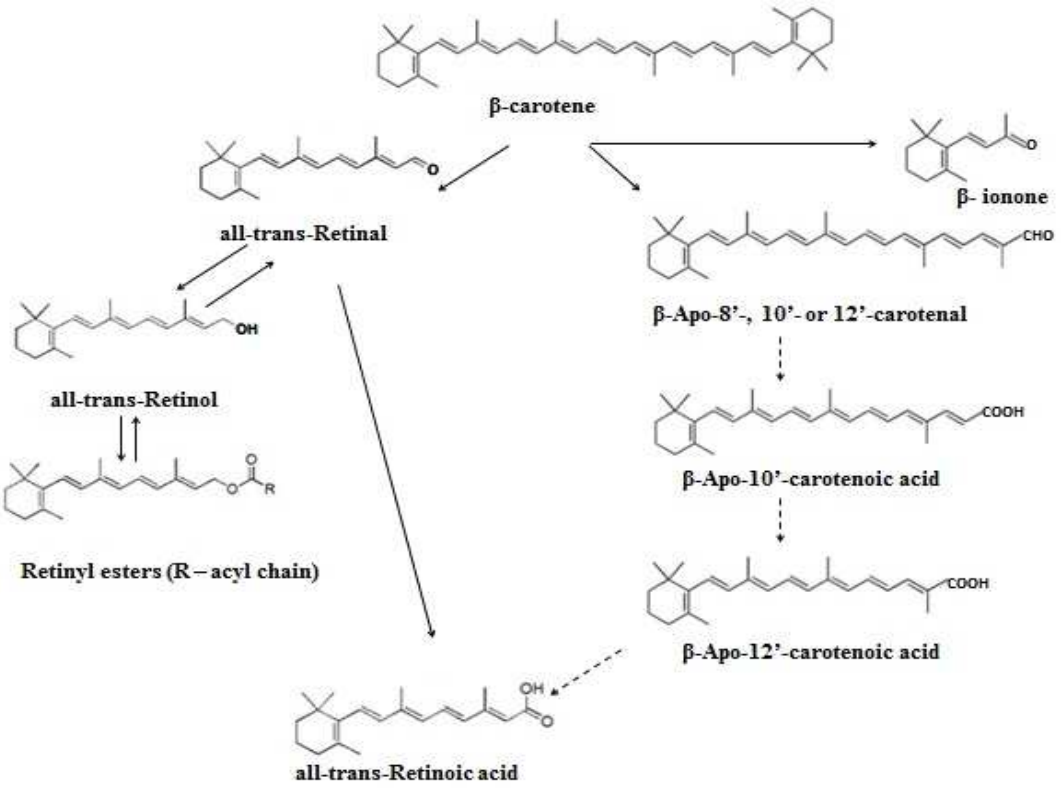

Figure 2.1: Schematic overview of vertebrate β-carotene and retinoid metabolism (modified from Kiefer et al., 2001 and Bachmann et al., 2002).

In addition of cleaving βc, both the enzymes are also capable to cleave other non- and pro-vitamin A carotenoids. BCO-I is also capable of catalyzing the symmetric cleavage of carotenoids with an un-substituted β-ionone ring, such as α-carotene and β-cryptoxanthin (Redmond et al., 2001; Lindqvist and Andersson, 2002). BCO-II enzyme also catalyzes the asymmetric cleavage of xanthophylls (e.g. zeaxanthin and lutein) and *cis*-lycopene (Kiefer et al., 2001; Hu et al., 2006; Amengual et al., 2011; Wang, 2012). The retinal pigment epithelium protein of 65 kDa (RPE65) also belongs to the same protein

family, although it is not implicated in carotenoids metabolism. Instead this last protein catalyzes the hydrolysis and isomerization of all-*trans*-REs to 11-*cis*-ROL for photo-pigment regeneration in the vision cycle (Mata et al., 2004; Jin et al., 2005). BCOs and RPE65 might have different function at retinoid metabolism though they share similar amino acid sequence and having evolved from a common ancestor (Wyss, 2004).

The asymmetrical cleavage of carotenoids has been discussed as an alternative route for vitamin A or RA production in the absence of the “classical retinoid pathway” (Simões-Costa et al., 2008; von Lintig, 2010). However, it seems that at least in mammals the BCO-I and not BCO-II has a predominant role in retinoids synthesis and homeostasis (Hessel et al., 2007). BCO-/- mice developed normally and are fertile. When maintain on a diet supplemented with βc, BCO-/- mice accumulated the carotenoid in large amounts in many tissues accompanied by a decrease in vitamin A levels even in the presence of BCO-II, suggesting a different physiological role for this enzyme (Hessel et al., 2007). Indeed, it has been demonstrated that BCO-II seems to play a role to maintain carotenoid homeostasis by metabolizing both carotenes and xanthophylls to apocarotenoid breakdown products, preventing against carotenoid accumulation in mitochondria that can result in oxidative stress and disease (Amengual et al., 2011).

The carotenoids oxidative cleavage might be an ancient pathway for retinoid synthesis with a basal evolutionary origin (Simões-Costa et al., 2008; Theodosiou et al., 2010). The presence of members of the BCO family has been reported in several metazoan species from Porifera to deuterostomes phyla (Theodosiou et al., 2010; Müller et al., 2012). For instance putative BCO family members have been *in silico* identified in the genome and/or isolated in the echinoderm *Strongylocentrotus purpuratus*, ecdysozoan *Drosophila melanogaster*, in two lophotrochozoans, *Lottia gigantea* and *Capitella sp*, and in the cnidarian *Nematostella vectensis* (Theodosiou et al., 2010). In addition, their isolation and functional characterization were performed in invertebrate species such as in the sponge *Suberites domuncula*, the tunicate *Ciona intestinalis*, and the insect *D. melanogaster* (von Lintig and Vogt, 2000; Müller et al., 2012; Poliakov et al., 2012). These evidence clearly points that retinoid synthesis through BCO might be present in all metazoans, representing an ancient pathway. However, their isolation and functional characterization were performed in a reduced number of invertebrate species and consequentiality it is missing for most metazoan phyla. For lophotrochozoan for instance, their presence was only reported in the genome and no isolation or functional characterization was performed to date.

Therefore, in the present study we aimed to provide evidences for the presence of an active pathway for retinoid synthesis through βc cleavage in protostome species. We

used as model species the annelid *Platynereis dumerilii*; in order to gain further insights into the evolutionary origin of the retinoid synthesis pathway in lophotrochozoans.

2.3 Material and Methods

2.3.1 Chemical compounds

All-*trans*-RAL (≥ 98%) and trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich. Methanol and *n*-hexane gradient grade for High performance liquid chromatography (HPLC) was purchased from VWR-Prolabo.

2.3.2 BCO isolation

Total RNA was extracted from a tissue mix of head and body of an sexually immature specimen using the Kit illustra RNAspin Mini RNA Isolation (GE Healthcare) following the manufacturer's instructions (animal tissues protocol), with on column DNAse I digestion. The integrity of isolated total RNA was confirmed by electrophoresis and concentrations were determined spectrophotometrically by absorbance at 260 nm and purity by the 260 / 280 nm ratio. The cDNA synthesis was performed with the iScript™cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions, using 1 µg of total RNA.

A set of degenerate primers (Table 2.1 and Fig. 2.2) were designed based on the highly conserved regions from BCO related gene of two lophotrochozoan species, *Capitella teleta* and *Lottia gigantea*, (Genome Portal database (JGI); http://genome.jgi.doe.gov/; accession numbers 20779 and 179518 respectively; Grigoriev et al., 2012), and from a mammal species, *Rattus norvegicus*, (National Center for Biotechnology Information (NCBI) GenBank; http://www.ncbi.nlm.nih.gov/; accession number EDL92648.1; Geer et al., 2010; Benson et al., 2013), using the software Consensus-degenerate hybrid–oligonucleotide primers (CODEHOP; http:// blocks.fhcrc.org/ codehop.html; Rose et al., 2003).

Table 2.1: Sequences of the primers used in this study.

Primer designation	Sequence 5'- 3'	Use
P1	CCACTCCTTCGGCATGacngaraayta	Degenerated PCR
P2	TGTGGCCGTCCATCTCRTANGCRTT	Degenerated PCR
P3	GAGCAACCTCTGGCCGTGAATGTC	RACE PCR
P4	TCGGCACCATGGCCtayccngaycc	Degenered + nested PCR
P5	GGATTTTGCCCGTTGCCTTCTCA	Degenered PCR
P6	TCCAGCGATTTGCAGAATGTTGAAT	Degenered nested PCR
P7	GAGTGGCTTTTCCTGGTTGAGAACG	RACE PCR
P8	TGCAACCATGACTTCTGGAG	ORF PCR
P9	TCTTAAAACAACTGTGCCAAATAATTT	ORF PCR

The polymerase chain reaction (PCR) was performed with NzyTaq Colourless Master Mix (Nzytech), using 100 ng cDNA template, 1.25 mM of Magnesium chloride ($MgCl_2$) and 0.4 µM primers at a final 25 µL reaction volume. Cycle parameters included an initial denaturation at 96ºC for 5 minutes (min), followed by 45 cycles of denaturation at 94ºC for 15 seconds (s), annealing at 55ºC for 5 s and extension at 72ºC for 15 s/1 Kb, and a final extension at 72ºC for 1 min. PCR products were then loaded onto 1% agarose gel stained with GelRed and run in 1 x Tris-acetate-EDTA (TAE) buffer at 90 Volt (V). Bands of the proper size were isolated and purified with illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and directly send for sequencing (Stabvida).

The full-length BCO cDNA was obtained by rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA amplification kit (Clontech) following the manufacturer's instructions. Briefly, RACE cDNA was prepared using 1 µg of total RNA. Gene specific primers were design using the software Primer3 (v.0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/; Koressaar and Remm, 2007; Untergasser et al., 2012) following the SMART RACE cDNA amplification kit specifications. RACEs-PCR was performed using Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) and with a specific 10 x Universal Primer A Mix (UPM) kit provided. For the 3' end a single PCR reaction was conducted using a forward specific oligo-primer (P3; Table 2.1) with cycle parameters that included an initial denaturation at 98ºC for 10 s, followed by 40 cycles of denaturation at 98ºC for 1 s, annealing at 65ºC for 5 s and extension at 72ºC for 15 s/ 1 Kb, and a final extension at 72ºC for 1 min. In the case of 5' end RACE we used a different methodology since it was difficult to obtain. In a first PCR the combination in reaction of a RACE reverse specific oligo-primer (P5; Table 2.1 and Fig. 2.2) with a degenerate forward oligo-primer (P4; Table 2.1 and Fig. 2.2) was used followed by nested PCR with other RACE reverse specific primer (P6; Table 2.1 and Fig. 2.2). The first PCR cycle parameters included an initial denaturation at 98ºC for 10 s,

followed by 40 cycles of denaturation at 98ºC for 1 s, annealing at 53ºC for 5 s and extension at 72ºC for 15 s/ 1 Kb, and a final extension at 72ºC for 1 min. Next, for hemi-nested cycle parameters included an initial denaturation at 98ºC for 10 s, followed by 40 cycles of denaturation at 98ºC for 1 s, annealing at 53ºC for 5 s and extension at 72ºC for 15 s/ 1 Kb, and a final extension at 72ºC for 1 min. Then with the obtain sequence another reverse primer (P7; Table 2.1 and Fig. 2.2) was design and used for a PCR in the following conditions: an initial denaturation at 98ºC for 10 s, followed by 40 cycles of denaturation at 98ºC for 1 s, annealing at 65ºC for 5 s and extension at 72ºC for 15 s/ 1 Kb, and a final extension at 72ºC for 1 min. All the bands of the proper size obtain were isolated and purified with illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and directly sequenced (Stabvida).

Next, the 5' and 3' sequences were used for primers designed to amplify full open reading frames (ORFs) (Table 2.1 and Fig. 2.2). PCR was performed using Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific), 100 ng of cDNA template and 0.5 µM primers concentration to a final 20 µL volume of reaction mixture. PCR parameters were as follows: initial denaturation at 98ºC for 10 s, followed by 40 cycles of denaturation at 98ºC for 1 s, annealing at 50ºC for 5 s and elongation at 72ºC for 25 s, and a final step of elongation at 72ºC for 1 min. PCR product were then loaded onto 1.2% agarose gel. Bands of the correct base pairs size were cut and purified with illustra GFX PCR DNA and Gel Band Purification Kit and directly sequenced (Stabvida).

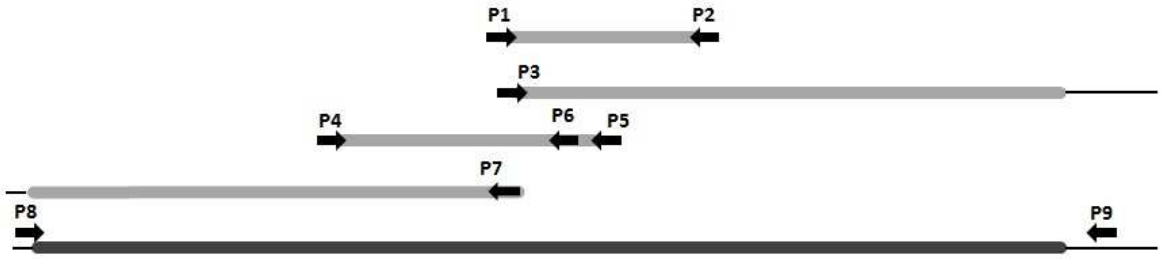

Figure 2.2: Schematic representation of the oligonucleotide primers strategy followed to isolate the complete cDNA coding sequence of BCO-like gene in the polychaeta *P. dumerilii*.

The coding sequence within the full length cDNA was identified based upon alignments with known full length BCO sequences derived from NCBI GenBank and JGI from related species. The molecular weight and amino acid sequence for the PdBCO protein was deduced using ExPASy bioinformatics resource software (http://www.expasy.org/; Artimo et al., 2012).

2.3.3 Phylogenetic analysis

The BCO-I and BCO-II protein and/ or nucleotide sequences from vertebrate and invertebrate species were retrieved from the NCBI databases (http://blast.ncbi.nlm.nih.gov/; Geer et al., 2010; Benson et al., 2013) and JGI Genome Portal (http://genome.jgi.doe.gov/; Grigoriev et al., 2012), via tBLASTn and BLASTp searches: *Homo sapiens* (Hs) BCO-I NP_059125.2, Hs BCO-IIa NP_114144.4, *Mus musculus* (Mm) BCO-Ia NP_067461.2, Mm BCO-Ib NP_001156500.1, Mm BCO-IIa NP_573480.1, *R. norvegicus* (Rn) BCOI NP_446100.2, Rn BCOII NP_001121184.1, *Danio rerio* (Dr) BCOI NP_956902.1, Dr BCOII1 NP_001035402.1, *Branchiostoma floridae* (Bf) BCOa XP_002604370.1, Bf BCOb XP_002605663.1, *Nematostella vectensis* (Nv) BCO XP_001620941.1, *L. gigantea* (Lg) BCOa XP_009055841.1, Lg BCOb 209535, *C. teleta* (Ct) BCOa JGI_212572, Ct BCOb JGI_20779.

A BCO-like from cnidarian *N. vectensis* was chosen to root the tree given the species basal position in metazoan evolution. Amino acid sequences were aligned with Clustal Omega online available software (http://www.ebi.ac.uk/Tools; Sievers et al., 2011) using default parameters. Neighbor-Joining phylogenetic analysis method (Saitou and Nei, 1987), with 1000 bootstrap replicates was performed using the MEGA 5 software (Felsenstein, 1985; Tamura et al., 2011). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965). The analysis involved 17 amino acid sequences.

2.3.4 *In vivo* assay for assess the *P. dumerilii* BCO-like enzymatic activity

To functional characterized PdBCO encoded enzyme we followed the previous published methodology by von Lintig and Vogt (2000) and Redmond et al. (2001) with some modifications. The plasmid for βc production and accumulation in *Escherichia coli* strains was kindly provided by Dr. Johannes von Lintig from the Department of Pharmacology, Case Western Reserve University (Cleveland, Ohio). Colonies of *E. coli* strain transformed with βc plasmid becomes orange-yellow due to *de novo* subtract synthesis. A color shift to white is expected upon expressing of a vector with cDNA coding for a metazoan βc cleaving enzyme meaning that the resultant strain became able to synthesize RAL or β-apocarotenal (von Lintig and Vogt, 2000).

A PCR was performed to obtain the complete ORF for ligation into pBAD-TOPO expression vector (Invitrogen). PCR was performed using Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific), 100 ng of cDNA template and 0.5 µM primers concentration to a final 20 µL volume of reaction mixture. The PCR cycle parameters were as follows: initial denaturation at 98ºC for 10 s, followed by 35 cycles of denaturation at 98ºC for 1 s, annealing for 5 s and elongation at 72ºC for 25 s, and a final

step of elongation at 72ºC for 1 min. PCR product obtained were isolated after separating on a 1.2% agarose gel and purified. A reaction with NZYTaq 2x Green Master Mix (Nzytech) at 70ºC for 30 min was performed to add 3' A-overhangs to the purified PCR product. The resultant product was directly ligated into the pBAD-TOPO expression vector using the TOPO-TA cloning kit (Invitrogen) and transformed into the TOPO 10 competent *E. coli* strain (Invitrogen). The ligation and transformation methods followed the manufacturer's protocol. Positive colonies were identified by PCR using pBAD-TOPO specific primers. A single positive colony was grown overnight at 37ºC with shaking in Luria-Bertani (LB) liquid medium contain ampicillin (100 µg/mL). Recombinant plasmid were isolated by NZYMiniprep (Nzytech) according the manufacturer's instructions and send for sequencing (GATC) to check for mutation and correct orientation of insert.

pBAD/PdBCO, pBAD/HsBCO-I (positive control), and pBAD/LacZ (negative control; Invitrogen) expression constructs were transformed into *E. coli* JM109 competent cells (Promega) together with the βc plasmid which makes the bacteria strain able to produces and accumulates βc. Bacteria were plated on LB agar with 100 µg/mL of ampicillin, 35 µg/mL of chloramphenicol and protein expression was induced with 0.5% L-arabinose (w/v). Bacteria were left to grow in the darkness at 28ºC for 48 h. Cultures of βc transformed *E. coli* cells with constructs were grown in 5 mL of LB broth supplemented with 100 µg/mL ampicillin and 35 µg/mL chloramphenicol at 28℃ over-night (~ 16 h) in darkness with shaking (250 rpm). One mL of the overnight culture was used to inoculate 50 mL of LB broth supplemented with the same antibiotics in 250 mL flasks. The culture was allowed to grow at 28℃ to mid-log phase (A_{600} ~ 0.4-0.6) and protein expression was induced with 0.5% (w/v) L-(+)-arabinose for 48 h in darkness with shaking. LB growth medium for each construct were split into 5 aliquots of 10 mL and the cells were collected by centrifugation at 4ºC, 4000 rpm for 20 min. Pellets were immediately frozen at -80℃ for up to 1 week before extraction.

To extract RAL, only 4 of the 5 aliquots were used, and for each the pellet was re-suspended with 200 µL of 6 M formaldehyde by vigorous vortexing, and incubated for 2 min at 30℃ followed by 2 mL of dichloromethane add ition. Then, RAL was extracted three times with 4 mL of *n*-hexane. Between *n*-hexane extractions and to facilitate the RAL available to organic phase the samples were vortexed and centrifuge at 4ºC for 3 min to 3000 rpm. The collected organic phases for the 4 aliquots of the same construct were put in a single tube and dried under a stream of atmosphere of nitrogen (N_2), and the resultant pellet were dissolved in 100 µL of pure methanol (MeOH).

2.3.5 Retinal HPLC detection

Retinal detection analyses were performed with a Hitachi LaChromELITE® HPLC System (VWR International, Darmstadt, Germany), equipped with a L-2130 pump, a L-2300 column oven, a L-2200 auto-sampler and a L-2455 diode array detector (DAD). The chromatographic column used was an Agilent ZORBAX SB-C18 rapid-resolution high-throughput (RRHT) column with 1.8-mm particles (4.6 mm x 5.0 mm) thermo-stated at 40ºC. The mobile phase consists in a 0.5 mL/min flow rate in a gradient: 0-3 min 100% ultrapure water (H_20)+0.1% TFA/ 3-6 min 20% ultrapure H_20 + 0.1% TFA + 80% MeOH/ 6-25 min 100% MeOH + 0.1% TFA/ 25-35 min 100% ultrapure H_20+0.1% TFA; The injection volume was 50 µL per sample.

The RAL chromatogram peaks were identified according to retention times and absorbance spectra at 383 nm compared to synthetic standards. Recoveries of the extraction procedure were evaluated by constructing calibration curves using the method of standard addition in the liquid extraction. Recovery teste (n = 3) were carried out using a 200 ng/mL of standard compound all-*trans*-RAL. The peak of each retinoid isomer was identified based on the retention time on the column compared to pure retinal synthetic standards.

2.4 Results and discussion

In the last decade several studies have been carried out to characterized BCO enzymes and get insights about its role on the vitamin A metabolism and biological implications in vertebrate species. It is recognize that these enzymes are essential in vertebrates, to keep a vitamin A homeostatic balance consequently maintain crucial physiological processes like vision, cell differentiation and development (Hessel et al., 2007; Lobo et al., 2010; Lietz et al., 2011; Kim et al., 2011; Shete and Quadro, 2013). In contrast, outside vertebrates information regarding BCO presence and functional roles remain very limited. *In silico* studies have revealed the presence of BCO-like family members in the genome of model and non-model invertebrate species, from cnidarian to protostomes, suggesting that the cascade of carotenoid cleavage implicated in retinoid biosynthesis has a more ancestral origin (Theodosiou et al., 2010; Albalat et al., 2011). Yet, most BCO orthologous genes have low sequence similarity with vertebrate counterparts; and once their functional characterization is still missing. They are mostly classified as BCOI/ BCOII/ RPE65 like (Albalat, 2009; Theodosiou et al., 2010; Albalat et al., 2011). For the protostomes lophotrochozoans (such annelids and mollusks), for instance, a BCO-like genes were only genome predicted (Albalat, 2009; Theodosiou et al., 2010). No functional characterization was made.

2.4.1 Isolation of a BCO-like orthologue in *P. dumerilii*

Through a combination of PCR strategies (Fig. 2.2) we were able to isolate the complete cDNA encoding for a *P. dumerilii* putative β-carotene oxygenase-like enzyme that we termed as PdBCO (Fig. 2.3). The retrieved sequence contains an ORF that is 1533 base pairs in length, encoding a 511 amino acid residues and has a calculated predicted molecular weight of 57.94 kilodaltons (kDa). The complete deduced amino acid sequence encoded by PdBCO has 36-41% amino acid identity to BCO like from available vertebrate, protostomes and cephalochordate sequences (Table 2.2).

Table 2.2: Amino acid percentage (%) identity between PdBCO and vertebrates and invertebrates BCO sequences. Percentage determine by sequence alignment in NCBI protein blastp tool. Abbreviation of used species names: *H. sapiens* (Hs), *M. musculus* (Mm), *R. norvegicus* (Rn), *D. rerio* (Dr), *B. floridae*, *L. gigantea* (Lg), *C. teleta* (Ct).

Vertebrates		**Invertebrates**	
HsBCOI	39%	BfBCOa	39%
HsBCOII	36%	BfBCOb	40%
DrBCOI	39%	CtBCoa	41%
DrBCOII	39%	CtBCOb	38%
MmBCOI	40%	LgBCOa	40%
MmBCOII	38%	LgBCOb	40%
RnBCOI	40%		
RnBCOII	40%		

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atgacttctggagaaccactttaccgaaacatgatgcgcttcaaacaggatcatcctgaa
 M  T  S  G  E  P  L  Y  R  N  M  M  R  F  K  Q  D  H  P  E
cctactgattgcaaagttaatgggactatcccagcttggctcaaaggaaatttattccga
 P  T  D  C  K  V  N  G  T  I  P  A  W  L  K  G  N  L  F  R
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 N  G  S  G  K  F  K  I  G  E  D  Q  F  Q  H  L  F  D  G  M
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 A  V  I  H  K  F  S  I  E  D  G  K  V  K  Y  Q  N  R  L  L
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 Y  A  L  T  E  T  D  R  V  T  R  I  D  K  D  T  L  K  T  V
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 D  R  A  A  Y  S  S  Y  A  T  V  N  S  G  T  A  H  P  H  M
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 L  I  F  H  V  V  E  K  A  T  G  K  I  Q  S  K  K  F  C  A
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 V  V  P  E  V  F  E  N  A  D  F  E  L  P  T  I  N  Y  S  H
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ttatttaagagtgattgggaaaccagagacataaagattttgaggaaaagaacaacaga
 L  F  K  S  D  W  E  T  R  D  I  K  I  F  E  E  K  N  N  R
attcttattgaaccaatcttcgttccaaacccagaaggcaaagctgaagacgatggcgta
 I  L  I  E  P  I  F  V  P  N  P  E  G  K  A  E  D  D  G  V
ttgctcgttcctgccatcagtccagaggatgacagtccatctcgattgtacattttggac
 L  L  V  P  A  I  S  P  E  D  D  S  P  S  R  L  Y  I  L  D
gccaaggatttgagtgaaattgcgaccgccgacgtgccacctcaaattgccatgcccttt
 A  K  D  L  S  E  I  A  T  A  D  V  P  P  Q  I  A  M  P  F
actttccatggactgtttttgcctaagatctga
 T  F  H  G  L  F  L  P  K  I  -
```

Figure 2.3: Complete *P. dumerilii* BCO-like cDNA encoding sequence including deduced protein amino acid sequence.

Studies have identified four histidine and five acidic amino acid residues (His172, His237, His308, His514, Asp52, Glu140, Glu314, Glu405 and Glu457; numeration according to *M. musculus* BCO-I) to be an important feature of BCO family members, that are related to the enzyme catalytic activity and iron co-factor coordinating (Poliakov et al., 2005). The reported PdBCO sequence covers all this key amino acid residues crucial for function including those of the catalytic domain and residues interacting with the co-factor of the BCO enzyme family, which are fully conserved when compared to its vertebrate counterparts (Fig. 2.4). In addition, there is a particularly well conserved region, of consensus sequence EDDGVVLSXVVS close to the C-terminus, which is considered a protein family signature sequence (Redmond et al., 2001; Poliakov et al., 2005) also present in *P. dumerilii* (Fig. 2.4). In addition, for mammalian BCO-I, two aromatics residues Tyr235 and Tyr326 located in the carotenoid binding cleft plays an important role in the catalytic mechanism (Poliakov et al., 2009). This aromatic residues are also conserved in PdBCO predicting that it might be a cleavage carotenoid enzyme of type one (Fig. 2.4).

```
HsBCO-I       ------MDIIFG------------RNRKEQLEPVRAKVTGKIPAWLQGTLLRNGPGMHTV
HsBCO-II      MGNTPQKKAVFGQCRGLPCVAPLLTTVEEAPRGISARVWGHFPKWLNGSLLRIGPGKFEF
MmBCO-I       ------MEIIFG------------QNKKEQLEPVQAKVTGSIPAWLQGTLLRNGPGMHTV
MmBCO-II      ---------MLGPKQSLPCIAPLLTTAEETLSAVSARVRGHIPEWLNGYLLRVGPGKFEF
PdBCO         ------------MTSGEPLYRNMMRFKQDHPEPTDCKVNGTIPAWLKGNLFRNGSGKFKI
                                       ::      .:* * :* **:* *:* * * . .

HsBCO-I       GESRYNHWFDGLALLHSFTIRDGEVYYRSKYLRSDTYNTNIEANRIVVSEFGTMAYPDPC
HsBCO-II      GKDKYNHWFDGMALLHQFRMAKGTVTYRSKFLQSDTYKANSAKNRIVISEFGTLALPDPC
MmBCO-I       GESKYNHWFDGLALLHSFSIRDGEVFYRSKYLQSDTYIANIEANRIVVSEFGTMAYPDPC
MmBCO-II      GKDRYNHWFDGMALLHQFRMERGTVTYKSKFLQSDTYKANSAGGRIVISEFGTLALPDPC
PdBCO         GEDQFQHLFDGMAVIHKFSIEDGKVKYQNRLLESQALAKNQGANRIVVSEFGTRAFPDPC
              *:.:::* ***:*::*.* :  * * *:.: *.*::   *    ***:***** * ****

HsBCO-I       KNIFSKAFSYLSH--TIPDFTDNCLINIMKCGEDFYATSETNYIRKINPQTLETLEKVDY
HsBCO-II      KNVFERFMSRFELPGKAAAMTDNTNVNYVRYKGDYYLCTETNFMNKVDIETLEKTEKVDW
MmBCO-I       KNIFSKAFSYLSH--TIPDFTDNCLINIMKCGEDFYATTETNYIRKIDPQTLETLEKVDY
MmBCO-II      KSIFERFMSRFEPP----TMTDNTNVNFVQYKGDYYMSTETNFMNKVDIEMLERTEKVDW
PdBCO         KNIFNRYFSHFFKM---GVLTDNDCVNIMAAGDALYALTETDRVTRIDKDTLKTVDRAAY
              *.:*.: :* :        :***  :* :      *  :**: : ::: : *:  ::. :

HsBCO-I       RKYVAVNLATSHPHYDEAGNVLNMGTSIVEKGKTKYVIFKIPATVPEGKKQGKSPWKHTE
HsBCO-II      SKFIAVNGATAHPHYDPDGTAYNMGNSFGPYGFSYKVI-R----VPPEKVDLGETIHGVQ
MmBCO-I       RKYVAVNLATSHPHYDEAGNVLNMGTSVVDKGRTKYVIFKIPATVPDSKKKGKSPVKHAE
MmBCO-II      SKFIAVNGATAHPHYDPDGTAYNMGNSYGPRGSCYNII-R----VPPKKKEPGETIHGAQ
PdBCO         SSYATVNSGTAHPHMEKDGTLYNLGVTYGKQ--MYNTIFRIPRSQP----GKATPLEGTH
               .: :** .*:*** :  *.  *:* :          * :     *          . ..

HsBCO-I       VFCSIPSRSLLSPSYYHSFGVTENYVIFLEQPFRLDILKMATAYIRRMSWASCLAFHREE
HsBCO-II      VICSIASTEKGKPSYYHSFGMTRNYIIFIEQPLKMNLWKIATSKIRGKAFSDGISWEPQC
MmBCO-I       VFCSISSRSLLSPSYYHSFGVTENYVVFLEQPFKLDILKMATAYMRGVSWASCMSFDRED
MmBCO-II      VLCSIASTEKMKPSYYHSFGMTKNYIIFVEQPVKMKLWKIITSKIRGKPFADGISWEPQY
PdBCO         VLAHIPSRWKMCHGYSHSFAMSENYVIYLEQPLAVNVMKLVTSHMRNSTFCKSLEYYKDE
              *:. * *      .* ***.::.**::::***. :.: *: *: :*   :.. : :  :
```

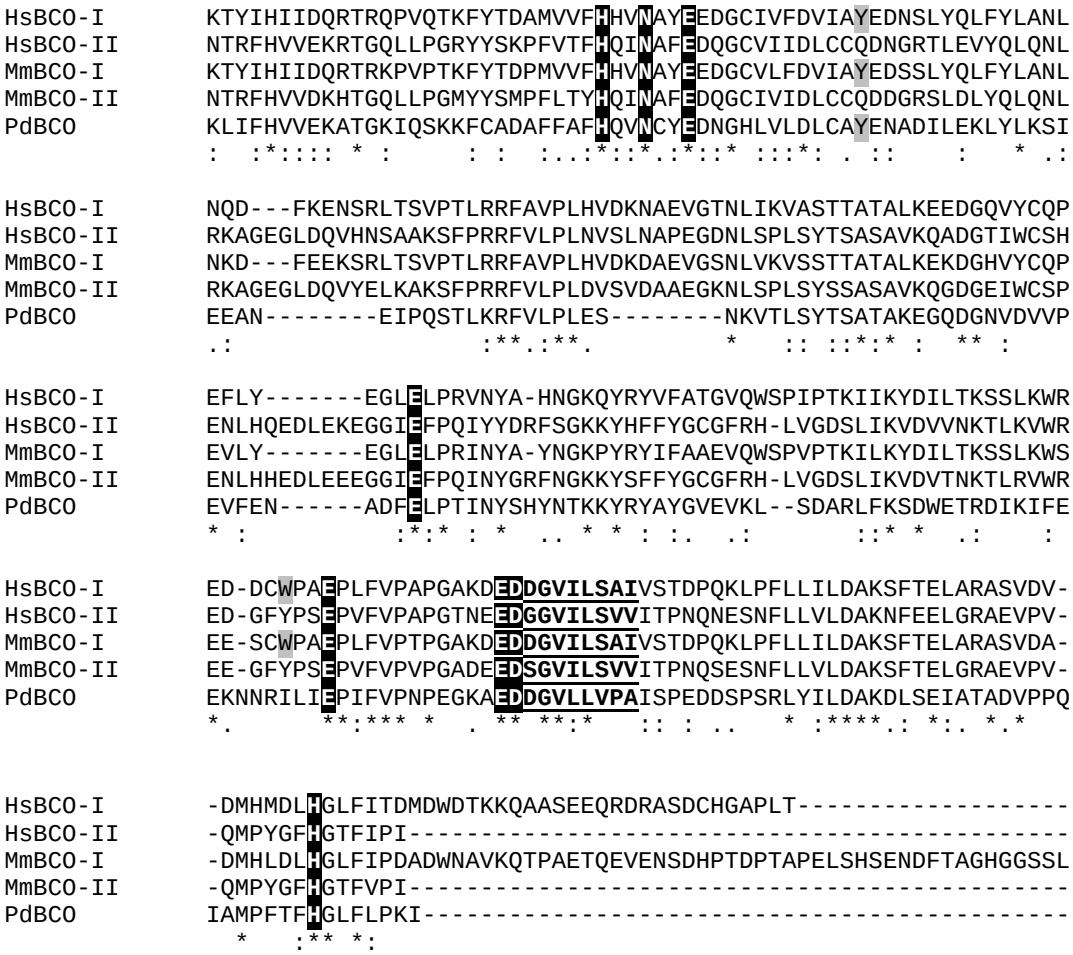

Figure 2.4: Alignment of PdBCO with BCO family members from *H. sapiens* and *M. musculus*. Histidine and acidic residues that participate in co-factor iron coordination and in catalytic activity conserved throughout the BCO superfamily are black shaded in bold letters; BCO family signature sequence is underlined with amino acids at bold; BCO-I key aromatics residues located in the carotenoid binding cleft are grey highlight with amino acids at bold. (*) identity; (:) strong similarity; (.) weak similarity. Sequence used GenBank sequence accession numbers as follows: *H. sapiens* (Hs) BCO-I AAI26211.1 and BCO-II NP_114144.4; *M. musculus* (Mm) BCO-I AF271298 and BCO-II AJ290392. Key amino acid residues inferred from: Redmond et al., 2001 and Poliakov et al., 2005, 2009.

The Neighbor-joining phylogenetic tree constructed with the MEGA5 program showed that PdBCO clustered within the lophotrochozoan branch as expected and near to the clade of BCO-I from vertebrates (Fig. 2.5).

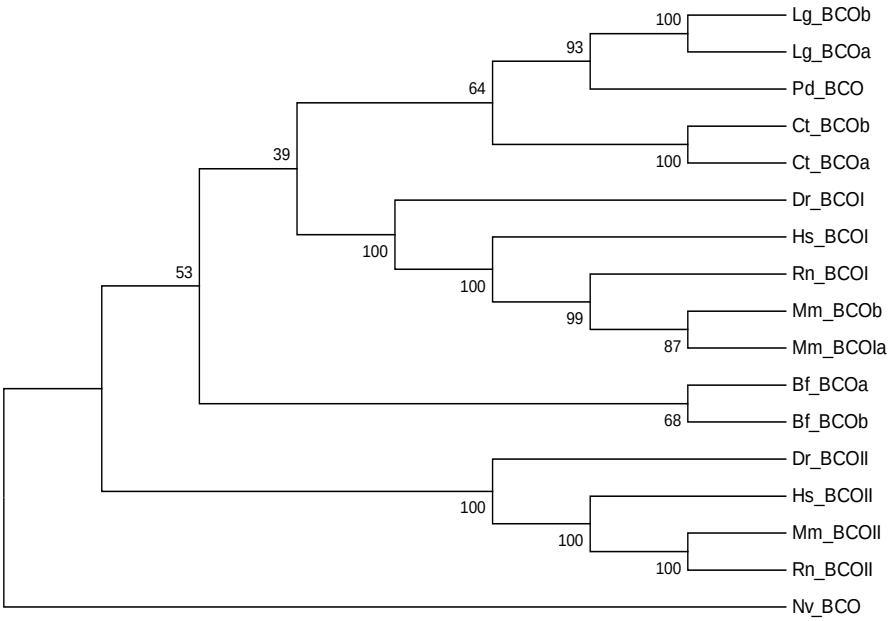

Figure 2.5: Neighbor joining phylogenetic tree derived from an amino acid alignment of BCO enzymes from vertebrate and invertebrate species. Gaps were excluded from the final dataset. Numbers at nodes are the percentages of 1000 bootstrap samples indicating the replicate trees in which the associated taxa clustered together. Abbreviation of used species names: *H. sapiens* (Hs), *M. musculus* (Mm), *R. norvegicus* (Rn), *D. rerio* (Dr), *B. floridae* (Bf), *L. gigantea* (Lg), *C. teleta* (Ct), *N. vectensis* (Nv).

2.4.2 *In vivo* β-carotene cleavage in producing and accumulating *E. coli* strains and retinoid detection

Sequence alignment comparisons suggested that PdBCO is a member of the carotenoid oxygenase family. To investigate its ability to cleave carotenoids, we next undertook a functional characterization based on the BCO gene ORF expression on βc accumulating and producing *E. coli;* a methodological approach, which was successfully used in previous studies (e.g. von Lintig and Vogt 2000, Redmond et al., 2001 and Poliakov et al., 2012). Hence, PdBCO protein was expressed into the modified *E. coli* cells using the pBAD/TOPO expression system and induction was performed with 0.5% (v/w) L-arabinose. As a positive control for βc cleavage into retinal we used the human BCO-I expressed into the pBAD/Topo expression vector transformed in the *E. coli*. As a negative control for carotenoid cleavage activity *E. coli* cells were transformed with the cloning vector containing an irrelevant insert, pBAD/LacZ, without carotene cleavage activity. As expected, the *E. coli* strain expressing the irrelevant insert present an orange coloration indicative of βc production and accumulation (n = 4; Fig. 2.6A). *E. coli* strain expressing the human BCO-I presented a white color (n = 4), due to the oxidative cleavage of bacteria βc produced into retinoids (Fig. 2.6C). As shown in Fig. 2.6B, the expression of PdBCO lead to obvious shift coloration (n = 4). Despite the color change in the PdBCO

expressing *E. coli*, in comparison to the *E. coli* expressing human BCO-I the cell pellet was bleached yellow, as depicted in Fig. 2.6B, suggesting that cleavage activity of βc to retinal or β-apocarotenals might have occurred in PdBCO expressing *E. coli* but at lower level in comparison with the positive control, human BCO-I (Fig. 2.6C).

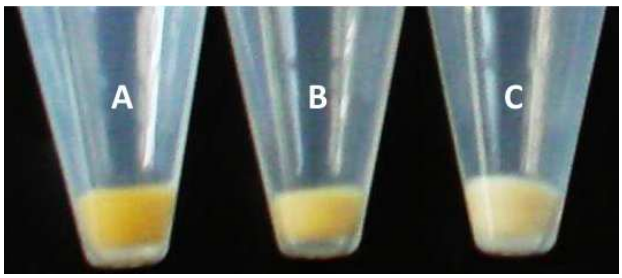

Figure 2.6: Color shift due to the cleavage of β-carotene in producing and accumulating *E. coli* colonies. The picture shows the color shift from yellow/orange, indicative of β-carotene biosynthesis to almost white (retinoids), indicative of subtract oxidative cleavage. A) refers to the negative control *E. coli* expressing β-carotene plasmid and pBAD-Lacz where colonies shows an orange color indicative of β-carotene synthesis; B) refers to *E. coli* strain expression subtract plasmid and pBAD-PBCO; a pellet colonies with / partially bleached color (intermediate color between yellow and white) has been obtained. C) Represents the positive colonies control; *E. coli* expression β-carotene and pBAD-human BCO-I that present a white coloration indicative of retinoids synthesis.

To identify the putative cleavage products, all-*trans*-retinal, retinoids were extracted from the cell pellets and subjected to HPLC analyses. From the *E. coli* strain expressing human BCO-I (the positive control) extracts we could detect all-*trans*-retinal (60.5 ng/mL; n = 1; Fig. 2.7B). For the negative control and PdBCO expressing cells no retinal could be detected (n = 1; Fig. 2.7C and 2.7D). We could not improve the recovery of the method above 40%, and that associated with the detection limit (28 ng/mL of limit of detection; 85 ng/mL limit of quantification) might have conditioned our detection capability, consequently affecting PdBCO functional characterization. Additional analytical determinations are warrant involving a more sensitive approach (Liquid chromatography–mass spectrometry) to demonstrate the capacity of PdBCO to synthesize all-*trans*-retinal or β-apocarotenals.

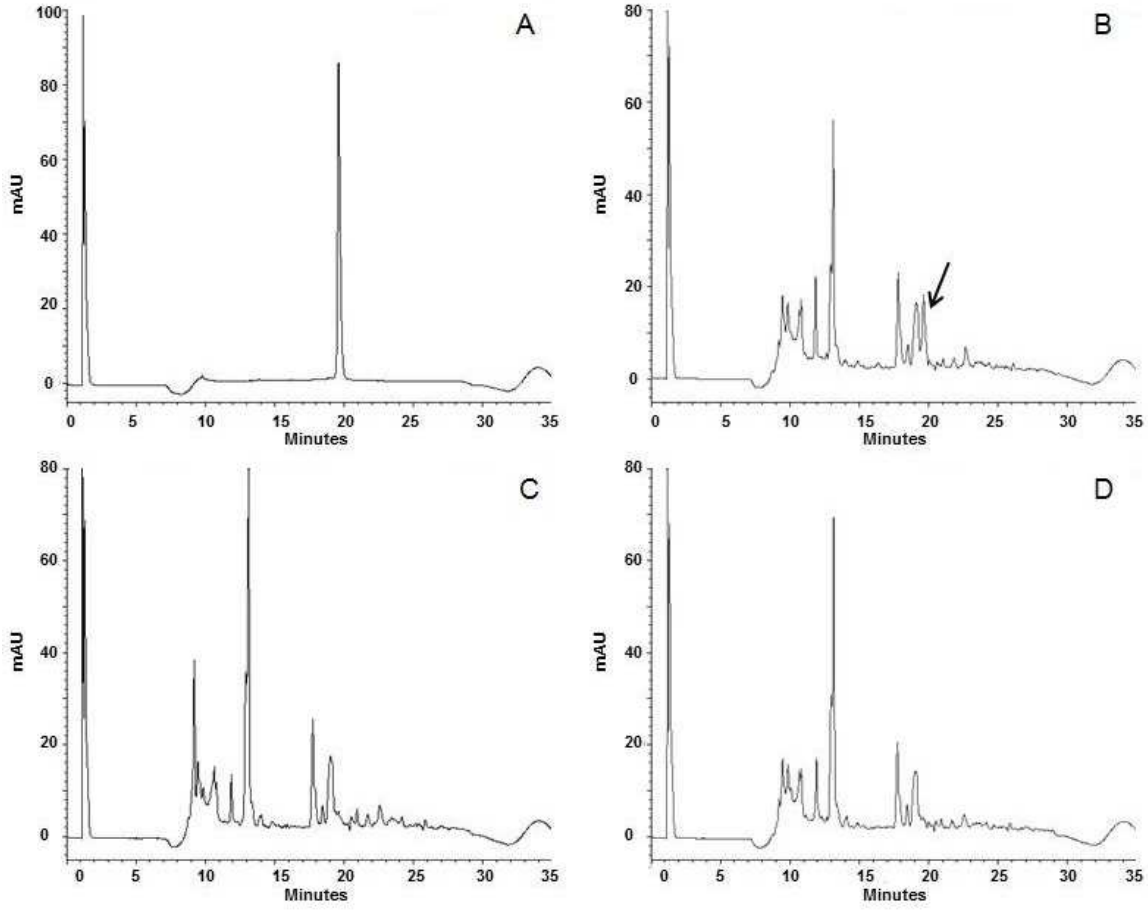

Figure 2.7: Detection of all-*trans*-retinal by HPLC analysis from *E. coli* β-carotene producing and accumulating transformed with BCO or LacZ inserted in pBAD expression vector extracts. The scale bars indicate an absorbance of 383 nm for milli absorbance units (mAU) per minute. A) Retinal standard dilution at 400 ng/mL, b) detection of RAL in organic extracts of *E. coli* cultures transformed with pBAD-Hs/BCOI transfected *E. coli* or positive function control for β-carotene cleavage into RAL; C) negative control *E. coli* transformed with pBAD-LacZ no RAL was detected; and D) detection of RAL in organic extracts of *E. coli* cultures transformed with the pBAD-Pd/BCO.

Additional studies should be performed to establish the identity and functional activity of this enzyme. One option involves the use of a similar experimental setup using also bacteria able to produce and accumulate another carotenoid, the lycopene, since previous data indicates that BCO-I does not use this carotenoid as a substrate whereas the BCO-II is capable of oxidative cleave it into β-apocarotenal (Kiefer et al., 2001; Amengual et al., 2011).

Platynereis dumerilii is an herbivorous polychaeta that selectively feeds on micro- and macro-algae (Gambi et al., 2000). Hence, the only source of retinoid precursors must be obtained from algae-derived carotenoids. The present work revealed the presence of a putative BCO enzyme, and suggests the ability to perform oxidative cleavage of

carotenoids indicating the existence of a pathway for synthesis of retinal for subsequence metabolism into non- and active retinoids or β-apocarotenoid retinoic acid precursors.

For *D. melanogaster* a single BCO-like homologue enzyme was isolated named as NinaB, and functional characterized by expressing it on *E. coli* strain able of producing and accumulate βc (von Lintig and Vogt, 2000). Subsequent biochemical analysis showed that RAL was detected revealing that the protein encoded a BCO enzyme able of catalyzes the symmetric cleavage of βc belonging to the type I (von Lintig and Vogt, 2000). Later, in another insect species, *Galleria mellonella*, a single NinaB gene was also cloned and functionally characterized. It had been shown that *G. mellonella* NinaB catalyze an oxidative cleavage at the C15, not only of βc into retinal, but also in others carotenoids. In addition, the authors also demonstrated that both NinaB genes from *D. melanogaster* and *G. mellonella* are capable to convert all-*trans*-RAL to 11-*cis*-RAL isomerization for the visual pigments and photoreceptor development (Oberhauser et al., 2008). Thus, the authors established a functional link between carotenoid oxygenases and retinoid isomerases by showing that both activities are combined in a single protein in insects (Oberhauser et al., 2008). For the tunicate *C. intestinalis* two encoding BCO-like enzymes were isolated and termed CiBCOa and CiBCOb (Takimoto et al., 2006; Poliakov et al., 2012). Initially, CiBCOa was suggested to be a RPE65 enzyme like. Later, it has been demonstrated that it has no isomerohydrolase activity (Poliakov et al., 2012). When ORF CiBCOa and CiBCOb were expressed into the modified lycopene or βc producing and accumulating *E. coli* a color shift was observed but no RAL was detected from bacteria extracts pointing to a BCO-II-like able of asymmetric carotenoid cleavage (Poliakov et al., 2012). A carotenoid cleavage metabolism seems to be also present in most basal metazoans. For the demosponge *S. domuncula* two BCO-like enzymes were isolated and initial termed based on sequence as SdCDO1 (carotene dioxygenase) and SdCDO2 (Müller et al., 2011, 2012). The two encoded BCO like enzymes were expressed into βc and lycopene-accumulating *E. coli*. The SdCDO1 encode an enzyme able of using both lycopene and βc. In contrast SdCDO2 does not encode an enzyme able to metabolize both carotenoids; no shift color was observed (Müller et al., 2012). From an evolutionary viewpoint, identification of BCO like enzymes in the demosponge *S. domuncula* (Müller et al., 2011, 2012), insects *D. melanogaster* and *G. mellonella* (von Lintig and Vogt 2000; Oberhauser et al., 2008), tunicate *C. intestinalis* (Takimoto et al. 2006; Poliakov et al., 2012) and in annelid *P. dumerilii* suggest the active carotenoid metabolism for retinoid synthesis might have emerged very early during evolution having a basal common ancestor that is conserved through metazoans. Yet, outside vertebrates, for most lineages it is still unclear the precise cascade involved in retinoid synthesis.

2.5 Conclusion

In conclusion, we have shown that the annelid *P. dumerilii* has a BCO like gene orthologue, that encodes a protein sequence with conserved key amino acid residues important for functional ability. In addition we demonstrated that the protein is able of using β-carotene and cleave it to retinoid precursors. Yet, additional studies are warrant to establish the identity and functional activity of this enzyme.

2.6 Acknowledgements

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CHAPTER 3

3 Diacylglycerol O-acyltransferase 1 (DGAT1) in lophotrochozoans: insights into the mechanisms of retinol esterification and storage

3.1 Abstract

In mammals lecithin retinol acyltransferase (LRAT) and diacylglycerol O-acyltransferase 1 (DGAT1) are the two main enzymes known to be involved in vitamin A (retinol) homeostasis by catalyzing retinol esterification and storage in various tissues. In model invertebrate genomes LRAT orthologues are missing, in contrast with DGAT1 which is found throughout. Previous studies have suggested that DGAT1 might be the main enzyme implicated in retinol storage and overall retinoid homeostasis outside vertebrates. In this context, no isolation and functional characterization of DGAT1 was performed in invertebrate phyla.

To elucidate the potential roles of DGAT1 in non-vertebrate retinoid homeostasis, we isolated DGAT1 orthologues in two protostome lophotrochozoan species, *Patella depressa* (a mollusk) and *Platynereis dumerilii* (an annelid). Our data shows that DGAT1 encoding sequences from both species exhibit high levels of sequence identity to that of vertebrates and other lophotrochozoans; relevant key residues for the enzymatic activity are present and fully conserved suggesting a possible conserved role. Next, through *in vivo* intramuscular injections and gene expression quantification (real-time PCR) approaches we addressed the DGAT1 involvement in retinol esterification in *P. depressa*. *Dgat1* gene expression on selected tissues from injected animals was not significantly different from the control. The implications of these findings are discussed and research priorities established.

3.2 Introduction

Vitamin A or retinol is an essential nutrient for biological functions such as vision, reproduction, embryonic development and immune system regulation; and therefore their endogenous levels should be tightly controlled (Mark et al., 2006; Moise et al., 2007; Mark et al., 2009). To achieve such homeostatic balance, an effective mechanism must exist, since vitamin A cannot be synthesized *de novo* by the organism; their immediate precursors or analogs, the so called retinoids, are obtained from the diet as preformed vitamin A and pro-vitamin A carotenoids from animal and plant-derived food sources, respectively (Blomhoff and Blomhoff, 2006).

Vertebrates retain an effective mechanism that provides an evolutionary advantage to maintain a vitamin A homeostatic balance. This mechanism relieves the organism from the obligate need to obtain retinoids regularly from its dietary intake by accumulating retinol storages as retinyl esters (mainly palmitate) in tissues (Albalat, 2009;

O'Byrne and Blaner, 2013; André et al., 2014; Wongsiriroj et al., 2014). In the body many tissues are capable of biosynthesizing and accumulating REs from dietary ROL. These include eye, lung, adipose tissue, testes, skin, spleen, intestine, liver and others (O'Byrne and Blaner, 2013). Nevertheless, REs are most abundant in the liver where approximately 80–90% of the body's retinoids are found mostly in hepatic stellate cells (Wongsiriroj et al., 2014). When, dietary vitamin A intake is insufficient, REs stores can be mobilized back to ROL, which is then secreted into the circulation bound to retinol-binding protein (RBP) in complex associated with transthyretin (TTR) to avoid elimination by the kidneys (Blomhoff and Blomhoff, 2006). Two distinct enzymatic routes are known for ROL esterification and storage in vertebrates. One is mediated by lecithin retinol acyltransferase (LRAT), an enzyme that catalyzes the transfer of the sn-1 fatty acid from membrane phosphatidyl choline to ROL bound to the cellular retinol-binding proteins (D'Ambrosio et al., 2011). Studies establish LRAT as the main enzyme implicated in REs stores in most of the body tissues, and the sole in the liver (Batten et al., 2004; O'Byrne et al., 2005; Wongsiriroj et al., 2008). LRAT-/- mice had no significant trace levels of REs stores in liver, eyes, lungs, testes, skin or spleen only in the adipose tissue (Batten et al., 2004; Liu and Gudas, 2005; O'Byrne et al., 2005). The diacylglycerol O-acyltransferase 1 (DGAT1) enzyme, known to be involved in the final step of triglyceride synthesis (Cases et al., 1998), is a multiple functional protein that also has a putative Acyl-CoA: retinol acyltransferase (ARAT) activity being able of esterifying ROL into REs (O'Byrne et al., 2005; Yen et al., 2005a, b; Wongsiriroj et al., 2008; Shih et al., 2009). In the intestine DGAT1 contributes for REs synthesis. DGAT1 activity only increases when large pharmacologic doses of ROL are administered exciding the capacity of LRAT (Wongsiriroj et al., 2008). Acting as an ARAT in murine skin DGAT1 helps keeping retinoic acid (RA) at homeostatic levels and prevents retinoid toxicity. When DGAT1-/- murine are maintain in a vitamin A rich diet, RA levels increased causing epidermis alopecia, which can only be rescue by removing ROL form the diet (Shih et al., 2009). Other enzymes with ARAT activity exists and also contribute for REs formation and storage by only esterifying "free" ROL but their identity remains unknown (Ross, 1982; Fortuna et al., 2001; Liu and Gudas, 2005; Shih et al., 2009).

Outside vertebrates, the mechanism by which retinoid levels are homeostatically maintained remains elusive (Albalat, 2009; Albalat et al., 2011; Gesto et al., 2012, 2013). Studies have been suggested an existence of REs endogenous levels in the tissues of sponge and mollusks species. Various findings challenge the hypothesis that ROL esterification and storage strategies are a vertebrate novelty, and strongly support an early metazoan origin (Biesalski et al., 1992; Albalat, 2009; Theodosiou et al., 2010; Albalat et al., 2011; Gesto et al., 2012, 2013, 2016). Still, the enzymatic cascade behind

REs storage in invertebrates remains unknown. The investigation into the genome sequences of various species has provided solid evidences that LRAT genes are vertebrate specific, while DGAT1 orthologues have an ample phylogenetic distribution (Albalat, 2009; Theodosiou et al., 2010; Albalat et al., 2011; Turchetto-Zolet et al., 2011). Thus, it has been proposed that in the absence of LRAT, ROL esterification might be catalyzed by DGAT1 orthologues, an enzyme that might have hence played a key role in the biosynthesis of REs in the last common metazoan ancestor (André et al., 2014). So far, isolation and functional studies were not performed in non-vertebrate phyla to support DGAT1 possible role in ROL esterification and tissue accumulation. Hence, to further investigate the possible involvement of DGAT1 ROL esterification and storage in invertebrates, in the present study we isolated a DGAT1 enzyme orthologue in two lophotrochozoan species: the annelid *Platynereis dumerilii* and the mollusk *Patella depressa*. With the goal to get insights about a possible ancestral pathway for retinol esterification, we also performed an *in vivo* injection of ROL with *P. depressa* and concomitantly evaluated *Dgat1* gene expression.

3.3 Material and methods

3.3.1 Chemicals

All-*trans*-retinol (ROL) (≥ 95%) was purchased from Sigma-Aldrich, whereas the Dimethyl sulfoxide (DMSO) was acquired from VWR.

3.3.2 Animals handling and RNA tissue extraction

Adult male and female *P. depressa* were captured at Homem do Leme (Foz, Porto, Portugal) rocky shore, and transported to the laboratory in plastic boxes without water. Then, animals were maintained in 30 L aquaria filled with natural sea water (salinity 35‰) with aeration for 48 h before sacrifice. During that period animals were not fed, and maintained at 15ºC with a photoperiod set for light: dark rhythm 12 h: 12 h, respectively. To sample the gonads and digestive gland for RNA extraction, limpets were sedated by immersion in a 7% magnesium chloride ($MgCl_2$) solution for 10 min before sacrifice.

Immature *P. dumerilii* specimens were collected at low tide in June of 2012 at Mindelo rocky shore (north, Portugal). After their transport to the laboratory in plastic boxes with seawater from the sampling site, organisms were acclimated in plastic boxes of 500 mL filled with natural seawater (salinity 35‰) with aeration. Temperature was set to 18ºC, light: dark rhythm 14 h: 10 h during acclimatization for 24 h before sacrifice. Then, animals were anesthetized in a 4% $MgCl_2$ solution for 10 min before sacrifice for tissue sampling.

For both animal tests, total RNA was extracted from the tissues using the Illustra RNAspin Mini RNA Isolation Kit animal tissues protocol (GE Healthcare) with column for DNAse I digest following the protocol instructions. The mRNA concentration quantification of samples was done by measuring absorbance at 260 nm using a spectrophotometer Jenway Genova. The first-strand cDNA synthesis was performed with the iScript cDNA synthesis kit (Bio-Rad) following the manufacture protocol recommendations using 1 µg of total RNA input.

3.3.3 DGAT1 isolation

Basic local alignment search tools (BLAST) were used to identify at the Join Genome Institute (JGI) website (http://genome.jgi.doe.gov/; Grigoriev et al., 2012) the sequences of the DGAT1 orthologues in *Capitella teleta* and *Lottia gigantea* genomes using the human DGAT1 sequence. The identified sequences from *L. gigantea*, *C. teleta*, and a previously small sequence obtain for *Perinereis marioni* and full sequence from *Nucella lapillus* (unpublished data) were used to design degenerate primers with the Consensus-degenerate hybrid-oligonucleotide primers (CODEHOP; http:// blocks.fhcrc.org/ codehop.html; Rose et al., 1998) (Table 3.1). The polymerase chain reactions (PCRs) were performed with Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific). Briefly, 2 µL of 10 x (v/v) diluted cDNA (for a 10 ng final concentration in reaction) was added to a reaction mixture using 1 µL of each primer to a concentration of 0.5 µM in final reaction volume of 20 µL. Cycle parameters and used primers are presented in table 3.1, 3.2 and 3.3. Bands of the appropriate size were isolated (illustra GFX PCR DNA and Gel Band Purification Kit) and sequenced directly (Stabvida) for confirmation.

Table 3.1: Oligonucleotide primer sequence used for DGAT1 isolation.

Specie	Designation	Sequence	Use
P. depressa	P1	5'-CGGGAGTTCTACCGGgaytggtggaa-3'	Degenerated PCR
	P2	5'-TGGACACCAGGTACTCGtgraaraangc-3'	Degenerated PCR
	P3	5'-TCGTAACGCCCACCTATGAACAGGA-3'	RACE PCR
	P4	5'-TCCTGTTCATAGGTGGGCGTTACGA-3'	RACE PCR
	P5	5'-TAGTAGTACATCGAGATGGCTTCT-3'	ORF PCR
	P6	5'-ATGTCATGGTTTGATTATGGAGT-3'	ORF PCR
P. dumerilii	P1	5'-TGATGCTGTGCCTGATCCArcartggat-3'	Degenerated PCR
	P2	5'-TCTCGGCGTTCCACCARTC-3'	Degenerated PCR
	P3	5'-GTCCATCTCCTTGAAAGGCACCAT-3'	RACE PCR
	P4	5'-CATGGTGCCTTTCAAGGAGATGGAC-3'	RACE PCR
	P5	5'-ATCCCTGTTCACAAGTGGGCTGTCA-3'	RACE PCR
	P6	5'-ATGGCGAGCAAACCAAGACTTC-3'	ORF PCR
	P7	5'-GGTAATAAATGCCATCGTCCTTTC-3'	ORF PCR

Table 3.2: PCR conditions for DGAT1 isolation and sequence confirmation for *P. depressa*

Primers	Denaturation	Denaturation	Annealing	Extension	Extension
P1 + P2	98ºC 10 s	98ºC 1 s	50ºC 5 s. 45 cycles	72ºC 5s	72ºC 1 min.
P3 + Up RACE		98ºC 1 s	63ºC 5 s 40 cycles	72ºC 15 s	
P4 + Up RACE		98ºC 1 s	63ºC 5 s 40 cycles	72ºC 9 s	
P5 + P6		98ºC1s	53ºC 5 s 40 cycles	72ºC 25 s	

Table 3.3: PCR conditions for DGAT1 isolation and sequence confirmation for *P. dumerilii*

Primers	Denaturation	Denaturation	Annealing	Extension	Extension
P1 + P2	98ºC 10 s	98ºC 1 s	50ºC 5 s 45 cycles	72ºC 5 s	72ºC 1 min.
P3 + RACE		98ºC 1 s	63ºC 5 s. 40cycles	72ºC 15 s	
P4 + Up RACE		98ºC1s	63ºC 5 s 40 cycles	72ºC 9 s	
P5 + nested RACE		98ºC1s	63ºC 5s 40 cycles	72ºC 15 s	
P6 + P7		98ºC1s	53ºC 5 s 40 cycles	72ºC 25 s	

Then, to obtain the full DGAT1 coding sequence, a 5' and 3' Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE-PCR) using gene specific primers strategy was followed. Gene specific primers were design within the known DGAT1 sequence using Primer3 (v.0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/; Rozen and Skaletsky, 2000). The SMART™ RACE cDNA amplification kit (Clonetech) was used to synthesize 5' and 3' RACE cDNA from total RNA, following the manufactures instructions. The RACE-PCRs was performed with the PCR Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) in the following reaction condition: 2 µL of 3' or 5' cDNA RACEs were used directly without dilution to respective reaction mixture (3' and 5' end), together with 1 µL of the corresponding specific RACE oligo primer to a final concentration of 0.5 µM, and 2 µL of Universal primer was used in final reaction volume of 20 µL. Primer sequences and cycle PCR conditions are presented in table 3.1, 3.2 and 3.3. This strategy was not successful regarding the isolation of 3' of the coding sequence for *P. dumerilii* DGAT1. To obtain the 3' end sequence a different approach was used consisting of a nested RACE-PCR where we have used as template the RACE-PCR first amplification product with other RACE primer design upstream of the first one (Fig. 3.1 and Table 3.3). All the retrieved bands of the appropriate size were isolated (illustra GFX PCR DNA and Gel Band Purification Kit) and sequenced directly (Stabvida).

The open reading frame (ORF) was identified with ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html; Geer et al., 2010; Benson et al., 2013). Specific PCR primers were design in Primer3 (v. 0.4.0) (Rozen and Skaletsky, 2000) to outflank the predicted full ORF. The PCR of the full ORF (Tables 3.1, 3.2 and 3.3; Fig. 3.1) was performed with the polymerase Phusion Flash high-fidelity polymerase PCR Master Mix under the following PCR conditions: 10 x diluted cDNA (v/v) 2 µL (final concentration of 100 ng) was added to reaction mixture, containing 1 µL of each specific ORF primer to a final concentration of 0.5 µM in a final 20 µL reaction volume. All the bands with expected size were isolated, purified with Nzytechgelpure (from Nzytech, Portugal) and sequenced directly (Stabvida) or first cloned into the pGEM®-T easy vector (Promega). Positive colonies were identified and selected for plasmid purification and sequencing at Stabvida.

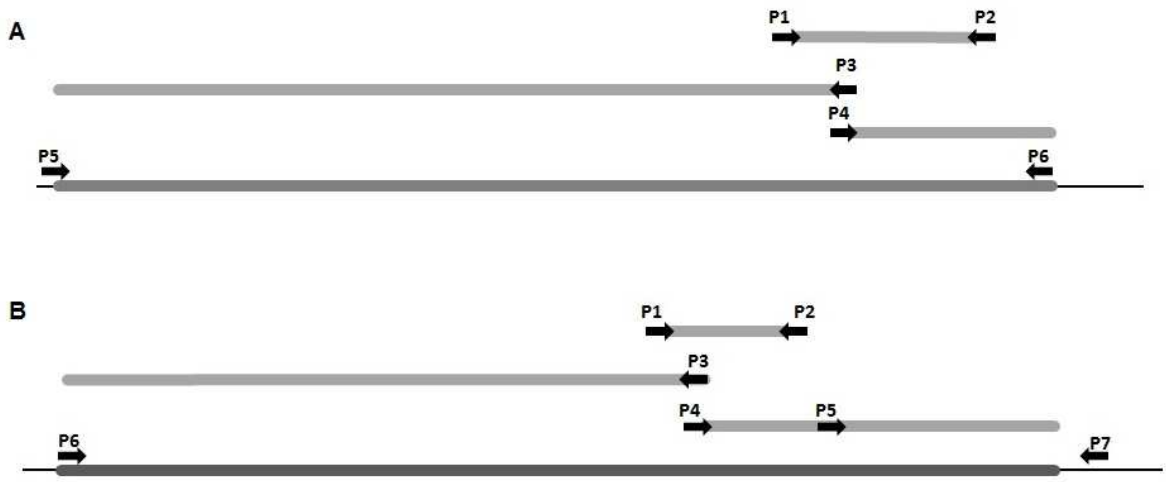

Figure 3.1: Schematic representation of the strategy followed for DGAT1 gene isolation in A) *P. depressa* and B) *P. dumerilii.*

3.3.4 Sequence analysis

The amino acid sequences and molecular weight of *P. depressa* and *P. dumerilii* DGAT1 were deduced using the ExPASy software (http://web.expasy.org/translate/; Artimo et al., 2012). Through BLAST search analyses in GenBank (http://www.ncbi.nlm.nih.gov/genbank/; Geer et al., 2010; Benson et al., 2013) and JGI databases (Grigoriev et al., 2012). DGAT1 amino acid sequences of different species were obtained and used for sequence alignment and phylogenetic analysis. Accession numbers and sequence identifiers from GenBank were as follows: *Homo sapiens* NP_036211.2, *Mus musculus* NP_034176.1, *Danio rerio*_a NP_956024.1, *D. rerio*_b NP_001002458.1, *Crassostrea gigas* EKC24153.1, *Apis mellifera* XP_624754.2, *Drosophila melanogaster* AAL78365.1 and *Nematostella vectensis* XP_001639351.1; and from JGI: *L. gigantea* estExt_231331 and *C. teleta* estExt_183593.

DGAT1 protein sequence alignment was generated using the CLUSTAL 2.1 multiple sequence alignment, with default parameters, to infer about conserved key residues (http://www.ebi.ac.uk/Tools/msa/; Chenna et al., 2003). Evolutionary analysis of DGAT1 was conducted in MEGA5 (Tamura et al., 2011). Thus, a phylogenetic tree was deduced using the Neighbor-joining (NJ) method (Saitou and Nei, 1987) after alignment of protein sequences with Clustal W method (Higgins et al., 1994). The percentage of evolutionary tree per replication grouping of related *taxa* was established by Bootstrap analysis (1000 replicates; Felsenstein, 1985), and the molecular evolutionary distances of the aligned sequences were calculated according to the Poisson Correction model (Zuckerkandl and Pauling, 1965).

3.3.5 Structural organization and transmembrane domain prediction

Predicted transmembrane structures (TrM) were obtained using the transmembrane prediction server TMHMM-2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) on-line provided by the Center for Biological Sequence Analysis of the Technical University of Denmark (von Heijne, 1992; Tusnady and Simon, 2001) using the complete protein sequences. Cellular localization prediction was obtained using the server Euk-mPLoc 2.0 server (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/; Chou and Shen, 2007, 2008 and 2010). Sequences used for comparison with *P. depressa and P. dumerilii* were from *H. sapiens*, *M. musculus*, *D.* rerio, *L. gigantea* and *C. teleta*.

3.3.6 Animal handle and experimental exposure

Individuals of *P. depressa* were collected in May 2013 at the beach Castelo de Queijo from Matosinhos (north Portugal). Specimens were brought to the laboratory and allowed to acclimate for 5 days prior to the onset of the experiment. After acclimatization, 20 animals per replicate (two replicas per condition) were assigned to 30 liters aquaria filed with in natural sea water (salinity 32‰), with aeration and maintained at 15℃ ± 1 in 12 h light: 12 h dark photoperiod condition. Water was change every day, and animals were not feed during the acclimation time.

After the acclimation period animals were sedated by immersion in a solution of 7% $MgCl_2$ for 5 to 10 minutes and injected into the foot with 2 µL, using a 5 µL syringe (Hamilton Bonaduz, Switzerland) with the fooling compounds, all-*trans*-ROL for 2.4 µg/g corresponding to 1.2 mg/mL and DMSO as a control solvent. Applied doses of the testing compounds were estimated according to their body mass in mg/wet weight ~1 g ± 0.42 (mean ± standard deviation), without the shell. The exposure time and applied doses were chosen on the basis of previously published work (Castro et al., 2007; Gesto et al., 2012). Un-injected limpets were also used as a control to assess potential vehicle effects.

Water from aquaria was change after 24 h after the injections. Then, 48 h after injection, animals were sacrificed after sedated by immersion in 7% $MgCl_2$ during 10 min. Shell size, weight with and without shell and gonad maturity stage was recorded. Gonads and digestive gland was collected and store in RNAlater at -80ºC until analysis. The mortality rate was below 25% in all test groups.

3.3.7 Tissue RNA extraction

Total RNA extraction from gonads and digestive gland was performed using the Illustra RNAspin Mini RNA Isolation Kit animal tissues protocol (GE Healthcare) with on-column DNase I digestion. For the extraction of male gonads RNA a different approach was used by combined the Purezol protocol (Bio-Rad) with Illustra RNAspin Mini RNA

Isolation Kit. The gonads were homogenized with Purezol reagent (Bio-Rad) and nucleic acids extracted with chloroform, according to the manufacturer's instructions. The resulting aqueous phase was used to isolate total RNA using the Illustra RNAspin Mini RNA Isolation Kit was used to clean up the RNA from the ethanol step onwards. RNA concentration was determined using the Nanodrop spectrophotometer, by measuring sample absorbance at 260 nm. First-strand cDNA synthesis was performed using the iScript^{TM} cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions, using 500 ng of total RNA from each sample.

3.3.8 DGAT1 expression by real-time PCR

The tissue expression of DGAT1 from *P. depressa* in the described experimental setup (control, DMSO and ROL injected specimens) was determined by real-time PCR. PCR oligonucleotide primers were designed using Primer3 (v. 0.4.0) (Rozen and Skaletsky, 2000), and secondary structures and annealing temperature were analyzed using Beacon Designer 5 free edition (http://www.premierbiosoft.com). Preliminary PCR reactions were run to determine optimal primer concentrations, cDNA volume and annealing temperatures. An initial dilution of the cDNA (1:20) was done, of which 4 µL were added to a reaction mixture containing 1 x iQ SYBR Green Supermix (Bio-Rad) and 2 mM of forward, 5´- AGCTCGCATCATTGGCTTAT -3', and reverse primer, 5'-TGCTGAGACCGTTTCAAGATT -3', in a final volume of 20 µL. A 'no-template control' (miliQ sterilized water) was included and all samples were run in duplicate in a 96-well plate. The real-time PCR profile had the following conditions: an initial denaturation step at 95ºC for 3 min, followed by 40 cycles of denaturation at 95ºC for 15 s, annealing at 59ºC for 30 s and extension at 72ºC for 30 seconds. A melting curve was generated for each run to confirm the specificity of the assays (55ºC-95ºC, for 15 s each with 5ºC of increment). The PCR efficiency for the gene of interest was determined through a standard curve, using six five-fold serial dilutions (efficiency of 94.8%). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001) and elongation factor alpha gene (EF-1α) was used as the housekeeping reference gene for normalization. For EF-1α real-time PCR amplification efficiency was calculated using seven five-fold serial dilutions (efficiency 95.1%) with the following PCR profile: 95ºC for 15 min, with 40 cycles at 95ºC for 15 s, 60ºC for 30 s and 72ºC for 30 s. A melting curve was generated for each run to confirm the specificity of the assays (55ºC-95ºC, for 15 s each with 5ºC of increment). The reference gene primers had the following sequence: forward 5´-GGCTGGGAAATAGAAAGAAA-3´, and reverse 5´-CACTGTTCCAATACCTCCAA-3´.

3.3.9 Statistical analysis

In order to apply the $2^{-\Delta\Delta Ct}$ method, internal control gene elongation factor, was properly validated for the experiment by determined that is expression was unaffected by the experimental treatment conditions (Livak and Schmittgen, 2001). After testing ANOVA assumptions (homogeneity of variances and normality of data), DGAT1 expression data of male and female gonads were analyzed through a one-way ANOVA. In the case of DGAT1 expression data of female and male digestive gland failed the ANOVA assumptions, in this case the Kruskal-Wallis H nonparametric test was used to test for statistically significant differences between treatments ($p < 0.05$). The values from all data are expressed in terms of mean ± standard error. Statistical analysis was accomplished using IBM Spss version 22.1 software, license and software provided by the University of Porto.

3.4 Results

3.4.1 Cloning and phylogenetic analysis

Through the combination of PCR strategies describe above, we were able to isolate the entire ORF of DGAT1 gene in *P. depressa* and *P. dumerilii* (Fig 3.2 and 3.3, respectively). Both sequences contain a total of 480 coding residues that in the case of *P. depressa* starts with "MASTTTNPRL" amino acid sequence and exhibited a predicted molecular weight of 56.49 kilodaltons (kDa); whereas in *P. dumerilii* contain an initiating amino acid sequence "MASKPRLRRV" and has a predicted molecular weight of 56.14 kDa. Sequence alignments at NCBI revealed high levels of sequence identity (Table 3.4); *P. depressa* DGAT1 has 71% sequence similarly with *L. gigantea*, while DGAT1 *P. dumerilii* sequences has only 56% similarity between *C. teleta*. Fifty six percent of identity is also observed between DGAT1 *P. dumerilii* and *P. depressa* (Table 3.4).

Table 3.4: Sequences percentage similarity predicted using NCBI protein-protein BLAST (Blastp) tool.

	P. depressa	*P. dumerilii*
H. sapiens	52%	49%
M. musculus	54%	53%
D. rerio a	50%	51%
D. rerio b	48%	48%
L. gigantea	76%	55%
C. gigas	61%	55%
P. depressa		56%
C. teleta	59%	56%
P. dumerilii	56%	

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atggcttctactactactaacccaaggcttcggcgcacaaaaagtatgtataaagtcgaa
 M  A  S  T  T  T  N  P  R  L  R  R  T  K  S  M  Y  K  V  E
gaaagaaaaggagaagagagaaaagaaatgttatggcgttcggacgatcctgtacataga
 E  R  K  G  E  E  R  K  E  M  L  W  R  S  D  D  P  V  H  R
catgcagattccttcttcagtacttcaagtggttttacaaattacagaggtttattaaat
 H  A  D  S  F  F  S  T  S  S  G  F  T  N  Y  R  G  L  L  N
ctgtgtttgatattattagtggtgtccaatagccgtttagttttggaaaatatcatcaaa
 L  C  L  I  L  L  V  V  S  N  S  R  L  V  L  E  N  I  I  K
tatggcatattagctaacccgattgactgggtacatctgtttctaaaacaaccatatagt
 Y  G  I  L  A  N  P  I  D  W  V  H  L  F  L  K  Q  P  Y  S
tggcctaatgttctactgtttctctctatcaatatcttcattttgttctcactttctctg
 W  P  N  V  L  L  F  L  S  I  N  I  F  I  L  F  S  L  S  L
gagcatctttacatcaagagatacttatcagagagattaggagccatacttcaagcattg
 E  H  L  Y  I  K  R  Y  L  S  E  R  L  G  A  I  L  Q  A  L
aatgtagctacattattatctttaccagctacaattatatacattttacatcctaaccct
 N  V  A  T  L  L  S  L  P  A  T  I  I  Y  I  L  H  P  N  P
atattttcatcagtttactgtcatttgtaacagtagtttcattaaaattaatctcatat
 I  F  S  S  V  L  L  S  F  V  T  V  V  S  L  K  L  I  S  Y
gcctgtgttaataaatggtgtcgtgataatcttaaaaatgatgccaagaaagattttaga
 A  C  V  N  K  W  C  R  D  N  L  K  N  D  A  K  K  D  F  R
aggaggaagactgttagtgtctcagaaaatggtgatcaaaggacgaaagtctcaaatgat
 R  R  K  T  V  S  V  S  E  N  G  D  Q  R  T  K  V  S  N  D
gagatgaaagattatctggttaaatatccagataatgtcaattttaaagatctttattat
 E  M  K  D  Y  L  V  K  Y  P  D  N  V  N  F  K  D  L  Y  Y
ttcataattgcacctacattggtttatgaactcaattttcccagatctgctagaataaga
 F  I  I  A  P  T  L  V  Y  E  L  N  F  P  R  S  A  R  I  R
aagaggtttctcgtcaaacggattgttgaaatgttgtttttgtctcaacttataatggct
 K  R  F  L  V  K  R  I  V  E  M  L  F  L  S  Q  L  I  M  A
ctaatacaacagtggattataccaactgtcaataatgccatgaaaccattagctgatatg
 L  I  Q  Q  W  I  I  P  T  V  N  N  A  M  K  P  L  A  D  M
gctttatacagagtagctgaaagactcttgaaattatctatacccaatcattttatatgg
 A  L  Y  R  V  A  E  R  L  L  K  L  S  I  P  N  H  F  I  W
ttgttatttttctactggttctttcattcaacattaaatgtgatagcagaagtacttaga
 L  L  F  F  Y  W  F  F  H  S  T  L  N  V  I  A  E  V  L  R
tttggtgatcgtgtgttctatagagattggtggaatgctgagaccgtttcaagattctgg
 F  G  D  R  V  F  Y  R  D  W  W  N  A  E  T  V  S  R  F  W
caagactggaatgttcctgttcataggtgggcgttacgacatttgtataagccaatgatg
 Q  D  W  N  V  P  V  H  R  W  A  L  R  H  L  Y  K  P  M  M
cgagctggattttcaaaagttaatgcatccattgcagttttttcttgtcagccttcttt
 R  A  G  F  S  K  V  N  A  S  I  A  V  F  F  L  S  A  F  F
catgagtatttgttatccataccctgagaatgtttaaaatttgggcattctctgctatg
 H  E  Y  L  L  S  I  P  L  R  M  F  K  I  W  A  F  S  A  M
attggacaggtgcctttagccttgttgacatccaagtataaatttctacaaggaacctgg
 I  G  Q  V  P  L  A  L  L  T  S  K  Y  K  F  L  Q  G  T  W
ggcaatgttattatgtggttgtctttaataatgggtcaacctattgctatattagcttat
 G  N  V  I  M  W  L  S  L  I  M  G  Q  P  I  A  I  L  A  Y
gtacatgactattatattattaacatgggaacactccataatcaaaccatgacatttgca
 V  H  D  Y  Y  I  I  N  M  G  T  L  H  N  Q  T  M  T  F  A
taa
 -
```

Figure 3.2: Complete *P. depressa* DGAT1 isolated nucleotide sequence including the deduced amino acid sequence.

```
atggcgagcaaaccaagacttcgtcgcgtgaatagttacagtaaatcagaagatagacaa
 M  A  S  K  P  R  L  R  R  V  N  S  Y  S  K  S  E  D  R  Q
gtggaggatagaaaatttcggtcgaacaggatagataaaccaattcacttgcatgcagac
 V  E  D  R  K  F  R  S  N  R  I  D  K  P  I  H  L  H  A  D
tccttattcagttcaacaagtggcttcacagactacagtggcttcttcaacctctgtatg
 S  L  F  S  S  T  S  G  F  T  D  Y  S  G  F  F  N  L  C  M
atattactgatattgtcaaatttacgagtagctcttgaaaatatcatcaagtatggtatt
 I  L  L  I  L  S  N  L  R  V  A  L  E  N  I  I  K  Y  G  I
cttatccagccgttgtcatggttacaatatatgataaaagaaccctacagctggccaagt
 L  I  Q  P  L  S  W  L  Q  Y  M  I  K  E  P  Y  S  W  P  S
tctgttctgtttttgtgttgtaatacctttatacttgtcacattctgtgtggaacgagca
 S  V  L  F  L  C  C  N  T  F  I  L  V  T  F  C  V  E  R  A
ttagcaaaggatctgatatcagaaaaactggggatgattttgcacattgcgaaccttgct
 L  A  K  D  L  I  S  E  K  L  G  M  I  L  H  I  A  N  L  A
gctgagatcagttttccggctgctgtggtgtggattcgccacccttttcccaatattttca
 A  E  I  S  F  P  A  A  V  V  W  I  R  H  P  F  P  I  F  S
tcagtcaccctcgccgtctatgttgtgatcttcctgaaactcatctcttacattcaagtg
 S  V  T  L  A  V  Y  V  V  I  F  L  K  L  I  S  Y  I  Q  V
aatgcctggtgccgtcaagcattgctcaacgtaagcaaaagacggttccgctcgagaaat
 N  A  W  C  R  Q  A  L  L  N  V  S  K  R  R  F  R  S  R  N
aagtcctattctgctgccctcaatacgtatgcagaagaatgggaaaataacgaaagtgct
 K  S  Y  S  A  A  L  N  T  Y  A  E  E  W  E  N  N  E  S  A
ttgattgggaaaagcaatggatatttggaaaaattggaagtcaacaagtatccaaaaaac
 L  I  G  K  S  N  G  Y  L  E  K  L  E  V  N  K  Y  P  K  N
ctcacttttagtgatctctattacttcatggttgtgccaactctgtgttatgaacttcac
 L  T  F  S  D  L  Y  Y  F  M  V  V  P  T  L  C  Y  E  L  H
tttccgatgtctgccaggattcgaatgagatttttgctcagaagatttgctgaaatgata
 F  P  M  S  A  R  I  R  M  R  F  L  L  R  R  F  A  E  M  I
ttcctagctgccttaataggaggccttgttcaacagtggatattacccacagtgaataat
 F  L  A  A  L  I  G  G  L  V  Q  Q  W  I  L  P  T  V  N  N
tccatggtgcctttcaaggagatggacatcggaagaatatttgaaagacttcttaaactt
 S  M  V  P  F  K  E  M  D  I  G  R  I  F  E  R  L  L  K  L
gcaattccaaaccacatcatctggttgatatttttctactgcttctttcatagtgtttta
 A  I  P  N  H  I  I  W  L  I  F  F  Y  C  F  F  H  S  V  L
aatgtggtcggtgagatcactctttttggtgatcgaacgttttatcaggattggtggaat
 N  V  V  G  E  I  T  L  F  G  D  R  T  F  Y  Q  D  W  W  N
gccaccaccgtcgagggattctggcagtcatggaatatccctgttcacaagtgggctgtc
 A  T  T  V  E  G  F  W  Q  S  W  N  I  P  V  H  K  W  A  V
aggcatctttacaaacccatgcttgccaacaaattctccaagaaacatgctacggttgct
 R  H  L  Y  K  P  M  L  A  N  K  F  S  K  K  H  A  T  V  A
gttttcatattgtctgcatttttcatgagtatttagtgtcaattcctttacggatgttc
 V  F  I  L  S  A  F  F  H  E  Y  L  V  S  I  P  L  R  M  F
cggatatgggctttcagtgccatgttatcacaggtgcctatggcctggataacgcggaag
 R  I  W  A  F  S  A  M  L  S  Q  V  P  M  A  W  I  T  R  K
tatctccctggccgttatggcaacatagctgtctggttctccttgataatcggacagcca
 Y  L  P  G  R  Y  G  N  I  A  V  W  F  S  L  I  I  G  Q  P
attgccctgctcatgtattaccatgactacttcttgattaacaagactgtggggtcagta
 I  A  L  L  M  Y  Y  H  D  Y  F  L  I  N  K  T  V  G  S  V
tga
 -
```

Figure 3.3: *P. dumerilii* DGAT1 isolated nucleotide sequence including the deduced amino acid sequence.

The cellular localization of DGAT1 of both species predicted by Euk-mPLoc 2.0 (Table 3.5) indicates that this protein localizes at the Endoplasmic reticulum (ER) which agrees with previous descriptions (Mcfie et al., 2010).

Table 3.5: DGAT1 protein cellular localization prediction.

Species	Analysis software	Cellular localization
P. depressa	Euk-mPLoc 2.0	Endoplasmic reticulum
P. dumerilii		Endoplasmic reticulum

P. depressa and *P. dumerilii* DGAT1 predictions of transmembrane domain (TrM) structures were performed, and compared with *H. sapiens*, *M. musculus*, *D. rerio*, *L. gigantea* and *C. teleta*. For each metazoan DGAT1 sequence examined, nine hydrophobic regions that corresponds to nine high probability regions for TrM sequences were observed, each located at similar positions (Fig. 3.4 and 3.5).

The analysis of DGAT1 amino acid sequence alignments revealed that key residues relevant for the biochemical activity (substrate recognition and catalysis) are fully conserved (Fig. 3.5) (Holmes et al., 2010).

Next, we performed a NJ analysis to clarify the phylogenetic relationship between our DGAT1 isolated sequences with other metazoan species from four distinct lineages (cnidarian, chordates, ecdysozoa and lophothrochozoa). NJ phylogenetic analysis resulted in a well-resolved tree and as expected, DGAT1 from *P. depressa* and *P. dumerilii* were cluster together to other lophotrochozoan species (Fig. 3.6).

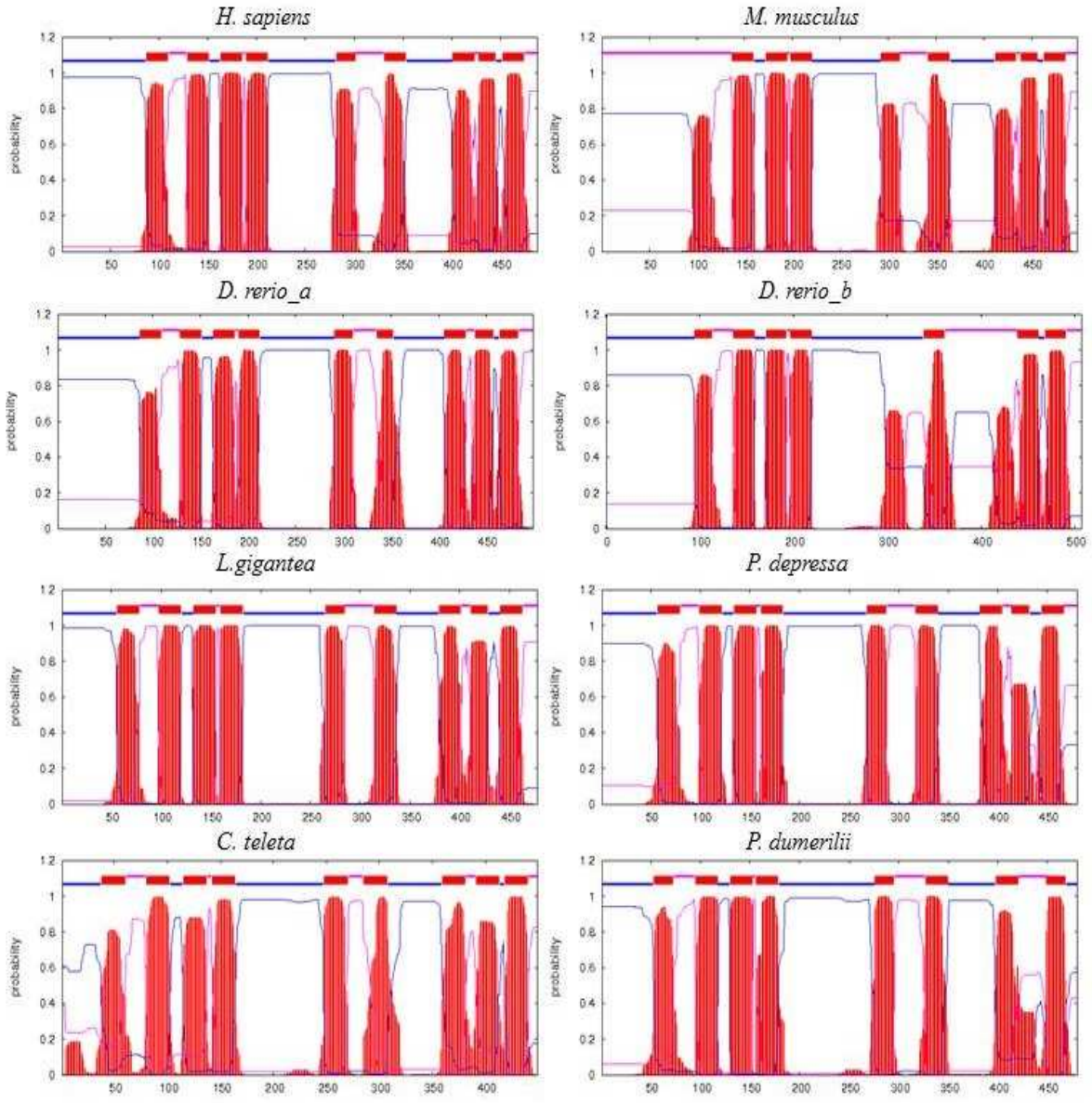
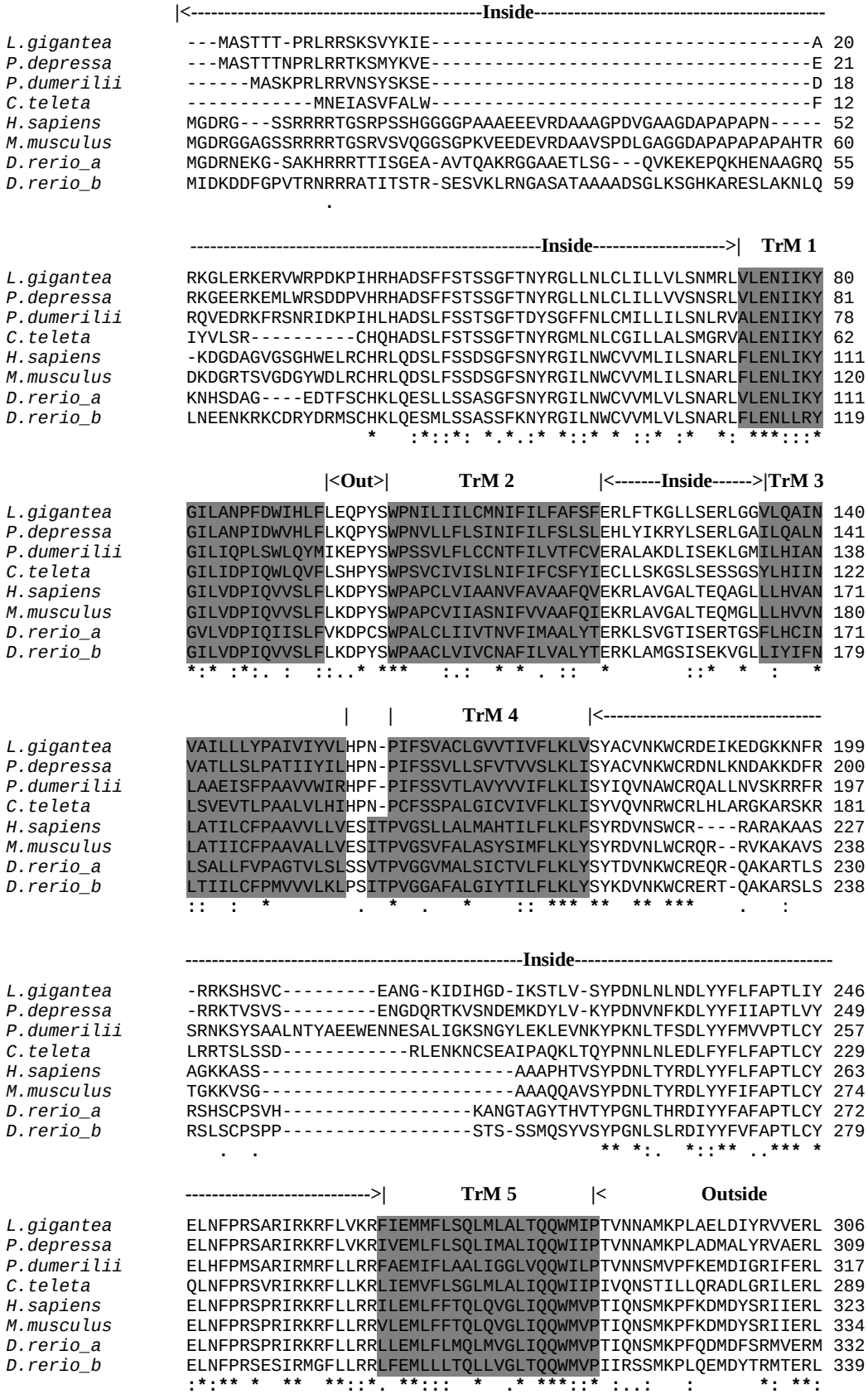


Figure 3.4: Predicted transmembrane domain for DGAT1 *P. depressa*, *P. dumerilii*, *L. gigantea*, *C. teleta*, *H. sapiens*, *D. rerio*, and *M. musculus*. The TMHMM Server plots the probability of the ALDH sequence forming a transmembrane helix (0-1.0 on the y-axis) shown in red for the relevant amino acid sequences. The x-axis describes the amino acid sequence length from the N-terminus to the C-terminus in each case. For each species tested nine transmembrane helices were identified for DGAT1 sequences. Regions of all sequences predicted to be located inside or outside the endoplasmic reticulum membrane are shown in blue and pink, respectively.


|<----------Inside----------
L.gigantea ---MASTTT-PRLRRSKSVYKIE----------A 20
P.depressa ---MASTTTNPRLRRTKSMYKVE----------E 21
P.dumerilii ------MASKPRLRRVNSYSKSE----------D 18
C.teleta ------------MNEIASVFALW----------F 12
H.sapiens MGDRG---SSRRRRTGSRPSSHGGGGPAAAEEEVRDAAAGPDVGAAGDAPAPAPN----- 52
M.musculus MGDRGGAGSSRRRRTGSRVSVQGGSGPKVEEDEVRDAAVSPDLGAGGDAPAPAPAHTR 60
D.rerio_a MGDRNEKG-SAKHRRRTTISGEA-AVTQAKRGGAAETLSG---QVKEKEPQKHENAAGRQ 55
D.rerio_b MIDKDDFGPVTRNRRRATITSTR-SESVKLRNGASATAAAADSGLKSGHKARESLAKNLQ 59
----------Inside---------->| TrM 1
L.gigantea RKGLERKERVWRPDKPIHRHADSFFSTSSGFTNYRGLLNLCLILLVLSNMRLVLENIIKY 80
P.depressa RKGEERKEMLWRSDDPVHRHADSFFSTSSGFTNYRGLLNLCLILLVVSNSRLVLENIIKY 81
P.dumerilii RQVEDRKFRSNRIDKPIHLHADSLFSSTSGFTDYSGFFNLCMILLILSNLRVALENIIKY 78
C.teleta IYVLSR----------CHQHADSLFSTSSGFTNYRGMLNLCGILLALSMGRVALENIIKY 62
H.sapiens -KDGDAGVGSGHWELRCHRLQDSLFSSDSGFSNYRGILNWCVVMLILSNARLFLENLIKY 111
M.musculus DKDGRTSVGDGYWDLRCHRLQDSLFSSDSGFSNYRGILNWCVVMLILSNARLFLENLIKY 120
D.rerio_a KNHSDAG----EDTFSCHKLQESLLSSASGFSNYRGILNWCVVMLVLSNARLVLENLIKY 111
D.rerio_b LNEENKRKCDRYDRMSCHKLQESMLSSASSFKNYRGILNWCVVMLVLSNARLFLENLLRY 119
|<Out>| TrM 2 |<-------Inside------>|TrM 3
L.gigantea GILANPFDWIHLFLEQPYSWPNILIILCMNIFILFAFSFERLFTKGLLSERLGGVLQAIN 140
P.depressa GILANPIDWVHLFLKQPYSWPNVLLFLSINIFILFSLSLEHLYIKRYLSERLGAILQALN 141
P.dumerilii GILIQPLSWLQYMIKEPYSWPSSVLFLCCNTFILVTFCVERALAKDLISEKLGMILHIAN 138
C.teleta GILIDPIQWLQVFLSHPYSWPSVCIVISLNIFIFCSFYIECLLSKGSLSESSGSYLHIIN 122
H.sapiens GILVDPIQVVSLFLKDPYSWPAPCLVIAANVFAVAAFQVEKRLAVGALTEQAGLLLHVAN 171
M.musculus GILVDPIQVVSLFLKDPYSWPAPCVIIASNIFVVAAFQIEKRLAVGALTEQMGLLLHVVN 180
D.rerio_a GVLVDPIQIISLFVKDPCSWPALCLIIVTNVFIMAALYTERKLSVGTISERTGSFLHCIN 171
D.rerio_b GILVDPIQVVSLFLKDPYSWPAACLVIVCNAFILVALYTERKLAMGSISEKVGLLIYIFN 179
| | TrM 4 |<----------
L.gigantea VAILLLYPAIVIYVLHPN-PIFSVACLGVVTIVFLKLVSYACVNKWCRDEIKEDGKKNFR 199
P.depressa VATLLSLPATIIYILHPN-PIFSSVLLSFVTVVSLKLISYACVNKWCRDNLKNDAKKDFR 200
P.dumerilii LAAEISFPAAVVWIRHPF-PIFSSVTLAVYVVIFLKLISYIQVNAWCRQALLNVSKRRFR 197
C.teleta LSVEVTLPAALVLHIHPN-PCFSSPALGICVIVFLKLISYVQVNRWCRLHLARGKARSKR 181
H.sapiens LATILCFPAAVVLLVESITPVGSLLALMAHTILFLKLFSYRDVNSWCR----RARAKAAS 227
M.musculus LATIICFPAAVALLVESITPVGSVFALASYSIMFLKLYSYRDVNLWCRQR--RVKAKAVS 238
D.rerio_a LSALLFVPAGTVLSLSSVTPVGGVMALSICTVLFLKLYSYTDVNKWCREQR-QAKARTLS 230
D.rerio_b LTIILCFPMVVVLKLPSITPVGGAFALGIYTILFLKLYSYKDVNKWCRERT-QAKARSLS 238
----------Inside----------
L.gigantea -RRKSHSVC---------EANG-KIDIHGD-IKSTLV-SYPDNLNLNDLYYFLFAPTLIY 246
P.depressa -RRKTVSVS---------ENGDQRTKVSNDEMKDYLV-KYPDNVNFKDLYYFIIAPTLVY 249
P.dumerilii SRNKSYSAALNTYAEEWENNESALIGKSNGYLEKLEVNKYPKNLTFSDLYYFMVVPTLCY 257
C.teleta LRRTSLSSD-----------RLENKNCSEAIPAQKLTQYPNNLNLEDLFYFLFAPTLCY 229
H.sapiens AGKKASS----------AAAPHTVSYPDNLTYRDLYYFLFAPTLCY 263
M.musculus TGKKVSG----------AAAQQAVSYPDNLTYRDLYYFIFAPTLCY 274
D.rerio_a RSHSCPSVH----------KANGTAGYTHVTYPGNLTHRDIYYFAFAPTLCY 272
D.rerio_b RSLSCPSPP----------STS-SSMQSYVSYPGNLSLRDIYYFVFAPTLCY 279
----------->| TrM 5 |< Outside
L.gigantea ELNFPRSARIRKRFLVKRFIEMMFLSQLMLALTQQWMIPTVNNAMKPLAELDIYRVVERL 306
P.depressa ELNFPRSARIRKRFLVKRIVEMLFLSQLIMALIQQWIIPTVNNAMKPLADMALYRVAERL 309
P.dumerilii ELHFPMSARIRMRFLLRRFAEMIFLAALIGGLVQQWILPTVNNSMVPFKEMDIGRIFERL 317
C.teleta QLNFPRSVRIRKRFLLKRLIEMVFLSGLMLALIQQWIIPIVQNSTILLQRADLGRILERL 289
H.sapiens ELNFPRSPRIRKRFLLRRILEMLFFTQLQVGLIQQWMVPTIQNSMKPFKDMDYSRIIERL 323
M.musculus ELNFPRSPRIRKRFLLRRVLEMLFFTQLQVGLIQQWMVPTIQNSMKPFKDMDYSRIIERL 334
D.rerio_a ELNFPRSPRIRKRFLLRRLLEMLFLMQLMVGLIQQWMVPTIQNSMKPFQDMDFSRMVERM 332
D.rerio_b ELNFPRSESIRMGFLLRRLFEMLLLTQLLVGLTQQWMVPIIRSSMKPLQEMDYTRMTERL 339

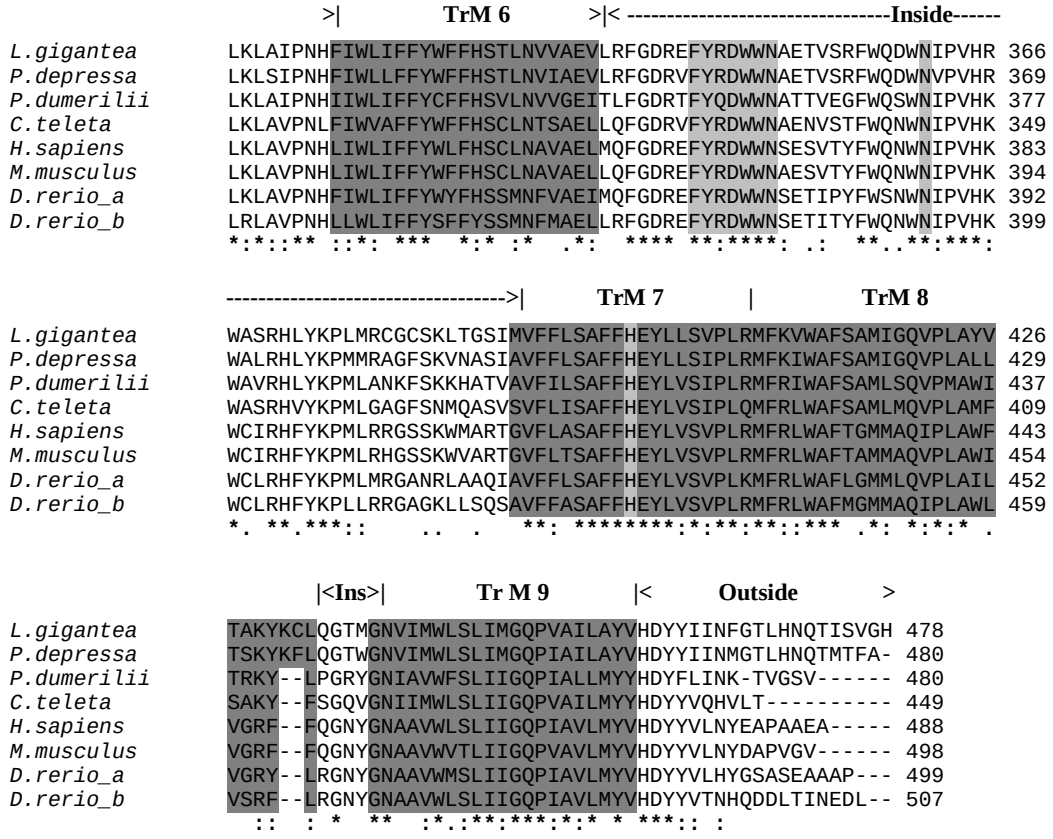


Figure 3.5: DGAT1 amino acid sequence alignments constructed with CLUSTAL 2.1 multiple sequence alignment. Dark grey shading shows the predicted transmembrane domains (TrM); key histidine (H) and Asparagine (N) residues (active site within TrM 7), and fatty acyl CoA binding site (FXRDWWN) are shown in grey; the predicted topology for the polypeptide chains are represented as inside the endoplasmic reticulum (ER) (the lumen) and outside the ER (cytosol). (*) - shows identical residues; (:) - shows one conservative amino acid substitution; and (.) shows two conservative amino acid substitutions. Prediction of key residues has been inferred in previously works (Holmes et al., 2010; Mcfie et al., 2010).

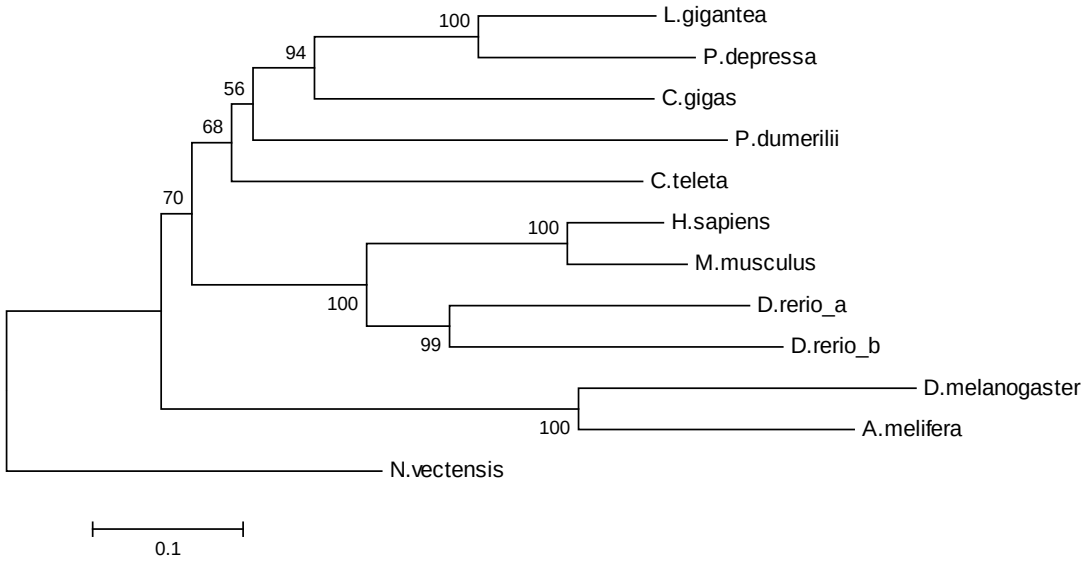


Figure 3.6: DGAT1 evolutionary history was inferred using the Neighbor-Joining (NJ) method. The percentage of replicate trees in which the associated *taxa* clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree was rooted with DGAT1 sequence from *N. vectensis*.

3.4.2 Expression of DGAT1 in *P. depressa* injected with ROL

Given that gene transcription of mammalian DGAT1 orthologues can be upregulated by ROL, we performed an exposure experiment with *P. depressa* and carried out gene expression quantification in the gonads and digestive gland from *in vivo* ROL-injected male and female *P. depressa* using Real-time PCR. However, no significant differences in the *Dgat1* gene expression were found between treatments ($P > 0.05$).

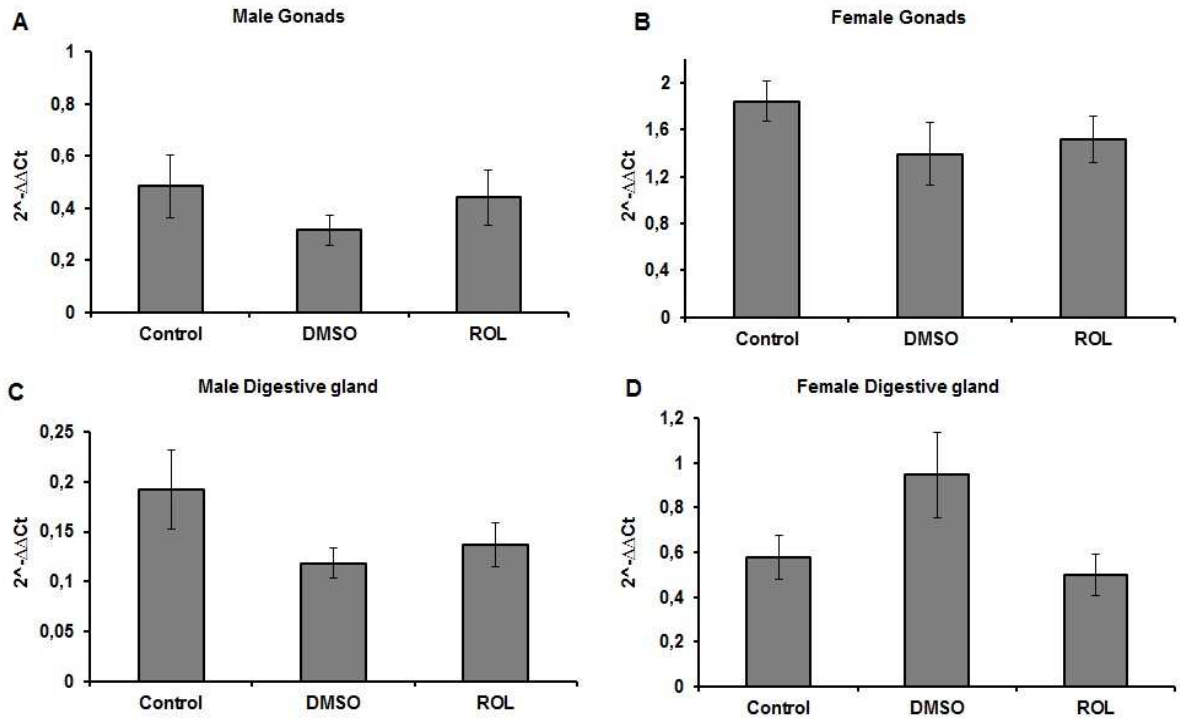


Figure 3.7: Tissues *Dgat1* expression data normalized with the reference gene elongation factor in *P. depressa* from the *in vivo* intramuscular injection assay. Values are presented as mean ± standard error (n = 10-16). *Dgat1* expression on A) male and B) female gonads for the test conditions (no injected, control DMSO and ROL injected groups); DGAT1 expression on C) male and D) female digestive gland in the three test conditions.

3.5 Discussion

In vertebrates, retinoid levels are tightly controlled by an effective homeostatic mechanism involving two major enzymatic pathways for retinol storage into REs, LRAT and DGAT1 (D'Ambrosio et al., 2011). The presence of retinoid storage forms in the invertebrate mollusk species *Osilinus lineatus*, *P. depressa* and *P. vulgata* (Gesto et al., 2012, 2013, 2016) as well in a demosponge species *Geodia cydonium* (Biesalski et al., 1992) suggest that the ability to store retinoids as REs is not a vertebrate innovation and supports an earlier evolutionary origin for the ability to maintain retinoid homeostasis through fatty acid esterification (Albalat et al., 2011; André et al., 2014). Nevertheless, the exact enzymatic cascade involved in the strict control of retinoid levels through retinol storage remains to be demonstrated.

DGAT1, recently described to be a multifunction enzyme, capable of esterifying ROL into REs in some vertebrate tissues (Yen et al., 2005a; Wongsiriroj et al., 2008; Shih et al., 2009) has been proposed to be in invertebrates the major protein responsible to keep a vitamin A homeostatic balance (Albalat et al., 2011; André et al., 2014). Nonetheless, this hypothesis has only been postulated through the observation that LRAT

is vertebrate specific while DGAT1 orthologues are present throughout the genomes of metazoans (Albalat et al., 2011; Turchetto-Zolet et al., 2011; André et al., 2014). No isolation and functional characterization was performed so far. In this work, we aimed to isolate DGAT1 in two marine lophotrochozoan species, *P. depressa* (mollusks) and *P. dumerilii* (annelid) and evaluated if key residues implicated in the catalytic activity were conserved. In addition, we aimed to address the possible role DGAT1 in ROL esterification and mobilization in *P. depressa* through a combination of *in vivo* injection approach and gene expression quantification.

First, we isolated the DGAT1 gene. DGAT1 belongs to a large family of membrane-bound O-acyltransferases (MBOAT) that also includes acyl-CoA: cholesterol acyltransferase-1 and -2 (ACAT-1 and -2), which catalyze cholesterol ester biosynthesis (Mcfie et al., 2010). A characteristic of protein MBOAT family members is the presence of a conserved "signature" residue motif, FYXDWWN that is implicated in the binding of fatty acyl-CoA, a common substrate (Oelkers et al., 1998; Hofmann, 2000; Chang et al., 2001; Guo et al., 2001). This motif is present in both DGAT1 sequences from *P. depressa* (Phe346-Tyr347-Arg348-Asp349-Trp340-Trp341-Asn342) and *P. dumerilii* (Phe354-Gln-355-Arg356-Asp357-Trp358-Trp359-Asn360) (Fig. 3.5), suggesting a putative conserved ability of recognizing and using fatty acyl CoA as a substrate. In vertebrates DGAT1, a highly conserved histidine residue is known to be essential for enzyme function (Mcfie et al., 2010). Mutation of His-426 of murine DGAT1 resulted in a non-functional enzyme lacking the ability to synthesize triacylglycerols, retinyl and wax esters in an *in vitro* acyltransferase assay (Mcfie et al., 2010). This histidine residue is present in the isolated DGAT1 coding sequences (Fig. 3.5), thus suggesting its involvement in triacylglycerols, waxes and retinyl esters synthesis pathways. The use of TMHMM analysis software indicated that *P. depressa* and *P. dumerilii* DGAT1 had nine potential transmembrane domains located in similar conserved positions to that of other species (Oelkers et al., 1998; Holmes et al., 2010). Taken together this data suggested that DGAT1 structurally are evolutionary conserved, at least between vertebrates and lophotrochozoans.

To assess whether DGAT1 might be in part responsible for ROL esterification and storage in lophotrochozoa we performed a preliminary approach based on ROL *in vivo* intramuscular injections, previously successfully used in *O. lineatus*, to provide insights into the ability of ROL esterification to REs (Gesto et al., 2012). Then, we analyzed through RT-PCR the DGAT1 expression on selected tissues of *P. depressa* after ROL injection. We selected the gonads and digestive gland since these were the tissues where REs and ROL levels were detected at higher levels in *P. depressa* (Gesto et al., 2013) and other gastropods, the *O. lineatus* (Gesto et al., 2012) and *P. vulgata* (Gesto et al., 2016). Non-polar retinoids, retinol and REs (mainly the palmitate form), were detected

only in the limpet *P. depressa* male digestive gland–gonad complex, whereas in females gland–gonad complex were below detection limit (Gesto et al., 2013). Hence, previous evidences indicate that an enzymatic pathway for ROL esterification must be present and active, at least, in male. Therefore, this exploratory approach hypothesizes that a gene expression upregulation could be expected in DGAT1, particularly in males, following ROL injection. Yet, no significant changes in *Dgat1* transcription levels were observed. This data does not give further support to the hypothesis of DGAT1 involvement in ROL metabolism in mollusks. However, we cannot rule out the hypothesis that DGAT1 involvement is such process occurs *in vivo* at a very small scale being the major role performed with other ARAT enzyme whose identity remains unknown. Alternatively, DGAT1 expression dynamic might not be directly regulated by ROL levels as their vertebrates' counterparts. Wongsiriroj et al., (2008) using *in vivo* approach with *Dgat1* deficient mice models, showed that in times of normal dietary retinoid intake LRAT accounts for the preponderance of ROL esterification and storage in the intestine, having DGAT1 a major role only when large retinoid amounts are present. DGAT1 is expressed in hepatocytes and hepatic stellate cells, but in LRAT null mice does not catalyze retinol esterification in both types of hepatic cells, since no retinyl esters formation were detected (O'Byrne et al., 2005).

3.6 Conclusion

Further studies focusing in the enzyme functionality should be performed to demonstrate lophotrochozoans DGAT1 capability to esterify ROL. Since in annelids and mollusks the main functional residues are present in the coding sequence we can predict, in addition to ROL esterification that it may also be involved in other metabolic pathways such of triacylglycerol and/or wax esters synthesis.

3.7 Acknowledgements

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CHAPTER 4

4 Aldehyde dehydrogenase type 1 enzyme in lophotrochozoans and their implication in retinoic acid synthesis: an evolutionary perspective

4.1 Abstract

Aldehyde dehydrogenase type 1 (ALDH1) are members of the family of proteins that catalyze the oxidation of toxic aldehyde. In vertebrates ALDH1 enzyme have a crucial role on retinoid metabolism, being responsible for the conversion of retinaldehyde (retinal or RAL) into the retinoid active form, the retinoic acid (RA). Genome investigations have hinted the presence of orthologues from this protein outside chordates. To date, no cloning and/or functional characterization of ALDH1 has been performed in protostomes.

In the present study we report the isolation, phylogenetic analysis and functional characterization of ALDH1-like enzymes in two marine lophotrochozoan species: the annelid *Platynereis dumerilii* and the mollusk *Patella depressa*. Through an *in vitro* approach assay we have demonstrated that the isolated ALDH1s for both studied species are not able to use RAL has a substrate since no RA production could be detected. The proteins substrate entry channel (SEC) revealed a signature residue's substitutions that suggest a role in processing small aldehydes similar to vertebrates ALDH2, other than processing retinal for RA synthesis.

4.2 Introduction

Vitamin A (retinol or ROL) is an essential fat-soluble micronutrient which has long been known to be indispensable for several crucial functions, such as vision, embryonic development, reproduction and immunity system regulation (Blomhoff and Blomhoff, 2006). The physiological functions of ROL are mainly mediated via its main biological active metabolite, retinoic acid (RA). Generally, RA acts as a ligand for two transcription factors of the nuclear receptors superfamily (NR), the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) (Blomhoff and Blomhoff, 2006). A growing number of studies have revealed that a fine tuned regulation of RA levels and tissue distribution is required for maintaining the biological functions, which is achieved through a balance between synthesis and degradation routes (Clagett-Dame and DeLuca, 2002; Theodosiou et al., 2010). Both RA insufficiency and excess can cause several negative effects in vertebrates. During embryonic development, for instance, RA imbalance might results in severe abnormalities in the offspring including fetal congenital malformations, affecting the heart, ocular tissues and the central nervous system (CNS) that ultimately might lead to death (Maden et al., 1998; Zile, 1998, 2001; Clagett-Dame and DeLuca, 2002; Zile, 2004; Clagett-Dame and Knutson, 2011). Regarding RA synthesis pathway, in vertebrates it depends on two major steps: 1) ROL is first reversibly oxidized to retinaldehyde (Retinal

or RAL) by the action of members of both the cytosolic alcohol dehydrogenases (ADH) and microsomal short-chain dehydrogenase/reductases (SDR) families, which are also proposed to play a role in the oxidation of RAL reduction back to ROL (Duester et al., 2003; Theodosiou et al., 2010); 2) Next, RAL is subsequently irreversibly oxidized to RA a reaction that can be mediated by three aldehyde dehydrogenase (ALDH) family members: ALDH1a1, ALDH1a2 and ALDH1a3 (Duester et al., 2003). The ALDH1s are cytosolic enzymes that display quite distinct spatiotemporal expression patterns and are crucial for maintaining RA levels in tissues mainly during embryonic development. For instance, in vertebrates ALDH1a1 is expressed during early embryogenesis particularly in developing lung, stomach and intestine epithelial, and in dorsal retina; whereas in the adult is expressed in the dorsal retina, stomach and small intestine (McCaffery et al., 1992; Ang and Duester, 1999; Frota-Ruchon et al., 2001; Niederreither et al., 2002; Fan et al., 2003). Regarding ALDH1a2, it has been demonstrated that *Aldh1a2*-/-null mice exhibit rhombomeres segmentation impairment, heart, hindbrain and neural crest defects (Niederreither et al., 1999, 2000, 2001). In their turn the ALDH1a3 has also a crucial role during embryonic development mainly related to the normal eye formation olfactory pit and forebrain, tissues where it is expressed (Dupé et al., 2003; Molotkova et al., 2007).

Like other key players of the retinoid metabolic and signaling cascade, the ALDH1 enzymes were initially thought to be a vertebrate-specific novelty (Simões-Costa et al., 2008; Albalat, 2009; Theodosiou et al., 2010). Conversely, genome searches and phylogenetic analysis of non-model invertebrate species revealed otherwise, suggesting a more basal origin of the RA synthesis pathway mediated by ALDH1s (Cañestro et al., 2006; Albalat and Cañestro, 2009; Sobreira et al., 2011). Interestingly, in invertebrates the number of ALDH1-like enzymes seems to be quite variable comparing to vertebrates, in which tetrapods have three and fish have only two main ALDH1 genes paralogs (Cañestro et al., 2006; Albalat, 2009; Albalat and Cañestro, 2009; Sobreira et al., 2011). For instance, genome search predicts the presence of six ALDH1 in the cephalochordate *Brachiostoma floridae*, four and two putative ALDH1 genes in the urochordate *Ciona intestinalis* and *C. savignyi*, respectively; five in the hemichordate *Saccoglossus kowalevskii*; and three in lophotrochozoan mollusk *Lottia gigantea* and annelid *Capitella teleta* (Cañestro et al., 2006; Albalat and Cañestro, 2009; Sobreira et al., 2011). The variable number of ALDH1s orthologues in different *taxa* suggests that the enzyme underwent independent multiple gene duplications within lineage-specific during evolution (Cañestro et al., 2006; Sobreira et al., 2011). However, an *in silico* study revealed that not all invertebrate ALDH1 enzymes identified might be capable of using RAL as a substrate to synthesis RA according to their substrate entry channels (SECs) size and amino acid signature (Sobreira et al., 2011). Some resemble a feature SECs signature typical of

ALDH2 (Sobreira et al., 2011) another member of the ALDH protein family with high sequence similarity to ALDH1 suggesting that might share a common origin (Duester, 1996; Cañestro et al., 2006). In contrast, to ALDH1, ALDH2 are not involved on the RA metabolic synthesis pathway instead process the catalytic oxidation of small toxic aldehydes playing a crucial role in cell protection and detoxification (Yoshida et al., 1998). Apart from genome identification and *in silico* evidences, ALDH1 orthologues isolation and functional characterization outside vertebrate is missing, hampering our knowledge on RA synthesis metabolic routes evolution. So far, only in the mollusk *Lymnaea stagnalis* was reported the presence of an ALDH1-like enzyme activity in central nervous system (CNS) extracts (Dmetrichuk et al., 2008). Latter, ALDH1-like enzyme was isolated (Charter, 2011) and demonstrated that has an *in vivo* function on *L. stagnalis* long term memory (Rothwell and Spencer, 2014). The authors exposed specimens to two inhibitors, Citral and 4-diethylaminobenzaldehyde, of the ALDH1 enzyme. Following exposure to these inhibitors for 72 h, they noticed that long-term memory formation of mollusks was inhibited (Rothwell and Spencer, 2014). Thus, together these evidences suggest a possible conserved role of ALDH1s in RA synthesis in mollusks.

In order to obtain insights into the evolutionary diversification of RA biosynthesis through ALDH1 enzymatic activity, we describe here the isolation and functional characterization of two ALDH1-like sequences in two marine lophotrochozoans, the mollusk *Patella depressa* and the annelid *Platynereis dumerilii.* The functional characterization has revealed that the isolated enzymes are not able to use RAL as a substrate to produce RA, suggesting that must have another role. We propose that the inability to perform the irreversible oxidation of RAL into RA is due to the substitution of key residues on the proteins SECs.

4.3 Material and Methods

4.3.1 Compounds

The all-*trans*-retinal (≥ 98%), 9-*cis*-retinal, 13-*cis*-retinal, all-*trans*-RA (≥ 98%), 9-*cis*-RA (≥ 98%), 13-*cis*-RA (≥ 98%), *N,N*-Dimethylformamide, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Potassium chloride (KCl), Ethylenediaminetetraacetic acid (EDTA) DL-Dithiothreitol (DTT), Bovine serum albumin (BSA), trifluoroacetic acid (TFA) and β-nicotinamide adenine dinucleotide phosphate (NADP+) sodium salt hydrate were all purchased from Sigma-Aldrich. Methanol, *n*-hexane High performance liquid chromatography (HPLC) grade gradient and nicotinamide adenine dinucleotide (NAD+) lithium salt were purchased from VWR.

4.3.2 RNA extraction and cDNA synthesis in *Platynereis dumerilii* and *Patella depressa*

Immature specimens of *P. dumerilii* (Pd) were collected at low tide in June 2012 in Mindelo rocky shore (north, Portugal). After transport to the laboratory in plastic boxes with seawater from the sampling site, organisms were acclimatized by placing plastic boxes, filled with 500 mL of natural seawater with aeration. Temperature was set to 18ºC, light: dark rhythm 14 h: 10 h during acclimatization for a day before sacrifice. Then, animals were anesthetized in a 4% magnesium chloride ($MgCl_2$) solution for 10 min before sacrifice to collect tissues for RNA extraction. Total RNA, from the annelid, was extracted from a tissue mix of the head and body using the Kit illustra RNAspin Mini RNA Isolation (GE Healthcare, animal tissues protocol), with on column DNAse I digestion.

Adult limpet *P. depressa* animals (Lp) were collected at Homem do Leme (Foz, Porto, Portugal) in March of 2013. The limpets were transported to the laboratory in plastic boxes. Animals were maintained in 30 L aquaria filled with natural sea water with aeration for 2 days, at 15ºC with a photoperiod set for light: dark rhythm 12 h: 12 h. Animals were not fed during the acclimation period. Then, specimens were sacrificed and gonads excised for RNA extraction, after being sedated in a 7% magnesium chloride ($MgCl_2$) solution for 10 min. Total RNA was extracted from the gonad of adult males using a protocol combination of PureZOL RNA Isolation Reagent (Bio-Rad), to homogenize the tissues and isolate the RNA from proteins and genomic DNA; and used of the Kit illustra RNAspin Mini RNA Isolation (GE Healthcare, animal tissues protocol) to subsequent RNA extraction from the ethanol step onwards.

For both test organisms the cDNA synthesis was performed with the iScript™cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions, using 1 µg of total RNA.

4.3.3 Gene isolation in *P. dumerilii* and *P. depressa*

Sets of degenerate oligonucleotide primers were designed with the software Consensus-degenerate hybrid–oligonucleotide primers (CODEHOP; http://blocks.fhcrc.org/codehop.html; Rose et al., 1998) (Table 4.1, Fig. 4.1, 4.2 and 4.3), based on the alignment of amino acid gene sequences from related species retrieved from Joint Genome Institute Portal (JGI; http://genome.jgi.doe.gov/; Grigoriev et al., 2012). The Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) was used to perform the polymerase chain reaction (PCR) with primers and cDNA for a final concentration of 0.5 µM and 10 ng/µL, respectively. Cycle parameters included an initial denaturation at 98ºC for 10 seconds (s), followed by 45 cycles of denaturation at 98ºC for 1 s, annealing at 50ºC for 5 s and extension at 72ºC for 15 s/1 Kb, and a final extension

step at 72º for 1 min. Bands of the appropriate size were excised from the gel and purified using NzytechGel Pure Kit (Nzytech). Purified products were cloned into the pGEM-Easy vector system I (PROMEGA), using Speedy ligase and ligation buffer from Nzytech, and transformed into Nzy5α competent cells (Nzytech). Product sequencing was performed by GATC Biotech.

To obtain 5' and 3' of ALDHs, specific Rapid Amplification of cDNA Ends (RACE) cDNA was prepared using the SMART RACE cDNA amplification kit (Clontech) following the manufacturer's instructions. The 3' and 5' PCR RACE was performed with gene-specific oligonucleotide primers designed in the software Primer3 (v. 0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/; Rozen et al., 2000; Table 4.1, Fig. 4.1, 4.2 and 4.3). Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) was used for PCR that was performed using a 0.5 µM concentration of the specific primer. Cycle parameters included an initial denaturation at 98ºC for 10 s, followed by 40 cycles of denaturation at 98ºC for 1 s, annealing at 63ºC for 5 s and extension at 72ºC for 15 s/1 Kb, and a final extension step at 72º for 1 min. Fragments of interest were cut-off from the gel and cloned into the pGEM-Easy vector system (Promega). Sequencing analyses were performed by GATC Biotech. In the case of *PdAldh1b* isolation was accomplished in a different manner, since a first sequence was obtained when 3' end RACE was performed for *PdAldh2*. From this initial sequence specific oligonucleotide primer was designed to obtain the 5' end through RACE (Table 4.1 and Fig. 4.1).

Table 4.1: List of primers used for gene isolation in the test organisms.

Gene	Primer designation	Sequence 5'- 3'	Use
PdAldh1b	P1	AAGCCCTTGTCTCCCACTCTGTTGC	RACE PCR
	P2	CATAGCTGGTGTCGTTGGCCCTTTT	RACE PCR
	P3	ATGGACTTCAAATTCCCTCCT	ORF PCR
	P4	TTATGAATTCTTTTCTGAAAT	ORF PCR
	P5	GTTTCAAATTTATGAATTCTT	ORF PCR
	P6	GTTTGCGCTCAAATCATCCCT	ORF PCR
PdAldh1c	P1	CGCCGCCATCTCCgarcayatgga	Degenerated PCR
	P2	CGAAGGTCCGGGAGccngcrcarca	Degenerated PCR
	P3	CCCAAGTTCCAGGGAGACTCGTTTG	RACE PCR
	P4	AACTTGGGGGAAAGAGTCCGCTGAT	RACE PCR
	P5	CCTGCTCCAGAGAAACCACTAA	ORF PCR
	P6	TGCGTGAATACAGACAATAGGG	ORF PCR
PdAldh2	P1	AAGATCGCGCAGGAGgarathttygg	Degenerated PCR
	P2	CGGTGTAGTTCTCCAGGccrtaytcncc	Degenerated PCR
	P3	CGTGGAGGACAAGATCTACgaysarttygt	Degenerated PCR
	P4	AAGCCCTTGTCTCCCACTCTGTTGC	RACE PCR
	P5	TCTGACCACAAACGCCAACAGGTTC	RACE PCR
	P6	CGAACAGGGACCACAGATTGACCAG	RACE PCR
	P7	ATGGCGGCTGTATTACGTGTGTT	ORF PCR
	P8	TTATGAGTTCTTCTGCGGCAC	ORF PCR
LpAldh1b	P1	CAACATGCAGATCCAGAAGgargarathtt	Degenerated PCR
	P2	CGGTGTAGTTCTCCAGGccrtaytcncc	Degenerated PCR
	P3	CTGCTGCTCCAAGTCCATAATGTG	RACE PCR
	P4	AGGCGTCTGTTCGGCAGGTTTTAGA	RACE PCR
	P5	CACATTATGGACTTGGAGCAGCAG	RACE PCR
	P6	TAATAATGCCACCACCACCCATC	ORF PCR
	P7	TAGGAATTCTTTTGTGGAATCTT	ORF PCR
LpAldh1c	P1	CGCCGCCATCTCCgarcayatgga	Degenerated PCR
	P2	CGAAGGTCCGGGAGccngcrcarca	Degenerated PCR
	P3	CCAGCACAGCAGTTTTGACCCATGT	RACE PCR
	P4	AGCCTGGAGCTGGGAGGAAAAAGTC	RACE PCR
	P5	TGAGACGAAATTAACCGATGTC	ORF PCR
	P6	TCTTCTGCGGTATTTTAACAGTCA	ORF PCR
LpAldh2	P1	GCCAGATCATCCCCtggaayttycc	Degenerated PCR
	P2	GGGAGCCGGCGcarcarcaytg	Degenerated PCR
	P3	TCCCATGGCAAGAGCTGGACCTAGT	RACE PCR
	P4	TTGTTCCAGGTTACGGGCCTACAGC	RACE PCR
	P5	AGTTTATTACACCAGCAGAAGG	ORF PCR
	P6	CTAAGAATTTTTCTGTGGGAT	ORF PCR

The full-length transcript encoding the open reading frame (ORF) was then obtained by PCR using a specific primer pair for the 5' and 3' ends (Table 4.1, Fig. 4.1, 4.2 and 4.3). Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) was used for PCR that was performed with a 0.5 µM of each primer and 10 ng/µL cDNA, final reaction concentration. The PCR parameters conditions encompassed an initial

denaturation at 98ºC for 10 s, followed by 40 cycles of 98ºC for denaturation at 1 s, annealing at 50ºC for 5 s and extension at 72ºC for 15 s/1 Kb, and a final extension step at 72º for 1 min. The resultant amplicons were cloned into the pGEM-easy vector System I using Speedy ligase (Nzytech), transformed into Nzy5α competent cells (Nzytech), and gene ORF sequences were confirmed by sequence analysis performed by GATC Biotech.

The Aldhs amino acid sequences were inferred by ExPASy software (http://www.expasy.org/; Artimo et al., 2012). The molecular weight and the theoretical isoelectric point (pI) of ALDHs proteins were also calculated using ExPASy software (http://www.expasy.org/; Artimo et al., 2012).

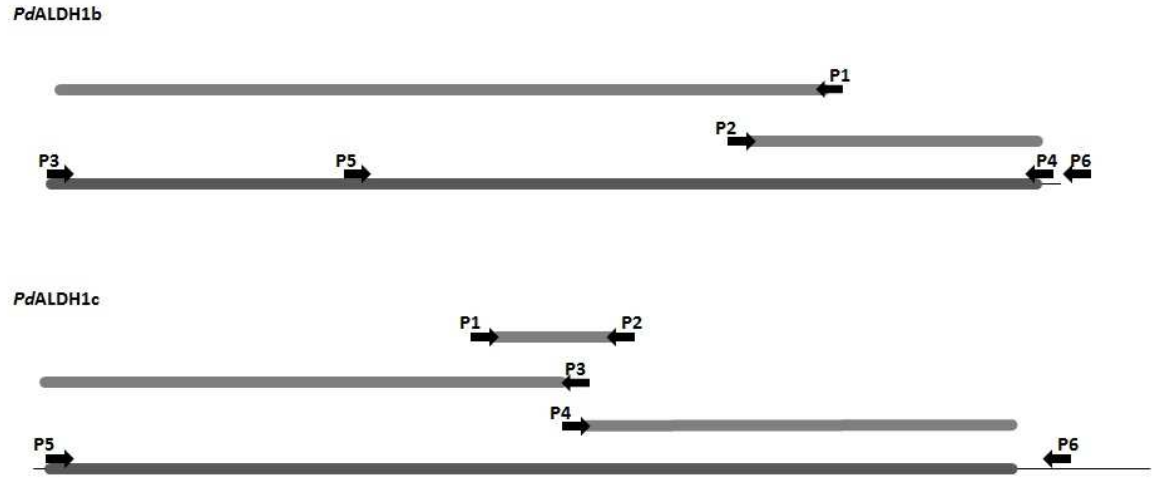

Figure 4.1: Schematic representation of the oligonucleotide primers strategy followed to isolate the complete cDNA coding sequence of *Aldh*1-like gene in the polychaeta *Platynereis dumerilii*.

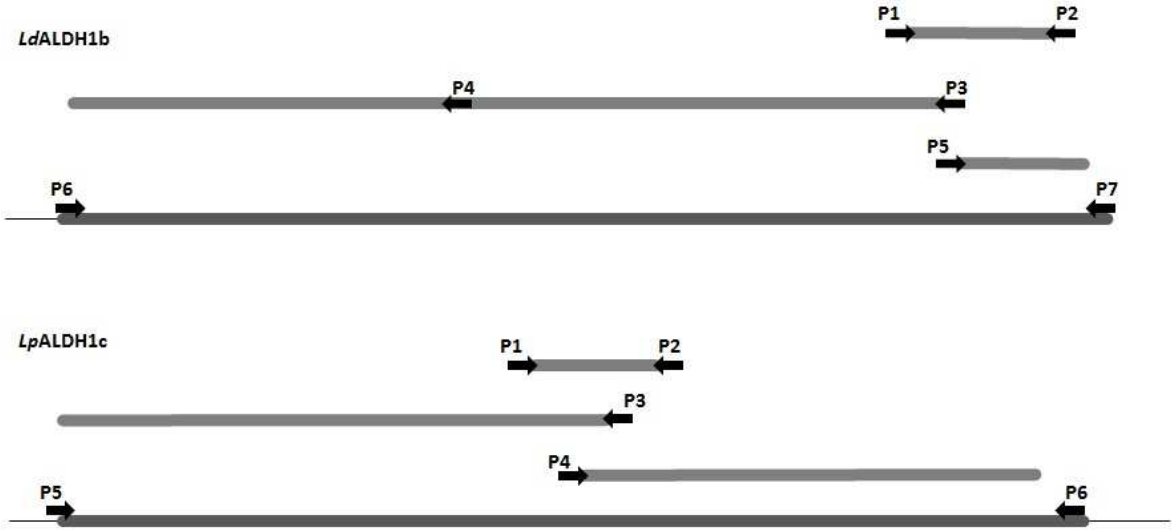

Figure 4.2: Schematic representation of the oligonucleotide primers strategy followed to isolate the complete cDNA coding sequence of *Aldh1*-like gene in the limpet *Patella depressa*.

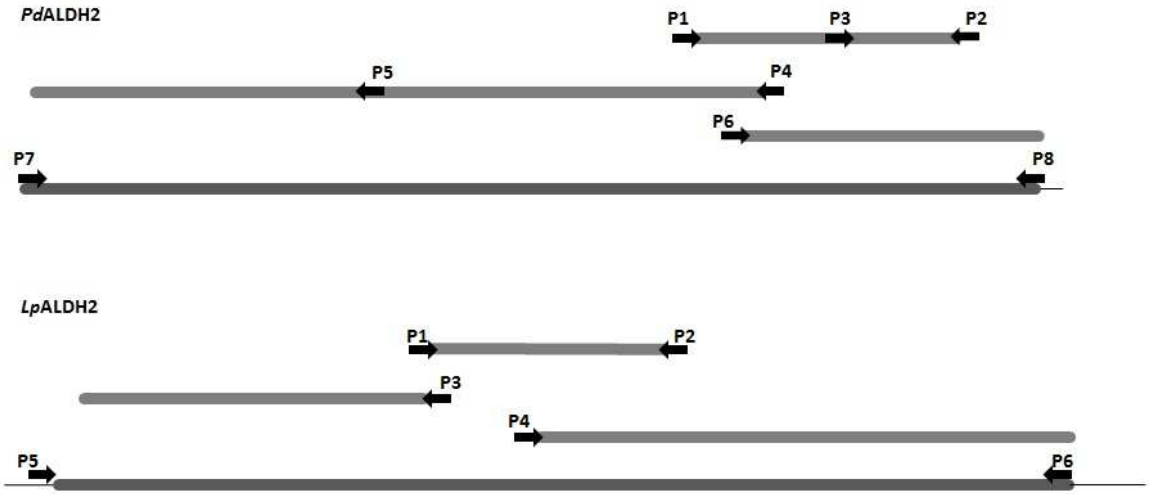

Figure 4.3: Schematic representation of the oligonucleotide primers strategy followed to isolate the complete cDNA coding sequence of *Aldh2* orthologues in the study specimens.

4.3.4 Phylogenetic analysis

Protein sequence alignment was generated with Clustal Omega from European Molecular Biology Laboratory web tool (http://www.ebi.ac.uk/Tools/msa/clustalo/; Sievers et al., 2011). Conserved parts of the amino acid sequences relevant for enzymatic activity were marked according to previous work from Liu et al. (1997), Marchitti et al. (2008), Sobreira et al. (2011) and Koppaka et al. (2012). The MEGA 5 software (Tamura et al., 2011) was used to construct a Neighbor-Joining phylogenetic trees (Saitou and Nei, 1987) corrected by a Poisson distribution of amino acid substitutions for evolutionary distance determination (Zuckerkandl and Pauling, 1965). A thousand repetitions were run for bootstrap support that is shown next to the branches (Felsenstein, 1985). Sequence used for alignment and/or phylogenetic analysis were retrieved from the Ensembl (http://www.ensembl.org/index.html), NCBI databases, and JGI: *Homo sapiens* (Hs) ALDH1a1 NP_000680.2, HsALDH1a2 O94788, HsALDH1a3 NP_000684.2, HsALDH2 CAG33272, *Rattus norvegicus* (Rn) ALDH1a1 NM_022407, RnALDH1a2 NP_446348.2, RnALDH1a3 NM_153300, RnALDH1a7 NM_017272.15, RnALDH2 NM_032416, *Ovis aries* (Oa) ALDH1a1 NP_001009778.1, *Mus musculus* (Mm) ALDH1a1 NM_013467, MmALDH1a2 NM_009022, MmALDH1a3 AAG38488.1, Mm ALDH1a7 AAH46315, *Gallus gallus* (Gg) ADLH1a3 NP_990000.1, GgALDH1a1 NP_989908.1, GgALDH1a2 NP_990326.1, *Xenopus tropicalis* (Xt) ALDH1a1 NP_001027486.1, XtALDH1a2 NP_001039196.1, XtALDH1a3 XP_002939310.1, *Takifugu rubripes* (Tr) ALDH1a2 NP_001028811.1, TrALDH1a2 NP_001153657.1, *Danio rerio* DrALDH1a2 AAI63897.1, DrALDH1a3 NP_001038210.11, *B. floridae* (Bf) ALDH1a JGI_261794, BfALDH1b

JGI_118947, BfALDH1c JGI_86762, BfALDH1d JGI_124720, BfALDH1e JGI_113974, BfALDH1f JGI_206892, BfALDH2 JGI_106815, *C. intestinalis* (Ci) ALDH1a Ensembl ENSCING00000007939, CiALDH1b Ensembl ENSCING00000007835, CiALDH1c Ensembl ENSCING00000007853, CiALDH1d XM_002123383, CiALDH2 AK112485, *C. savignyi* (Cs) ALDH1a Ensembl ENSCSAVG00000003997, CsALDH1b Ensembl ENSCSAVG00000001325, CsALDH2 Ensembl ENSCSAVG00000001705, *L. gigantea* (Lg) ALDH1a JGI_218707, LgALDH1b JGI_192810, LgALDH1c JGI_207312, LgALDH2 JGI_ 177987, *Crassostrea gigas* (Cg) ALDH1b XP_011436817.1, *L. stagnalis* (Ls) ACL79834.1, *C. teleta* (Ct) ALDH1a JGI_151890, CtALDH1b JGI_ 159056, CtALDH1c JGI_145966, CtALDH2 JGI_183731, *Anopheles gambiae* (Ag) ALDH2 XP_313425, *Drosophila melanogaster* (Dm) ALDH2 NP 609285, *Tribolium castaneum* (Tc) ALDH2 XP_970835 and *Nematostella vectensis* (Nv) ALDH2 XP_001639716.1.

4.3.5 Construction of expression vectors

The ALDHs expression vectors were constructed by PCR amplification of the ORF using gene specific primers containing a restriction enzyme site (Table 4.2). Then, purified PCR products were restricted digested, using the specific restriction enzymes, and inserted into the pcDNA3.1 (+) mammalian expression vector (Invitrogen), previously digested with the same restriction enzymes was the respective insert. The ligation of insert into pcDNA3.1 (+) vector was carried out using Seepdy ligase (Nzytech). Then, the plasmids were transformed into Nzy5α competent cells (Nzytech), purified and sequenced by GATC (German) in order to check if the insert was in the correct orientation, reading frame and mutation free.

Table 4.2: List of primers used for subcloned ALDH sequences into pcDNA3.1 (+) mammalian expression vector for the *in vitro* protein functional characterization. "F indicates the forward primer and "R" indicates the reverse primer. Bold nucleotides at the primer sequence shows the restriction enzyme sequence used for restriction digestion.

Gene	Sequence 5'- 3'	Restriction enzyme
*Pd*ALDH1b	F: GCATG**GCGGCCG**Catggacttcaaattccct	NotI
	R: GCATG**CTCGAG**ttatgaattcttttctga	XhoI
*Pd*ALDH1c	F: GCATG**AAGCTT**atgcctgctccagagaaa	HindIII
	R: GCATG**TCTAGA**ctaagaatttttctgaggaat	XbaI
*Lp*ALDH1b	F: GCATG**GCTAGC**atgggggcaggtaggcct	NheI
	R: GCATGT**CTAGA**ttaggaattcttttgtggaat	XbaI
*Lp*ALDH1c	F: GCATG**GCGGCCGC**atgtcggattccgaagca	NotI
	R: GCATG**CTCGAG**ctaggaattcttctgcggtat	XhoI
*Hs*ALDH1a2	F: GCATG**GCGGCCGC**atgacttccagcaagatagag	NotI
	R: GCATG**CTCGAG**ttaggagttcttctgggggat	xhoI

4.3.6 Cell culture conditions

COS-1 cells were maintained Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) with phenol red and supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). The cells were cultured at 37℃ in a humidified atmosphere containing 5% CO_2.

4.3.7 *In vitro* ALDH activity assays

COS-1 cells were transfected using Lipofectamine® 2000 Transfection Reagent (Invitrogen, Thermo Fisher) with the vector pcDNA3.1 (+) (Invitrogen) containing the entire coding region for ALDH1s or with an empty vector. Briefly, the day before transfection, 2 mL of cells were seeded on 12-well culture plates at a density of 2 x 10^5 cells per mL, cultured in DMEM (PAN-Biotech) supplemented with 10% fetal bovine serum (Invitrogen, Thermo Fisher), and 1% penicillin/streptomycin (Invitrogen, Thermo Fisher). After 24 h, the cells were transfected with 1.5 µg construct expression vector pcDNA3.1 (+)/ALDH Lipofectamine® 2000 Transfection Reagent (Invitrogen, Thermo Fisher) and Opti-MEM reduced serum medium (Gibco, Thermo Fisher) according to manufacturers' instruction. Briefly, 4 µL of Lipofectamine® 2000 was added to Opti-MEM for a final volume of 50 µL and let incubate for 5 min at room temperature. Then total 1.5 µg pcDNA3/ALDH diluted with Opti-MEM for a final volume of 50 µL, then was mixed with the Lipofectamine® 2000 and incubated for 20 min at room temperature. After complexes were formed 650 µL more of Opti-MEM was added. The seeded cells were washed once with 1 mL of Phosphate Buffer Saline (PBS; PAA Biotech) to eliminate DEMEM serum traces, and the mixture (total of 750 µL) was added to the cells and incubated at 37℃ in a humidified atmosphere containing 5% CO_2 for 5 h. After 5 h of incubation, cells were washed once with Phosphate Buffer Saline (PBS; PAA Biotech) and 2 mL of DMEM (Invitrogen) with phenol red supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) were add to the cells. At 48 h after transfection, cells were washed with 1 mL of PBS and lysed by sonication at 50 amplitude for 5 seconds in ALDH activity buffer contain 20 mM HEPES (pH 8.5) with 150 mM KCl, 1 mM EDTA and 2 mM DTT (see Farjo et al., 2011) and stored at −80℃ until use.

To measure ALDH1s activity *in vitro* (see Farjo et al., 2011), the cell lysates were used as a source of the enzymes. Protein quantification determination was done with the Pierce BCA Protein Assay Kit (Fisher Scientific) following the manufacture's protocol for microplate quantification. A total of ~200-250 µg of cell lysate protein was suspended for a final volume of 200 µL of ALDH1s activity buffer containing 0.5% BSA, and 2 mM NAD+ or NADP+ (Farjo et al., 2011). Two microliters of all-*trans*-, 9-*cis*- or 13-*cis*-retinal, dissolved in DMF at a final concentration of 5 µM, was added to initiate the reaction and left for

incubation at 37℃ for 30 min with gentle agitation (50 rpm). Then, the reaction was stopped by the addition of 200 µL of methanol (Farjo et al., 2011). Standard all-*trans*-, 9-*cis*- and 13-*cis*-retinal were prepared in DMF and stored at -80ºC under an azote atmosphere.

4.3.8 Retinoids handle

Since retinoids are sensitive to light, all experiments (sample and standards preparation as well as the HPLC analysis) were carried out under light protection (Gundersen et al., 2007). Synthetic standard stock solutions of the retinoids were prepared by dissolving 1 mg of each substance in 1 mL of pure ethanol, and the final concentration was calculated according to Lambert–Beer's law using their reported molar extinction coefficients after measuring it´s absorbance spectrophotometrically. Standard dilution for performing a calibration curve was prepared in pure methanol. The multilinear calibration was carried out by measuring direct injection of methanolic standard solutions of five different concentrations (e.g. 50, 200, 500, 1000, 1500 ng/mL). All stock solutions were stored in the dark at -20ºC under an azote atmosphere.

4.3.9 Retinoid extraction and analysis

Retinoids were extracted according to Farjo et al. (2011) with little modifications. Briefly, non-polar retinoids were extracted by adding 4 µL of 0.1 M sodium hydroxide and 600 µL of *n*-hexane. After removal of non-polar retinoid fraction (retinal isomers), polar retinoids (RA isomers) were extracted by adding 16 µL of 5 M HCl followed by addition 600 µL of *n*-hexane. Samples were evaporated under nitrogen and resuspended in 100 µL of 100% methanol for HPLC analysis.

Retinoic acid isomers detection were performed using a chromatographic equipment consisted of a Hitachi LaChromELITE® HPLC System (VWR International, Darmstadt, Germany), equipped with a L-2130 pump, a L-2300 column oven, a L-2200 auto-sampler and a L-2455 diode array detector (DAD). The chromatographic column used was a reverse-phase Agilent ZORBAX SB-C18 rapid-resolution high-throughput (RRHT) column with 1.8 mm particles (4.6 mm x 50 mm). The mobile phase consists in a 0.5 mL/min flow rate in a gradient: 0-3 min 100% ultra-pure water + 0.1% TFA; 3-6 min 20% ultra-pure water + 0.1% TFA + 80% methanol; 6-25 min 100% methanol + 0.1% TFA and 25-35 min 100% ultra-pure water + 0.1% TFA. The injection volume was 50 µL per sample. Column oven was set at 40℃ and the auto-sa mpler at 4℃. The DAD was set to record absorbance data for wavelengths between 250 and 450 nm. The retinoid chromatogram peaks were identified according to retention times and absorbance spectra of synthetic standards (Table 4.3).

Table 4.3: Chromatographic parameters for separations of retinoic acid isomers, recoveries percentages, limit of detection (LOD) and limit of quantification (LOQ). Determination of LOD and LOQ were based on the comparison of standard deviation of the peak area and the slope of calibration curve (Shrivastava and Gupta, 2011).

Compound	Mean retention time	ʎ nm	% recovery	LOD (ng/mL)	LOQ (ng/mL)
All-*trans*-RA	19.06	354	45.89	42.5	140
9-*cis*-RA	18.98	347	50.27	80	263
13-*cis*-RA	18.64	357	43.50	36	118.5

4.4 Results

4.4.1 ALDH1- and ALDH2-like gene isolation

In this work we have cloned two aldehyde dehydrogenases related to vertebrate ALDH type 1 in two lophotrochozoan marine species, the gastropod *P. depressa* and the polychaeta *P. dumerilii* (Fig. 4.1 and 4.2). We named the isolated cDNA coding protein according to their similarity to other ALDH1 from taxonomic related species (Table 4.4), i.e., an *LpAldh1b* and *LpAldh1c* for *P. depressa*, whereas for *P. dumerilii PdAldh1b* and *PdLpAldh1c*. For *PdAldh1b* (Fig. 4.4) deduced protein contain an initiating amino acid sequence "MDFKFPPPIT", with a total of 496 coding residues and has a predicted molecular weight (Mw) of 54.17 kilodaltons (kDa) and a theoretical pI 6.13; while *PdAldh1c* (Fig. 4.5) deduced protein sequence initiates “MPAPEKPLIR”, contains a total of 491 coding residues, a theoretical pI of 5.27 with a calculated Mw of 53.34 kDa. In the case of *P. depressa*, *LpAldh1b* (Fig. 4.6) deduced protein sequence initiates “MGAGRPNSEI”, contains a total of 508 coding residues, a theoretical pI of 5.62 and Mw of 55.24 kDa; whereas the *LpAldh1c* (Fig. 4.7) 454 coding residues with an initiating amino acid sequence "MSDSEANPNQ" and has a predicted Mw of 53.82 kDa a theoretical pI 5.26. The deduced amino acid sequence of isolated ALDH1-like genes on both lophotrochozoan species showed 59% to 67% homology identifies with a human ALDH1a1, 2, 3 and the Sheep ALDH1a1; and a 63% to 65% homology identity to human ALDH2 (Table 4.4). In comparison to phylogenic related species *P. depressa* ALDH1s share sequence similarity of 60% to 85% with *L. gigantea* and 56% to 70% with *C. teleta*. The LpALDH1c share high sequence identity (85%) with LgALDH1c, while LpALDH1b share 79% sequence identity to LgALDH1b (Table 4.4). In contrast, *P. dumerilii* ALDH1s shares a sequence similarity of 57% to 73%, with the highest level of similarity observed with *L. gigantea* compared to *C. teleta* (Table 4.4).

Table 4.4: Sequences percentage homology similarity predicted using NCBI protein-protein BLASTp tool.

	LpALDH1b	LpALDH1c	LpALDH2	PdALDH1b	PdALDH1c	PdALDH2
HsALDH1a1	61%	64%	66%	65%	61%	67%
HsALDH1a2	62%	66%	65%	64%	63%	64%
HsALDH1a3	61%	62%	64%	63%	59%	64%
HsALDH2	63%	66%	72%	65%	63%	71%
OaALDH1a1	62%	64%	65%	65%	62%	64%
LgALDH1a	60%	60%	61%	60%	57%	58%
LgALDH1b	79%	65%	63%	67%	62%	62%
LgALDH1c	66%	85%	61%	64%	73%	62%
LgALDH2	61%	61%	85%	64%	60%	73%
CtALDH1a	61%	61%	57%	61%	62%	59%
CtALDH1b	57%	56%	57%	57%	59%	59%
CtALDH1c	62%	70%	61%	62%	67%	62%
CtALDH2	63%	62%	73%	63%	60%	70%
LpALDH1b		67%	62%	66%	62%	62%
LpALDH1c	67%		61%	64%	72%	62%
LpALDH2	62%	61%		65%	61%	71%
PdALDH1b	66%	64%	65%		64%	63%
PdALDH1c	62%	72%	61%	64%		61%
PdALDH2	62%	62%	71%	63%	61%	

```
atggacttcaaattccctcctcccatcaccaaccccacagtgaagaatactcagattttt
 M  D  F  K  F  P  P  P  I  T  N  P  T  V  K  N  T  Q  I  F
atcaacaatgaatttgtcaactctgcaagtggaaaaacattccctaccattaatccaagc
 I  N  N  E  F  V  N  S  A  S  G  K  T  F  P  T  I  N  P  S
acaggggaaaccatttgccagatccaagagggagacaaggctgatatcaacaaggcggtt
 T  G  E  T  I  C  Q  I  Q  E  G  D  K  A  D  I  N  K  A  V
gaagtggcaagaaaagcttttgaactaggatcgacatggcgacgaatggacgcatcggac
 E  V  A  R  K  A  F  E  L  G  S  T  W  R  R  M  D  A  S  D
agaggacgacttgtgaacaaatttgccgatcttgtcgagagggatgctgtttacattgct
 R  G  R  L  V  N  K  F  A  D  L  V  E  R  D  A  V  Y  I  A
agtttagagactttggacaatggcaaaccttacacaaatgcatttttggatgttcatttc
 S  L  E  T  L  D  N  G  K  P  Y  T  N  A  F  L  D  V  H  F
ggcgtgaaagttttacgatactatgctggaatggccgacaagaattcaggacagacaatt
 G  V  K  V  L  R  Y  Y  A  G  M  A  D  K  N  S  G  Q  T  I
ccagttgatggagaccacttcacttacacccgtcatgagcccattggagtttgcgctcaa
 P  V  D  G  D  H  F  T  Y  T  R  H  E  P  I  G  V  C  A  Q
atcatcccttggaatttcccaatggtgatgtggatctggaagtttgctcctgctgttgca
 I  I  P  W  N  F  P  M  V  M  W  I  W  K  F  A  P  A  V  A
accggaaatgtagttgtgttgaaaccagctgagcagaccccactcacagccttgtacatg
 T  G  N  V  V  V  L  K  P  A  E  Q  T  P  L  T  A  L  Y  M
gccgctcttaccaaagaagccggtttcccaccaggtgtcgtcagtgtggtacctggatat
 A  A  L  T  K  E  A  G  F  P  P  G  V  V  S  V  V  P  G  Y
ggtcctacagccggtgctgccctctcagaacacatggatgttgacaaaattgcattcaca
 G  P  T  A  G  A  A  L  S  E  H  M  D  V  D  K  I  A  F  T
ggctcgactgaggttggcaaactcatccaaattgcatcaggaaaaacaaatctgaagaat
 G  S  T  E  V  G  K  L  I  Q  I  A  S  G  K  T  N  L  K  N
gtcacattggaattaggcggaaaaagcccaaacatcgtgttcgatgatgtcgatttggat
 V  T  L  E  L  G  G  K  S  P  N  I  V  F  D  D  V  D  L  D
gctgccgtagaaggggcccacttcgccctcttcaccaacatgggtcaagtctgcacagcc
 A  A  V  E  G  A  H  F  A  L  F  T  N  M  G  Q  V  C  T  A
ggatcaagaacatttgtccatgaggcgatctacgatgaatttgtgaagaaggcaaccgcg
 G  S  R  T  F  V  H  E  A  I  Y  D  E  F  V  K  K  A  T  A
agagcccagaaaagaactgtgggaagtccatgggatttgaccactgaatctggaccacag
 R  A  Q  K  R  T  V  G  S  P  W  D  L  T  T  E  S  G  P  Q
attgaccaggagcagatggaaaaaatcttggagatgatcgaaagtgggaaaaaagacgga
 I  D  Q  E  Q  M  E  K  I  L  E  M  I  E  S  G  K  K  D  G
gcaaaattgcaaacaggaggagtgagaaagggagacaagggttacttcattgaatcaaca
 A  K  L  Q  T  G  G  V  R  K  G  D  K  G  Y  F  I  E  S  T
gttttctctgatgtcacagacaatatgaggattgccagggaagaaattttcgggccagtg
 V  F  S  D  V  T  D  N  M  R  I  A  R  E  E  I  F  G  P  V
caacaaatcatcaagttcaaggacgtcaatgatgtcatcaaaagggccaacgacaccagc
 Q  Q  I  I  K  F  K  D  V  N  D  V  I  K  R  A  N  D  T  S
tatggattagcggcgtccgtgtataccaaagatctggataaggcaataattgtctcaaac
 Y  G  L  A  A  S  V  Y  T  K  D  L  D  K  A  I  I  V  S  N
agtctacagtctggtactgtttgggtgaacacctaccatgctgtgcatacccaggctcca
 S  L  Q  S  G  T  V  W  V  N  T  Y  H  A  V  H  T  Q  A  P
tttggaggattcaagatgtctggtattggaagagagctgggcagctatggactgagtcag
 F  G  G  F  K  M  S  G  I  G  R  E  L  G  S  Y  G  L  S  Q
tacacccaagtcaagacggtcacaatgaagatttcagaaaagaattcataa
 Y  T  Q  V  K  T  V  T  M  K  I  S  E  K  N  S  -
```

Figure 4.4: Nucleotide and predicted amino acid sequence of an ALDH1 orthologue for *Platynereis dumerilii* named as ALDH1b. In italic and underlined is the variant Rossman Fold sequence corresponding to the cofactor binding domain. Amino acids highlighted in gray refer to the SEC key residues implicated in subtract recognition and accommodation.

```
atgcctgctccagagaaaccactaattagatatacacagattttattaacaatgagttt
 M  P  A  P  E  K  P  L  I  R  Y  T  Q  I  F  I  N  N  E  F
gtaaactcggaaagtgggaaggtgtttgctacggttgacccgtgctcaggagaaaagatt
 V  N  S  E  S  G  K  V  F  A  T  V  D  P  C  S  G  E  K  I
tgtgacatacaggaaggagataaggtggacatagatgcagctgttgtcgcagccagaaat
 C  D  I  Q  E  G  D  K  V  D  I  D  A  A  V  V  A  A  R  N
gccattaaatttgggtccccatggaggaccctggatgcctcccagaggggcaggctcatt
 A  I  K  F  G  S  P  W  R  T  L  D  A  S  Q  R  G  R  L  I
gacaagtttgccagtttaatggagagggatctggactacattgccgccttggacacattg
 D  K  F  A  S  L  M  E  R  D  L  D  Y  I  A  A  L  D  T  L
gacaatgggaagcctttctcagatgccaaggaggacattgaattttcaatcaatgtcatg
 D  N  G  K  P  F  S  D  A  K  E  D  I  E  F  S  I  N  V  M
aagtactatgctgggtggtgcgacaagataactggcaaaaccattccagtcgatggagac
 K  Y  Y  A  G  W  C  D  K  I  T  G  K  T  I  P  V  D  G  D
tatttcacgttcactcgcattgaacctgtcggggtcgttggtcaaattataccttggaac
 Y  F  T  F  T  R  I  E  P  V  G  V  V  G  Q  I  I  P  W  N
tatcctacggccatggtgtcatggaagtggggaccagcattggcctgtggttgcaccatt
 Y  P  T  A  M  V  S  W  K  W  G  P  A  L  A  C  G  C  T  I
gtgatgaaacctgctgaacagacacctctgtctgccatctacctatgtgcattaattaag
 V  M  K  P  A  E  Q  T  P  L  S  A  I  Y  L  C  A  L  I  K
gaggctggctttcctcctggagtgatcaatgtggtgcctggatttggacccacggctgga
 E  A  G  F  P  P  G  V  I  N  V  V  P  G  F  G  P  T  A  G
gcagcagtcgcagaacatatggacattgacaaagtggccttcactggatccacagaaatt
 A  A  V  A  E  H  M  D  I  D  K  V  A  F  T  G  S  T  E  I
ggcagaattgtgatggctgcagcagccaagtctaacctcaaacgagtctccctggaactt
 G  R  I  V  M  A  A  A  A  K  S  N  L  K  R  V  S  L  E  L
gggggaaagagtccgctgatcatattttctgacactgatttggacaatgccgtggagtgg
 G  G  K  S  P  L  I  I  F  S  D  T  D  L  D  N  A  V  E  W
gcccatgcagccatcatgaacaatcacggccagaactgctgtgctgggtcacgcaccttc
 A  H  A  A  I  M  N  N  H  G  Q  N  C  C  A  G  S  R  T  F
gtacaagaggagatatacgatgcatttgtggcaaaagccaaagcaatagctgacaagagg
 V  Q  E  E  I  Y  D  A  F  V  A  K  A  K  A  I  A  D  K  R
gtagtggggagtccatgggccgatgggacccagcaaggccctcaggttgacgatgcccaa
 V  V  G  S  P  W  A  D  G  T  Q  Q  G  P  Q  V  D  D  A  Q
ttcaagaaaattttatcattcgttgagagtgggaagtctgaaggtgcaaagctgcagtcg
 F  K  K  I  L  S  F  V  E  S  G  K  S  E  G  A  K  L  Q  S
ggaggagtgcgagttggagacaaagggtattttgtgagaccaaccgtcttctcagatgtg
 G  G  V  R  V  G  D  K  G  Y  F  V  R  P  T  V  F  S  D  V
acagacaacatgaagattgcaactgaagagatatttggtccggtgcagagcatcctgaag
 T  D  N  M  K  I  A  T  E  E  I  F  G  P  V  Q  S  I  L  K
ttcaaaacgatggaggaggtgatcgagagagccaacaagacgagctacggattggcagca
 F  K  T  M  E  E  V  I  E  R  A  N  K  T  S  Y  G  L  A  A
ggtgtattcactaacgacataagcaaggccatgatgatggtgcaggcgctgcaggggggc
 G  V  F  T  N  D  I  S  K  A  M  M  M  V  Q  A  L  Q  G  G
agtgtgtgggtcaattgctacgacattgtcacccaacagactccctttggaggattcaaa
 S  V  W  V  N  C  Y  D  I  V  T  Q  Q  T  P  F  G  G  F  K
cagtctggaattggcagagaactgggagaatatgcattgaaggaatattcagaggtcaaa
 Q  S  G  I  G  R  E  L  G  E  Y  A  L  K  E  Y  S  E  V  K
acggtgacaatgaagattcctcagaaaaattcttag
 T  V  T  M  K  I  P  Q  K  N  S  -
```

Figure 4.5: Nucleotide and predicted amino acid sequence of an ALDH1 orthologue for *Platynereis dumerilii* named as ALDH1c. In italic and underlined is the variant Rossman Fold sequence corresponding to the cofactor binding domain. Amino acids highlighted in gray refer to the SEC key residues implicated in subtract recognition and accommodation.

The aldehyde dehydrogenase family also includes the ALDH2 enzymes. Whereas ALDH1s is a cytosolic enzyme that process larger aldehydes, apart from RAL, ALDH2s is a mitochondrial enzyme that degrades small toxic aldehydes, such as the acetaldehyde derived from ethanol metabolism (Yoshida et al., 1998; Bhat and Samaha, 1999; Sobreira et al., 2011). Due to sequence similarity between members of ALDHs superfamily, during our attempt to isolate ALDH1s we also isolated putative ALDH2 sequences. The isolated *P. depressa* ALDH2 (named has LpALDH2) sequence analyses revealed that the cDNA encoded a protein of 495 amino acid residues sequence starting "MSSLPSPILK" with a calculated Mw of 54.18 kDa and a pI 5.53 (Fig. 4.8). The deduced amino acid coding sequence of LpALDH2 shares a higher degree identity 85% similarity with other the limp *L. gigantea* ALDH2 and 71% with human ALDH2. In the case of *P. dumerilii* isolated ALDH2 sequence cDNA encoded a protein of amino acid 519 residues sequence starting "MAAVLRVLKP", a calculated Mw of 56.87 kDa and pI 5.84 (Fig. 4.9). The deduced amino acid sequence showed high identifies of 70-73% with a human and lophotrochozoan species (Table 4.4).

```
atgggggcaggtaggcctaacagtgaaatcaggagtgatataataatgccaccaccaccc
 M  G  A  G  R  P  N  S  E  I  R  S  D  I  I  M  P  P  P  P
atcaaaaaccctgatgttaaatatactcaggctttataaacaatgagtatgtaaactgc
 I  K  N  P  D  V  K  Y  T  Q  A  F  I  N  N  E  Y  V  N  C
gtcagtggtaaaactttccctacgattaatccgactacaaagcagaaaatagcagatgtg
 V  S  G  K  T  F  P  T  I  N  P  T  T  K  Q  K  I  A  D  V
caagaaggggacaaggctgatgttgataaggctgttgagtctgccaaggcagcgtttaaa
 Q  E  G  D  K  A  D  V  D  K  A  V  E  S  A  K  A  A  F  K
ataggatcagcatggaggcggatggatgccagtaaaaggggatgttgttaatgaagtta
 I  G  S  A  W  R  R  M  D  A  S  K  R  G  M  L  L  M  K  L
gcagatttaatagaaagagatatgagttacattggaagtttagaaacaatagataatggt
 A  D  L  I  E  R  D  M  S  Y  I  G  S  L  E  T  I  D  N  G
aaaccatataaaaatgcttgtgttgatattggttttgctgtagaagttatacgttattat
 K  P  Y  K  N  A  C  V  D  I  G  F  A  V  E  V  I  R  Y  Y
gctggatgggcagataaaataacaggcaaaaccattccaatcaatggtgattatttctgt
 A  G  W  A  D  K  I  T  G  K  T  I  P  I  N  G  D  Y  F  C
tatacacgtcatgaacctgttggaatctgtggacagattgttccttggaactatccgttc
 Y  T  R  H  E  P  V  G  I  C  G  Q  I  V  P  W  N  Y  P  F
atgttatttattttgaaactggcaccagctttagcatctgggtgcgttagtattctaaaa
 M  L  F  I  L  K  L  A  P  A  L  A  S  G  C  V  S  I  L  K
cctgccgaacagacgcctttaacttctttgtatgctggcgccttgattaaagatgctggg
 P  A  E  Q  T  P  L  T  S  L  Y  A  G  A  L  I  K  D  A  G
tttccaccaggtgtggtaaatgtaataccaggttatggtccaactgctggcgctgccatt
 F  P  P  G  V  V  N  V  I  P  G  Y  G  P  T  A  G  A  A  I
accaatcatccgggtatcgataaggtgtcttttactgggtctacagaggtaggacagtta
 T  N  H  P  G  I  D  K  V  S  F  T  G  S  T  E  V  G  Q  L
atccaacaagcttcgggtatgagtaacttaaagagaacatcattagaactgggtgggaaa
 I  Q  Q  A  S  G  M  S  N  L  K  R  T  S  L  E  L  G  G  K
tcgcctaatattgtgtttggagatgttgatttggacgccgctgttgcttggtctcatgct
 S  P  N  I  V  F  G  D  V  D  L  D  A  A  V  A  W  S  H  A
gctgtaatggagaacatgggtcagtgttgtgtagctggaaccagaacatttgtacatgaa
 A  V  M  E  N  M  G  Q  C  C  V  A  G  T  R  T  F  V  H  E
tctatttacgatgaatttgttaagaagagcgttgcaatggctaacaaccgagttgttggg
 S  I  Y  D  E  F  V  K  K  S  V  A  M  A  N  N  R  V  V  G
gatccatttgatgaaaaaacacaaaatggcccacagattgatgatgtacaatttgagaag
 D  P  F  D  E  K  T  Q  N  G  P  Q  I  D  D  V  Q  F  E  K
atattagatttaatagaaagtggtaaaaaagaaggtgctaaagtagaatgtggtggagaa
 I  L  D  L  I  E  S  G  K  K  E  G  A  K  V  E  C  G  G  E
aagattggtgataaaggttattatattcaaccaacagtattctctaatgtacaagataac
 K  I  G  D  K  G  Y  Y  I  Q  P  T  V  F  S  N  V  Q  D  N
atgcgtattgctaaagaagagatatttggaccagttcaacagattttgaagttttcagat
 M  R  I  A  K  E  E  I  F  G  P  V  Q  Q  I  L  K  F  S  D
gaagatgaagttatagaaagagctaataatacacattatggacttggagcagcagtattt
 E  D  E  V  I  E  R  A  N  N  T  H  Y  G  L  G  A  A  V  F
actaacgatatcaatagagcattaaaatttgccaacagccttcaagctggatcagtttgg
 T  N  D  I  N  R  A  L  K  F  A  N  S  L  Q  A  G  S  V  W
gttaactgtaatctagctataacaccacaagctccatttggtggatttaaaatgtccggt
 V  N  C  N  L  A  I  T  P  Q  A  P  F  G  G  F  K  M  S  G
attggtagagaatatggtgaatatggtctgcaagactatctcgaggttaaaaatgtggtg
 I  G  R  E  Y  G  E  Y  G  L  Q  D  Y  L  E  V  K  N  V  V
ataaagattccacaaaagaattcctaa
 I  K  I  P  Q  K  N  S  -
```

Figure 4.6: Nucleotide and predicted amino acid sequence of an ALDH1 orthologue gene isolated from *Patella depressa* ALDH1b. In italic and underlined is the variant Rossman Fold sequence corresponding to the cofactor binding domain. Amino acids highlighted in gray refer to the SEC key residues implicated in subtract recognition and accommodation.

```
atgtcggattccgaagcaaaccctaaccaagaaatagtctacaaacagatatttataaac
 M  S  D  S  E  A  N  P  N  Q  E  I  V  Y  K  Q  I  F  I  N
aatgaatggtgtaattctgtgagtggacgaacatttccaactataaatccagcaactggt
 N  E  W  C  N  S  V  S  G  R  T  F  P  T  I  N  P  A  T  G
gaaaaactatgtgatgttcaggaaggggataaggaagatgttgacaaagccgttgctgct
 E  K  L  C  D  V  Q  E  G  D  K  E  D  V  D  K  A  V  A  A
gctagagcagcctttaagataggttccccctggcggagaaaagatgcatcaaagagagga
 A  R  A  A  F  K  I  G  S  P  W  R  R  K  D  A  S  K  R  G
gaactcatgatgaaatttgtatctctttagaacgtgatataggtatattagcgaatttg
 E  L  M  M  K  F  V  S  L  L  E  R  D  I  G  I  L  A  N  L
gagacgttggataatggtaaaccgttcctggatgcagttgatgatgtaagagctacaata
 E  T  L  D  N  G  K  P  F  L  D  A  V  D  D  V  R  A  T  I
gatgtgtttcagtattatgctggttggtgtgataaaataactggaaaaaccataccagta
 D  V  F  Q  Y  Y  A  G  W  C  D  K  I  T  G  K  T  I  P  V
gacggtgattattttacctttacgagacatgaaccagtgggtgtatgtggacaaattatt
 D  G  D  Y  F  T  F  T  R  H  E  P  V  G  V  C  G  Q  I  I
ccttggaattatccagttgccatgctgtcatggaaattaggaccggcattagcatgtggg
 P  W  N  Y  P  V  A  M  L  S  W  K  L  G  P  A  L  A  C  G
aacactgtgatactaaaaccagctgagcagacgccgttgacagcactttattgttgtgct
 N  T  V  I  L  K  P  A  E  Q  T  P  L  T  A  L  Y  C  C  A
ttatttaaagaggcaggttttccgccgggtgttgtaaatgttgtaccaggctatggatca
 L  F  K  E  A  G  F  P  P  G  V  V  N  V  V  P  G  Y  G  S
acagcaggagctgccatatctgaacatatggacgtggataaagttgcctttactggatca
 T  A  G  A  A  I  S  E  H  M  D  V  D  K  V  A  F  T  G  S
acagaggttggtaaattaataatgcaagctgctggaagaagtaatttaaagcgaattagc
 T  E  V  G  K  L  I  M  Q  A  A  G  R  S  N  L  K  R  I  S
ctggagctgggaggaaaaagtcctttgatcatattagctgatgcagatgttgataatgct
 L  E  L  G  G  K  S  P  L  I  I  L  A  D  A  D  V  D  N  A
gccctttgggctcattctgctataatgaccaacatgggtcaaaactgctgtgccggttct
 A  L  W  A  H  S  A  I  M  T  N  M  G  Q  N  C  C  A  G  S
agaactcttgtccaagaagacatctatgatgaatttgtttcaaaagcaaaattgatggcc
 R  T  L  V  Q  E  D  I  Y  D  E  F  V  S  K  A  K  L  M  A
gaaaaccgttgtgttggagatccatttgatgccatgactgcacatggttcacagattgat
 E  N  R  C  V  G  D  P  F  D  A  M  T  A  H  G  S  Q  I  D
gaaacccagtttaagaagatattaagtcttattgatagtggtgtacaagaaggagctgta
 E  T  Q  F  K  K  I  L  S  L  I  D  S  G  V  Q  E  G  A  V
ttagaagctggtggaaaaagatatggtgataaaggttatttcataaaacccacagtattc
 L  E  A  G  G  K  R  Y  G  D  K  G  Y  F  I  K  P  T  V  F
agtaatgttcaagataatatgcgaatagctaaagaagagatatttggtcctgttcagtcc
 S  N  V  Q  D  N  M  R  I  A  K  E  E  I  F  G  P  V  Q  S
attattaagtttaagacaatagaagaggcaatagagagggctaataatacaaactatggt
 I  I  K  F  K  T  I  E  E  A  I  E  R  A  N  N  T  N  Y  G
ctggctgctggcattattaccaatgatatcaacaaagctctaatgtttgctcaaagtata
 L  A  A  G  I  I  T  N  D  I  N  K  A  L  M  F  A  Q  S  I
caggctggttctgtttgggttaattgttatgatattgttacaacacaaacaccgtttggt
 Q  A  G  S  V  W  V  N  C  Y  D  I  V  T  T  Q  T  P  F  G
ggattcaaacaatctgggcatggtagagaactgggagagtatgccttaaaagaatacacg
 G  F  K  Q  S  G  H  G  R  E  L  G  E  Y  A  L  K  E  Y  T
gaaataaaaacagtgactgttaaaataccgcagaagaattcctag
 E  I  K  T  V  T  V  K  I  P  Q  K  N  S  -
```

Figure 4.7: Nucleotide and predicted amino acid sequence of an ALDH1 orthologue gene isolated for *Patella depressa* ALDH1c. In italic and underlined is the variant Rossman Fold sequence corresponding to the cofactor binding domain. Amino acids highlighted in gray refer to the SEC key residues implicated in subtract recognition and accommodation.

```
atgtcatcattgccatcacctattttgaaaccagatgtgaaatacgctcagatatttata
 M  S  S  L  P  S  P  I  L  K  P  D  V  K  Y  A  Q  I  F  I
aataatgagtttgttaatgccgaaagtggtaagacgtttcaaaccatcaacccgacaaca
 N  N  E  F  V  N  A  E  S  G  K  T  F  Q  T  I  N  P  T  T
ggggaggcaatctgtgaagtagcagaaggcgacaaggcagatattgataaagccgttctt
 G  E  A  I  C  E  V  A  E  G  D  K  A  D  I  D  K  A  V  L
gcagctcgaaaggcctttcagcttggatcgccatggagaactatggatgcttcacagaga
 A  A  R  K  A  F  Q  L  G  S  P  W  R  T  M  D  A  S  Q  R
ggtctcctgctgtacagattagctgatctgattgaacgagatcgacagattctagcttct
 G  L  L  L  Y  R  L  A  D  L  I  E  R  D  R  Q  I  L  A  S
ttagaaacactggataatggtaaaccatataatgtagctattactgctgatttaggactg
 L  E  T  L  D  N  G  K  P  Y  N  V  A  I  T  A  D  L  G  L
gttgtcaagtgctacagatattatgctggatgggcaaataagcatcatggaaaaactatt
 V  V  K  C  Y  R  Y  Y  A  G  W  A  N  K  H  H  G  K  T  I
ccaatcgatggagattatcttgcgtatacccgacatgaacctgttggtgtttgtggacaa
 P  I  D  G  D  Y  L  A  Y  T  R  H  E  P  V  G  V  C  G  Q
attataccctggaactttcctttattaatgcaagcctggaaactaggtccagctcttgcc
 I  I  P  W  N  F  P  L  L  M  Q  A  W  K  L  G  P  A  L  A
atgggaaatactgttgtcatgaaaacggccgaacagacaccattaacagctctgcatgta
 M  G  N  T  V  V  M  K  T  A  E  Q  T  P  L  T  A  L  H  V
gcacaattagccactgaggccggttttcctcctggtgttataaatattgttccaggttac
 A  Q  L  A  T  E  A  G  F  P  P  G  V  I  N  I  V  P  G  Y
gggcctacagctggtgctgccttggccagacacccagatgttgataaagtcgcatttaca
 G  P  T  A  G  A  A  L  A  R  H  P  D  V  D  K  V  A  F  T
ggatctacagagattggacaacttgtagcccaagatgctgctagaaccaacttaaaacga
 G  S  T  E  I  G  Q  L  V  A  Q  D  A  A  R  T  N  L  K  R
gtaacgttagaacttggtggaaagagtcctaacatcattttagctgatgcagatttggat
 V  T  L  E  L  G  G  K  S  P  N  I  I  L  A  D  A  D  L  D
catgctgtggaacaggctcattttggtctgtttttcaatcaaggacagtgttgttgtgct
 H  A  V  E  Q  A  H  F  G  L  F  F  N  Q  G  Q  C  C  C  A
ggtaccagaatatatgttgaagataaaatatatgatgaatttgtagaaagaagcacagaa
 G  T  R  I  Y  V  E  D  K  I  Y  D  E  F  V  E  R  S  T  E
cgagccaaaaaaagggccgtaggtgatccctttaccaatgtagagcagggaccacaggtg
 R  A  K  K  R  A  V  G  D  P  F  T  N  V  E  Q  G  P  Q  V
gataaagaacaatttgataaagtattaaattatatcgacattggtaagaaagaaggagct
 D  K  E  Q  F  D  K  V  L  N  Y  I  D  I  G  K  K  E  G  A
aaactgacgactggcggaaacagagtgggtgatcgaggctacttcattgagccaacagta
 K  L  T  T  G  G  N  R  V  G  D  R  G  Y  F  I  E  P  T  V
ttcaccgacgtcaaggacaatatggttatttcacaagaagagatttttggaccagttatg
 F  T  D  V  K  D  N  M  V  I  S  Q  E  E  I  F  G  P  V  M
agtatttcaaaattttctagtttagacgaggttataaatagatcccacgataccatatat
 S  I  S  K  F  S  S  L  D  E  V  I  N  R  S  H  D  T  I  Y
ggtctggcagcagctgtctttactaaggatttagacagagccctttatttatcgaacagc
 G  L  A  A  A  V  F  T  K  D  L  D  R  A  L  Y  L  S  N  S
gtcagagctggaacaatatgggtaaactgctatgatgttttttgacgcatcagcaccattt
 V  R  A  G  T  I  W  V  N  C  Y  D  V  F  D  A  S  A  P  F
ggagggtataagatgtctggtaatggtcgtgaattgggagagtatggtttagataactac
 G  G  Y  K  M  S  G  N  G  R  E  L  G  E  Y  G  L  D  N  Y
acagaggtcaaaacggtcaccattaagatcccacagaaaaattcttag
 T  E  V  K  T  V  T  I  K  I  P  Q  K  N  S  -
```

Figure 4.8: *Patella depressa* ALDH2 complete isolated sequence in nucleotide including the deduced amino acid sequence. In italic and underlined is the variant Rossman Fold sequence corresponding to the cofactor binding domain. Amino acids highlighted in gray refer to the SEC key residues implicated in subtract recognition and accommodation.

```
atggcggctgtattacgtgtgttaaaaccgagaaacttctctggaatactcaggactcag
 M  A  A  V  L  R  V  L  K  P  R  N  F  S  G  I  L  R  T  Q
caggctttgctgtcaactgtgcctgaaccttccagaaaccctgatattcactacaacaag
 Q  A  L  L  S  T  V  P  E  P  S  R  N  P  D  I  H  Y  N  K
atctttatcaacaatgagtggcaagacgctgtcagtggtaagacctttccaacgataaat
 I  F  I  N  N  E  W  Q  D  A  V  S  G  K  T  F  P  T  I  N
cccacaactggaaatgttataacccaagtggctgaaggagataaggctgatgtagacaaa
 P  T  T  G  N  V  I  T  Q  V  A  E  G  D  K  A  D  V  D  K
gcagtgaaagccgcgaataatgcttttgcatttggttcggaatggcgcagaatggacgct
 A  V  K  A  A  N  N  A  F  A  F  G  S  E  W  R  R  M  D  A
gccaccaggggagactcatgtacaaactggccgacctgattgaacgagacaaggcttac
 A  T  R  G  R  L  M  Y  K  L  A  D  L  I  E  R  D  K  A  Y
cttgctgaattggagaccctggacaatggaaaaccttatcaggttgcctacaatgccgat
 L  A  E  L  E  T  L  D  N  G  K  P  Y  Q  V  A  Y  N  A  D
cttgaccttgtaataaaatgtatcagatactatgctggctgggcagacaaaaaccatgga
 L  D  L  V  I  K  C  I  R  Y  Y  A  G  W  A  D  K  N  H  G
aaagttattcctgttcatggcgacttccacacatacacccgccatgaacctgttggcgtt
 K  V  I  P  V  H  G  D  F  H  T  Y  T  R  H  E  P  V  G  V
tgtggtcagattatcccctggaattttccacttctgatgctggcttggaagtgggggcca
 C  G  Q  I  I  P  W  N  F  P  L  L  M  L  A  W  K  W  G  P
gctcttgccatgggaaacacgatcgtaatgaaagtagctgaacagactcctcttacagcg
 A  L  A  M  G  N  T  I  V  M  K  V  A  E  Q  T  P  L  T  A
ttatatgtggcacaactgacagctgaggcaggtttccctgctggtgtagtgaatgtcatt
 L  Y  V  A  Q  L  T  A  E  A  G  F  P  A  G  V  V  N  V  I
cctggctttggaccaacagcagggggtgccatcgcctctcacatgaggctggacaaagtt
 P  G  F  G  P  T  A  G  G  A  I  A  S  H  M  R  L  D  K  V
gctttcacgggctccactgaggtcggccaaatcatagcggaggcagccgccaagtctaac
 A  F  T  G  S  T  E  V  G  Q  I  I  A  E  A  A  A  K  S  N
ttgaagagggtgacccttgaactgggtggcaagagtcctaacatcgtcttgaaagatgcc
 L  K  R  V  T  L  E  L  G  G  K  S  P  N  I  V  L  K  D  A
gatttagcacaagctgtggagtggtcgcattttggacttttcttcaaccaagggcagtgt
 D  L  A  Q  A  V  E  W  S  H  F  G  L  F  F  N  Q  G  Q  C
tgttgcgctggcagtcgtgtctttgtggaagaggacatttacgatgaatttgttgagcgc
 C  C  A  G  S  R  V  F  V  E  E  D  I  Y  D  E  F  V  E  R
agtgtggagagagccaagtcaagaactgtaggagatccattcgaagctggcagcgaacag
 S  V  E  R  A  K  S  R  T  V  G  D  P  F  E  A  G  S  E  Q
ggaccacagattgaccaggaacagatggacaaaatcttgactttggtagagagtggcaag
 G  P  Q  I  D  Q  E  Q  M  D  K  I  L  T  L  V  E  S  G  K
aaagaaggagccaatctggtcgtaggaggcaacagagtgggagacaagggcttcttcatt
 K  E  G  A  N  L  V  V  G  G  N  R  V  G  D  K  G  F  F  I
gagccaacgatattcaaggatgttgaggacaacatgaggattgctaatgaagagatcttt
 E  P  T  I  F  K  D  V  E  D  N  M  R  I  A  N  E  E  I  F
ggaccagtcatgcaaatcctgaagttcaagtctgtagacgaggtcattgaacgagcgaac
 G  P  V  M  Q  I  L  K  F  K  S  V  D  E  V  I  E  R  A  N
aagaccatctatggcttggccgcttctgtaatgacaaaagacatggaacgagggctgcac
 K  T  I  Y  G  L  A  A  S  V  M  T  K  D  M  E  R  G  L  H
atagctcatggcgtccgtgctggcagtgtatgggtgaattgctttgatatcttcgatgcc
 I  A  H  G  V  R  A  G  S  V  W  V  N  C  F  D  I  F  D  A
gctactcccttcggaggctacaaaatgtcaggaaacggaagagaattgggcgaatatgga
 A  T  P  F  G  G  Y  K  M  S  G  N  G  R  E  L  G  E  Y  G
ctggaggcttacactgaagtcaaggcggtcaccatcaaagtgccgcagaagaactcataa
 L  E  A  Y  T  E  V  K  A  V  T  I  K  V  P  Q  K  N  S  -
```

Figure 4.9: ***Platynereis dumerilii*** **ALDH2 complete isolated sequence in nucleotide including the deduced amino acid sequence.** In italic and underlined is the variant Rossman Fold sequence corresponding to the cofactor binding domain. Amino acids highlighted in gray refer to the SEC key residues implicated in subtract recognition and accommodation.

The multiple alignment sequences analysis reveals conservation of key residues characteristic of ALDHs superfamily members relevant for enzymatic biochemical activity, for the catalysis, co-factor binding (NAD(P)+) and substrate binding (Liu et al., 1997; Marchitti et al., 2008; Koppaka et al., 2012) (Table 4.5 and Fig. 4.10).

Table 4.5: Substrate access channel amino acid key residues signature in ALDHs from Chordata and lophotrochozoan species indicating putative substitution leading to the enzyme ability or inability to perform retinal oxidation to retinoic acid. Table based on present work results and previously study from Sobreira et al., 2011. *H. sapiens* (Hs), *R. norvegicus* (Rn), *O. aries* (Oa), *M. musculus* (Mm), *C. intestinalis* (Ci), *C. savignyi* (Cs), *B. floridae* (Bf), *L. gigantea* (Lg), *P. depressa* (Lp), *C. teleta* (Ct) and *P. dumerilii* (Pd).

Chordates	124	303	459	RAL use	Lophotrochozoa	124	303	459	RAL use
HsALDH1a2	G	T	L	+	PdALDH1b	F	T	V	-
HsALDH2	M	C	F	-	PdALDH1c	F	C	V	-
OaALDH1a1	G	I	V	+	PdALDH2	L	C	F	-
RnALDH1a2	G	T	L	+	LpALDH1b	F	V	I	-
MmALDH1a3	G	T	F	+	LpALDH1c	A	C	V	-
BfALDH1a	G	T	I	+	LpALDH2	L	C	F	-
BfALDH1b	G	C	I	-	LgALDH1a	G	A	L	?
BfALDH1c	S	C	T	-	LgALDH1b	F	V	V	-
BfALDH1d	G	I	I	+	LgALDH1c	G	C	V	?
BfALDH1e	E	C	G	+	LgALDH2	L	C	L	-
BfALDH1f	E	C	G	+	CtALDH1a	A	I	S	-
BfALDH2	L	C	F	-	CtALDH1b	F	T	F	-
CsALDH1a	G	T	F	+	CtALDH1c	Y	C	V	-
CsALDH1b	A	C	M	-	CtALDH2	L	C	F	-
CsALDH2	L	C	F	-					
CiALDH1a	G	T	F	+					
CiALDH1b	S	C	F	-					
CiALDH1c	I	C	M	-					
CiALDH1d	G	C	L	+					
CiALDH2	L	C	F	-					

The Neighbor-joining tree shows that *P. depressa* ALDH1b and c as well as *P. dumerilii* ALDH1b and c were clustered together with other lophotrochozoan species adjacent to ecdysozoa species clade (Fig. 4.11), suggesting that this protein is more related to them. In relation to PdALDH2 and LpALDH2 they were robustly clustered with other lophotrochozoans ALDH2 sequences, nearby the Chordata clade (Fig. 4.12).

```
Hs_ALDH1a2      MTSSKIEMPGEVKADPAALMASLHLLPSPTPNLEIKYTKIFINNEWQNSESGRVFPVYNP 60
Hs_ALDH2        MLRAAARFGPRLGRRLLSA-AATQAVPAPNQQPEVFCNQIFINNEWHDAVSRKTFPTVNP 59
Oa_ALDH1a1      ----------------MSSSAMPDVPAPLTNLQFKYTKIFINNEWHSSVSGKKFPVFNP 43
Rn_ALDH1a2      MTSSEIAMPGEVKADPAALMASLQLLPSPTPNLEIKYTKIFINNEWQNSESGRVFPVCNP 60
Mm_ALDH1a3      MAT----TNGA—VENGQPDGKPPALPRPIRNLEVKFTKIFINNDWHESKSGRKFATYNP 54
Pd_ALDH1b       --------------------MDFKFPPPITNPTVKNTQIFINNEFVNSASGKTFPTINP 39
Pd_ALDH1c       -------------------------MPAPEKPLIRYTQIFINNEFVNSESGKVFATVDP 34
Lp_ALDH1c       ----------------------MSDSEANPNQEIVYKQIFINNEWCNSVSGRTFPTINP 37
Lp_ALDH1b       ---------MGAGRPNSEIRSDIIMPPPPIKNPDVKYTQAFINNEYVNCVSGKTFPTINP 51
Bf_ALDH1a       ------------------------------------------------------------
Bf_ALDH1d       --------------------MDDFQMPTPDPNPEIKYTQIFINNEFVNSVSGKTFPTINP 40

Hs_ALDH1a2      ATGEQVCEVQEADKADIDKAVQAARLAFSLGSVWRRMDASERGRLLDKLADLVERDRAVL 120
Hs_ALDH2        FTGEVICQVAEGDKEDVDKAVKAARAAFQLGSPWRRMDASHRGRLLNRLADLIERDRTYL 119
Oa_ALDH1a1      ATEEKLCEVEEGDKEDVDKAVKAARQAFQIGSPWRTMDASERGRLLNKLADLIERDRLLL 103
Rn_ALDH1a2      ATGEQVCEVQEADKVDIDKAVQAARLAFSLGSVWRRMDASERGRLLDKLADLVERDRATL 120
Mm_ALDH1a3      STLEKICEVEEGDKPDVDKAVEAAQAAFQRGSPWRRLDALSRGQLLHQLADLVERDRAIL 114
Pd_ALDH1b       STGETICQIQEGDKADINKAVEVARKAFELGSTWRRMDASDRGRLVNKFADLVERDAVYI 99
Pd_ALDH1c       CSGEKICDIQEGDKVDIDAAVVAARNAIKFGSPWRTLDASQRGRLIDKFASLMERDLDYI 94
Lp_ALDH1c       ATGEKLCDVQEGDKEDVDKAVAAARAAFKIGSPWRRKDASKRGELMMKFVSLLERDIGIL 97
Lp_ALDH1b       TTKQKIADVQEGDKADVDKAVESAKAAFKIGSAWRRMDASKRGMLLMKLADLIERDMSYI 111
Bf_ALDH1a       ------------------------------------MDASQRGRLLSKLADLIERDSMYL 24
Bf_ALDH1d       ATREKICDVQEAEKADVDKAVAAARAAFQLGSPWRNMDASQRGRLLLKLAGLMQRDGSYL 100
                                                     **  ** *: ::. *::**   :

Hs_ALDH1a2      ATMESLNGGKPFLQAFYVDLQGVIKTFRYYAGWADKIHGMTIPVDGDYFTFTRHEPIGVC 180
Hs_ALDH2        AALETLDNGKPYVISYLVDLDMVLKCLRYYAGWADKYHGKTIPIDGDFFSYTRHEPVGVC 179
Oa_ALDH1a1      ATMEAMNGGKLFSNAYLMDLGGCIKTLRYCAGWADKIQGRTIPMDGNFFTYTRSEPVGVC 163
Rn_ALDH1a2      ATMESLNGGKPFLQAFYIDLQGVIKTLRYYAGWADKIHGMTIPVDGDYFTFTRHEPIGVC 180
Mm_ALDH1a3      ATLETMDTGKPFLHAFFVDLEGCIKTFRYFAGWADKIQGRTIPTDDNVVCFTRHEPIGVC 174
Pd_ALDH1b       ASLETLDNGKPYTNAF-LDVHFGVKVLRYYAGMADKNSGQTIPVDGDHFTYTRHEPIGVC 158
Pd_ALDH1c       AALDTLDNGKPFSDAK-EDIEFSINVMKYYAGWCDKITGKTIPVDGDYFTFTRIEPVGVV 153
Lp_ALDH1c       ANLETLDNGKPFLDAV-DDVRATIDVFQYYAGWCDKITGKTIPVDGDYFTFTRHEPVGVC 156
Lp_ALDH1b       GSLETIDNGKPYKNAC-VDIGFAVEVIRYYAGWADKITGKTIPINGDYFCYTRHEPVGIC 170
Bf_ALDH1a       ASLDTLDNGKPFIQAMFVDLQGSMGTLRYFSGWADKIHGKTIPTDGPHFTYTRREPVGVC 84
Bf_ALDH1d       ASLETLDNGKPFVHSYFADVLGPIKDLTYFAGWCDKITGKTIPVDGPYFTYTRLEPIGVC 160
                . ::::: ** :  :   *:   :  : * :* .**  * *** :   . :** **:*:

Hs_ALDH1a2      GQIIPWNFPLLMFAWKIAPALCCGNTVVIKPAEQTPLSALYMGALIKEAGFPPGVINILP 240
Hs_ALDH2        GQIIPWNFPLLMQAWKLGPALATGNVVVMKVAEQTPLTALYVANLIKEAGFPPGVVNIVP 239
Oa_ALDH1a1      GQIIPWNFPLLMFLWKIGPALSCGNTVVVKPAEQTPLTALHMGSLIKEAGFPPGVVNIVP 223
Rn_ALDH1a2      GQIIPWNFPLLMFTWKIAPALCCGNTVVIKPAEQTPLSALYMGALIKEAGFPPGVVNILP 240
Mm_ALDH1a3      GAITPWNFPLLMLAWKLAPALCCGNTVVLKPAEQTPLTALYLASLIKEVGFPPGVVNIVP 234
Pd_ALDH1b       AQIIPWNFPMVMWIWKFAPAVATGNVVVLKPAEQTPLTALYMAALTKEAGFPPGVVSVVP 218
Pd_ALDH1c       GQIIPWNYPTAMVSWKWGPALACGCTIVMKPAEQTPLSAIYLCALIKEAGFPPGVINVVP 213
Lp_ALDH1c       GQIIPWNYPVAMLSWKLGPALACGNTVILKPAEQTPLTALYCCALFKEAGFPPGVVNVVP 216
Lp_ALDH1b       GQIVPWNYPFMLFILKLAPALASGCVSILKPAEQTPLTSLYAGALIKDAGFPPGVVNVIP 230
Bf_ALDH1a       GAIIPWNFPLMMAVWKLAPALCAGCTVVLKPAEQTPLSALYLAALIKEAGFPPGVVNIVP 144
Bf_ALDH1d       GGIIPWNFPINMFIWKLATALAAGNTVVIKPAEQTPLTALYLASLIKEAGFPPGVVNVLP 220
                . * ***:*  :   * . *:. * . ::* ******::::   * *:.******:.::*

Hs_ALDH1a2      GYGPTAGAAIASHIGIDKIAFTGSTEVGKLIQEAAGRSNLKRVTLELGGKSPNIIFADAD 300
Hs_ALDH2        GFGPTAGAAIASHEDVDKVAFTGSTEIGRVIQVAAGSSNLKRVTLELGGKSPNIIMSDAD 299
Oa_ALDH1a1      GYGPTAGAAISSHMDVDKVAFTGSTEVGKLIKEAAGKSNLKRVSLELGGKSPCIVFADAD 283
Rn_ALDH1a2      GYGPTAGAAIASHIGIDKIAFTGSTEVGKLIQEAAGRSNLKRVTLELGGKSPNIIFADAD 300
Mm_ALDH1a3      GFGPTVGAAISSHPQINKIAFTGSTEVGKLVREAASRSNLKRVTLELGGKNPCIVCADAD 294
Pd_ALDH1b       GYGPTAGAALSEHMDVDKIAFTGSTEVGKLIQIASGKTNLKNVTLELGGKSPNIVFDDVD 278
Pd_ALDH1c       GFGPTAGAAVAEHMDIDKVAFTGSTEIGRIVMAAAAKSNLKRVSLELGGKSPLIIFSDTD 273
Lp_ALDH1c       GYGSTAGAAISEHMDVDKVAFTGSTEVGKLIMQAAGRSNLKRISLELGGKSPLIILADAD 276
Lp_ALDH1b       GYGPTAGAAITNHPGIDKVSFTGSTEVGQLIQQASGMSNLKRTSLELGGKSPNIVFGDVD 290
Bf_ALDH1a       GYGPTAGAAISEHMDIQKVAFTGSTEVGKIIQQAAGKSNLKRVSLELGGKSPTIVFPDAD 204
Bf_ALDH1d       GYGPTCGAHIVEHMDVDKVAFTGSTEVGKIIQAAAGKSNLKRVSLELGGKSPLIVFSDAD 280
                *:* * ** : .*  ::*::******:*:::  *:. :***. :******.* *:  *.*
```

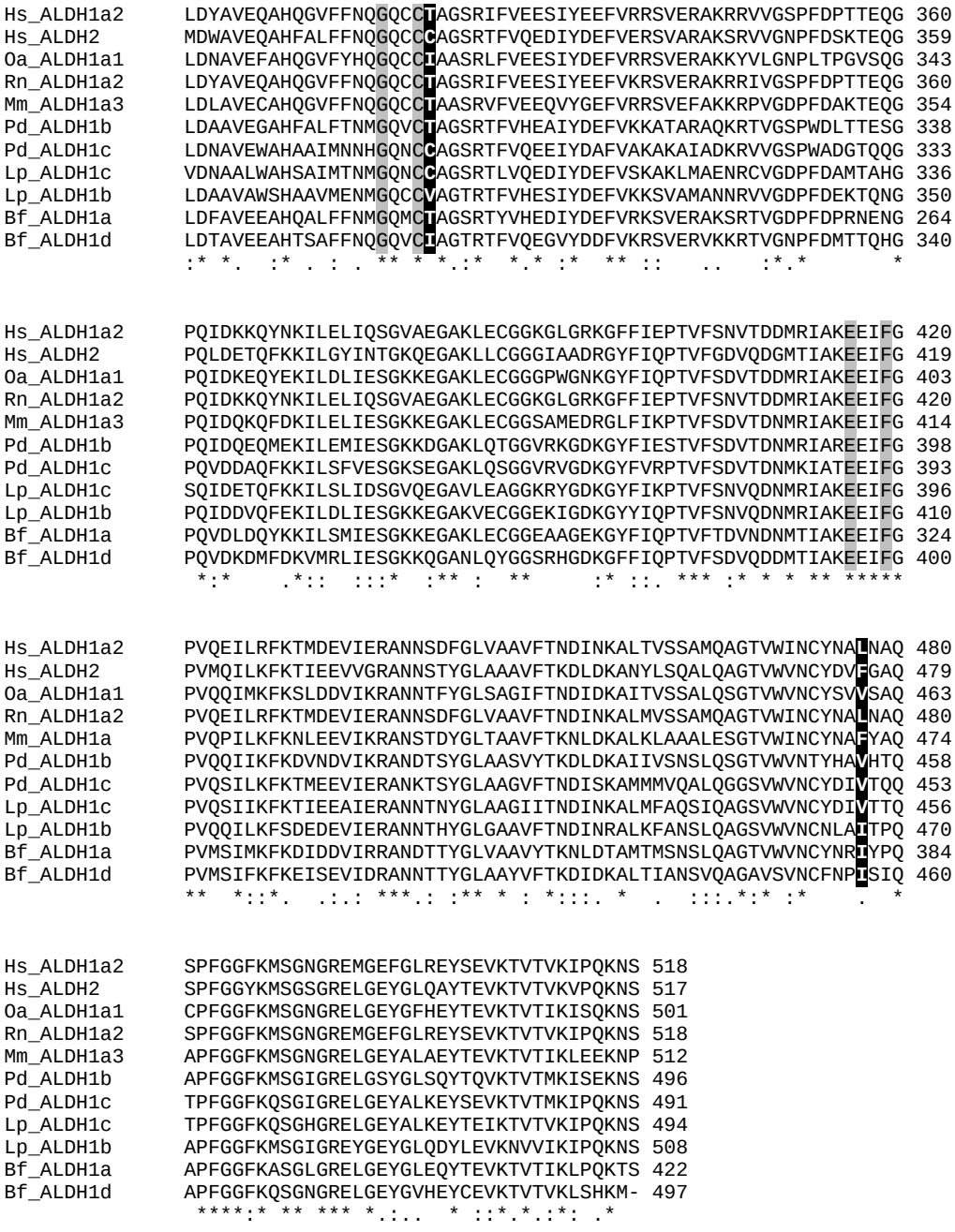

Figure 4.10: Alignment of the amino acid of ALDHs from the isolated study animals with relevant ALDHs from other selected species for key residues identification. Residues relevant for enzyme biochemical function highlighted according to the following works: Liu et al., 1997; Marchitti et al., 2008; Sobreira et al., 2011; Koppaka et al., 2012. Key amino acids directly involved in substrate accommodation in the substrate access channel are highlighted in black, corresponding to SEC entry position 124, SEC neck position 459 and SEC bottom position 303 (according to the sheep ALDH1 sequence). In italic and grey highlighted is the variant Rossman Fold (GxxxxG) corresponding to the cofactor binding domain; and grey highlighted are the main residues of the catalytic domain. (*) - denotes identical residues; (:) - denotes one conservative amino acid substitution; and (.) denotes two conservative amino acid substitutions.

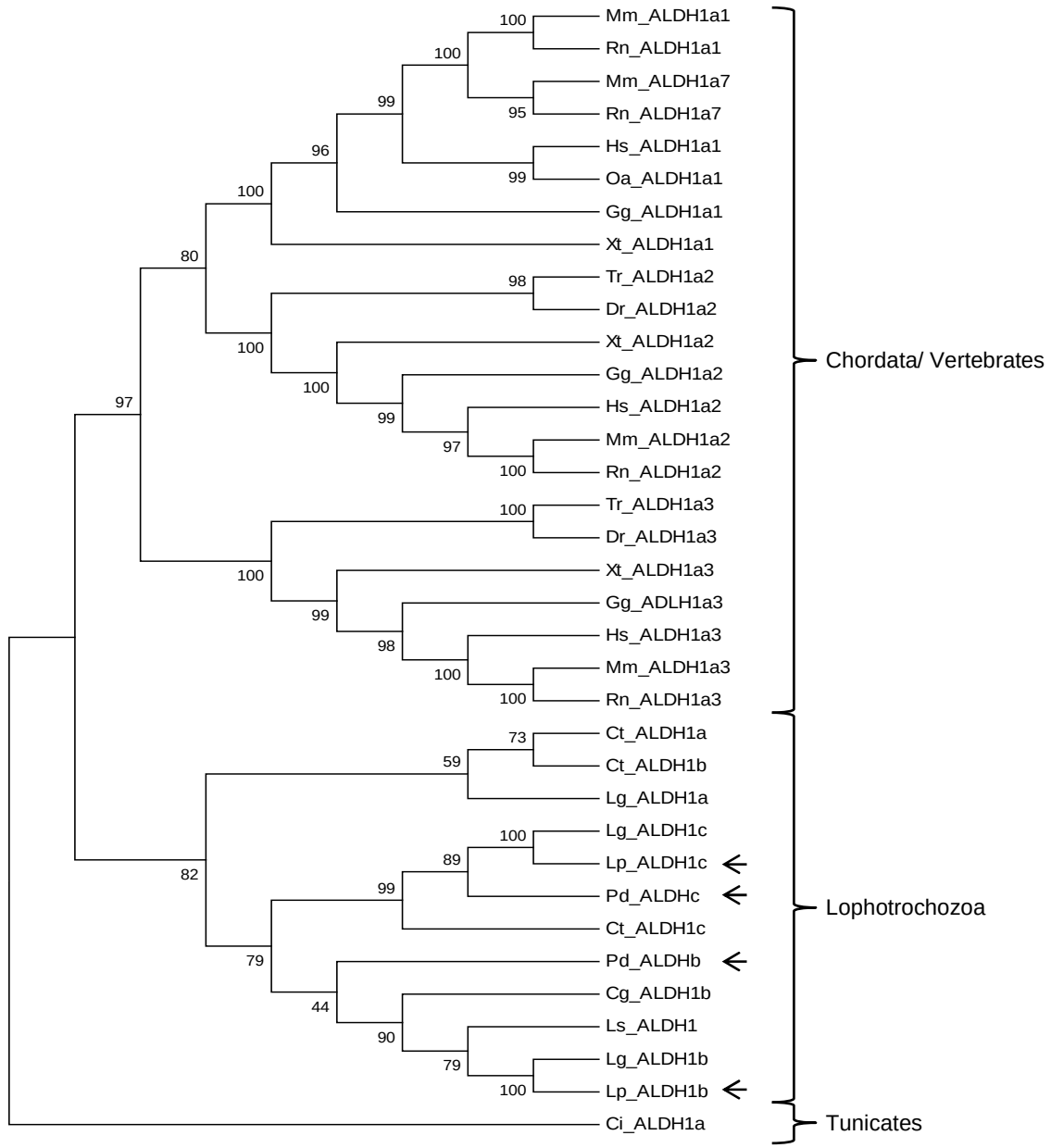

Figure 4.11: Protein phylogenetic analysis of ALDH1 by Neighbor joining method. Number at nodes represents percentage of bootstrap values related to 1000 replicates. Three was rooted with *C. intestinalis* ALDH1a sequence. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. *H. sapiens* (Hs), *R. norvegicus* (Rn), *O. aries* (Oa), *M. musculus* (Mm), *G. gallus* (Gg), *X. tropicalis* (Xt), *T. rubripes* (Tr), *D. rerio*, *C. intestinalis* (Ci), *L. gigantea* (Lg), *P. depressa* (Lp), *C. gigas* (Cg), *L. stagnalis* (Ls), *C. teleta* (Ct) and *P. dumerilii* (Pd). The arrows indicate the isolated genes in the present work.

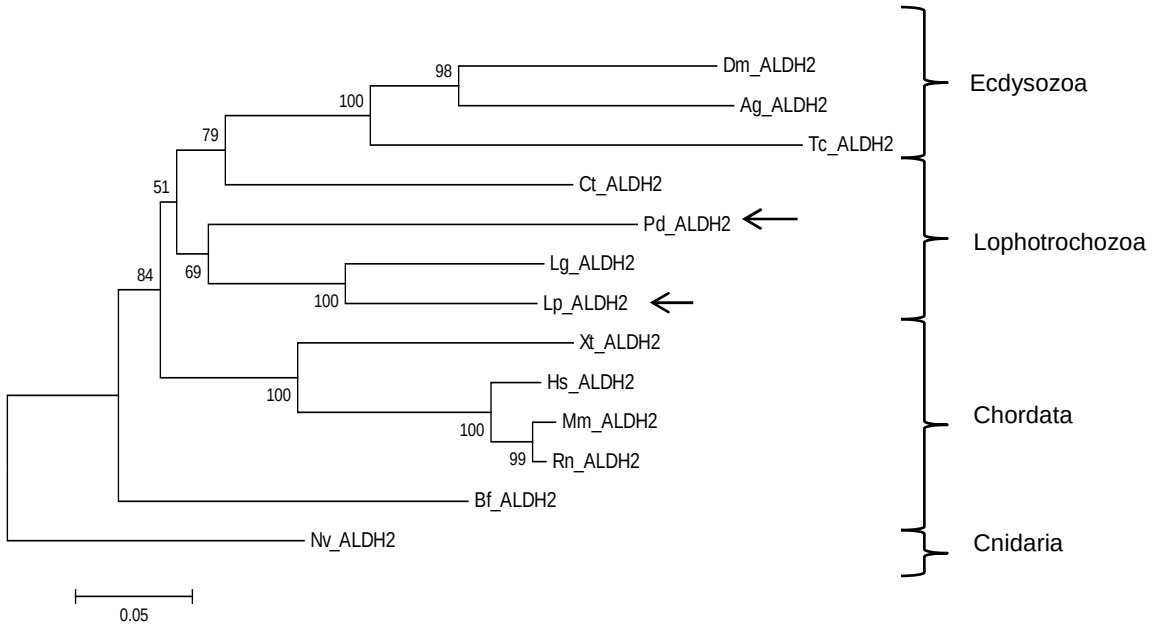

Figure 4.12: Protein phylogenetic analysis of ALDH2 by Neighbor-joining method. Number at nodes represents percentage of bootstrap values related to 1000 replicates. Three was rooted with *N. vectensis* ALDH2 sequence. The evolutionary distances were computed using the Poisson correction method. *H. sapiens* (Hs), *R. norvegicus* (Rn), *M. musculus* (Mm), *X. tropicalis* (Xt), *B. floridae* (Bf), *L. gigantea* (Lg), *P. depressa* (Lp), *C. teleta* (Ct), *P. dumerilii* (Pd), *A. gambiae* (Ag), *D. melanogaster* (Dm), *T. castaneum* (Tc), *N. vectensis* (Nv). The arrows indicate the isolated genes in the present work.

4.4.2 *In vitro* ALDH1 activity assays

To address the possible role of the putative isolated ALDH1s proteins, we performed *in vitro* assays to evaluate the capability of oxidation of retinal to RA. Recombinant *P. depressa* and *P. dumerilii* ALDH1s were expressed in COS-1 cells as well as human ALDH1a2 (as a positive control) and empty pcDNA3.1 (+) expression vector (as a negative control). Cell lysates were prepared to assay retinaldehyde dehydrogenase activity using all-*trans*-, 9-*cis*- and 13-*cis*-RAL as substrates and co-factor NAD(P)+. No RA isomers synthesis was detected for *P. dumerilii* and *P. depressa* ALDH1s-expressing cell lysates (Table 4.6).

In contrast, we detected synthesis of RA in the positive control, the recombinant human ALDH1a2 (Table 4.6). Our results are in agreement with the amino acid signature of the substrate access channel that predicted a preference for small aldehydes instead of retinal (Table 4.5 and Fig. 4.10). Most ALDHs enzymes prefer NAD+ over NADP+ as a cofactor (Yoshida et al., 1998). Indeed, for human ALDH1a2 we obtained almost 3 fold more RA synthesis when NAD+ was used as cofactor compared with NADP+. ALDH1a2 enzymes catalyzes the oxidation of all-*trans*-, 9-*cis*- and 13-*cis*-RAL to RA (Gagnon et al., 2002; Niederreither et al., 2002), but exhibit a highest specificity for all-*trans*-RAL due to

disordered loop in its active site that binds all-*trans*-retinal in a distinct manner (Lamb and Newcomer, 1999).

Table 4.6: Results from RAL isomers oxidation *in vitro* assay with COS-1 whole cell lysates after transfection with recombinant ALDHs. Retinoic acid isomers concentration is given at ng/mL mean ± standard deviation. All conditions were tested at least twice using retinal isomers precursors concentration of 5 µM. A) enzymatic activity in the presence of NAD+ as a co-factor and B) enzymatic activity in the presence of NADP+.

A	**NAD+**								
	All-*trans*-RAL			**9-*cis*-RAL**			**13-*cis*-RAL**		
Enzyme/vector	**RA**	**9cRA**	**13cRA**	**RA**	**9cRA**	**13cRA**	**RA**	**9cRA**	**13cRA**
HsALDH1a2	610±47.8	-	-	-	-	-	451±27.2	-	-
LpALDH1b	-	-	-	-	-	-	-	-	-
LpALDH1c	-	-	-	-	-	-	-	-	-
PdALDH1b	-	-	-	-	-	-	-	-	-
PdALDH1c	-	-	-	-	-	-	-	-	-
pcDNA3.1(+)	-	-	-	-	-	-	-	-	-

B	**NADP+**		
	All-*trans*-RAL		
Enzyme/vector	**RA**	**9cRA**	**13cRA**
HsALDH1a2	246±18.4	-	-
LpALDH1b	-	-	-
LpALDH1c	-	-	-
PdALDH1b	-	-	-
PdALDH1c	-	-	-
pcDNA3.1(+)	-	-	-

For human ALDH1a2 we could not detect any RA formation when 9-*cis*-RAL was provided as substrate, instated we have detected peaks at 352 nm for an unknown oxidative product (data not shown). Regarding the *P. dumerilii* and *P. depressa* ALDH2, we did not assay their possible implication in RAL oxidation since our phylogenetic analysis and SEC signature clearly indicates high homology to other ALDH2 (Fig. 4.10 and Table 4.5).

4.5 Discussion

The aldehyde dehydrogenases superfamily comprises two main enzymes types: ALDH1 which usually is localized in the cell cytosol and plays an important role in processing larger aldehydes, including retinaldehyde (RAL or retinal), one of the main vitamin A-derivatives precursor of the biological active form of retinoids, the RA (Yoshida et al., 1998; Sobreira et al., 2011); and the ALDH2 that has a mitochondrial localization and is involved in the metabolization of small toxic aldehydes, thus having a strong role in cellular detoxification and protection (Yoshida et al., 1998).

Although ALDH1 enzymes have been known for some time to be essential for RA synthesis in vertebrates, in other metazoan groups evidence for their putative involvement in such a process is very limited (Nagatomo and Fujiwara, 2003; Cañestro et al., 2006; Albalat and Cañestro, 2009; Theodosiou et al., 2010). Only in recent years, data for the presence of a retinoid metabolic and signaling system outside vertebrates has emerged (Albalat, 2009; Theodosiou et al., 2010; Sobreira et al., 2011; Gesto et al., 2012, 2013; André et al., 2014; Gesto et al., 2016). ALDH1-like orthologues have been genome predicted in various invertebrate phyla including cnidarians, hemichordates, urochordates, cephalochordates, arthropods, nematodes, mollusks and annelids (Nagatomo and Fujiwara, 2003; Cañestro et al., 2006; Albalat and Cañestro, 2009; Sobreira et al., 2011; André et al., 2014). In invertebrates, the ALDH1 gene underwent duplication, and hence for several *taxa* more than one isoform has been predicted (Cañestro et al., 2006; Albalat and Cañestro, 2009; Sobreira et al., 2011). Yet, a proper isolation and functional characterization is still missing, and since orthology does not necessarily imply functional conservation, ALDH1 enzymes outside vertebrates could have acquired different roles other than RA synthesis (Yoshida et al., 1998; Hsu et al., 1999). Indeed, only some of these ALDH1s might be capable of catalyzing the irreversible oxidation of RAL according to the SEC size and amino acid signature residues (Sobreira et al., 2011).

In the present study we have isolated ALDH1-like enzymes from two marine lophotrochozoan groups, the mollusk *P. depressa* and the annelid *P. dumerilii*, and functional characterized the capacity of such proteins to metabolize RAL into RA. The attributed nomenclature considers sequence similarity between related species, i.e., *L. gigantea* and *C. teleta* (Table 4.4).

Previous crystallographic studies revealed that all ALDH enzyme family members share a number of highly conserved residues necessary for catalysis, NAD(P)+ (the co-factor) and substrate binding (Liu et al., 1997; Graham et al., 2006; Marchitti et al., 2008; Sobreira et al., 2011; Koppaka et al., 2012). A catalytic cysteine Cys-302, Glu-268, Gly-299, and Asn-169 are essential for catalysis (numbering based on the Sheep ALDH1a1 protein see Fig. 4.10). For cofactor binding residues from Gly-245 to Gly-250 of ALDH

Rossmann Fold (GxxxxG) are crucial. The Lys-192, Glu-399, and Phe-401 are also expected to be indispensable for cofactor binding and may facilitate catalysis (Marchitti et al., 2008). All this amino acid are characteristic from ALDH superfamily are present in the isolated ALDH1s (Fig. 4.10). The ALDH SEC has three key variant residues signatures that define the channel volume/size concomitantly with enzyme substrate and function specificity (Moore et al., 1998; Sobreira et al., 2011). The first amino acid at position 124 is located on the channel entrance, and "performs" an aldehyde size selection role. In chordates ALDH2, a bulky Met124 or Leu124 (both with 124 $Å^3$) protrudes into the entry channel, allowing access of only small aldehydes, whereas in chordates ALDH1a small unobtrusive Gly124 (48 $Å^3$) allows entry of large aldehydes (Moore et al., 1998; Sobreira et al., 2011). The second signature amino acid at position 459 is founded in the SEC neck. In chordates ALDH2 usually displays a large Phe459 (135 $Å^3$), while ALDH1 display smaller Val (93 $Å^3$), or large Leu (124 $Å^3$) or Ile (124 $Å^3$). The last signature corresponds to the residue 303, near the catalytic Cys302, on the SEC end (Moore et al., 1998). All chordates ALDH2s the amino acid is a Cys303 (86 $Å^3$), whereas in ALDH1s can be a Thr303 (93 $Å^3$), Ile303 (124 $Å^3$) or a Val303 (105 $Å^3$). Hence, metazoan ALDH1s are often characterized by a large SEC with a wide open channel entrance and are known to be capable of using RAL (a large aldehyde) to RA synthesis. Whereas, ALDH2s exhibit a small SEC with a narrow channel entrance reflecting its functional ability to process small aldehydes important in detoxification processes (Moore et al., 1998; Sobreira et al., 2011). Our isolated ALDH1-like enzymes, for both *P. depressa* and *P. dumerilii*, were not able to use as substrate any of the RAL isomers provided in the *in vitro* assay since no RA isomer was detected (Table 4.6), in contrast with the positive control human ALDH1a2 (Table 4.6). This finding indicates that these enzymes are not able to metabolize large aldehydes such as RAL or similar molecules, and hence their function has yet to be determined. Based on the SEC characteristics we hypothesize that the natural subtract(s) might be molecules with small size, perhaps small-size aldehydes. The encoding amino acid sequence analysis revealed the inability to RA synthesis from RAL oxidation of *P. dumerilii* and *P. depressa* that lays in the key signature residues at the SEC. At position 124 corresponding to the channel entrance LpALDH1c have a slightly larger Ala (67 $Å^3$) instead of the vertebrates ALDH1 typical small Gly124 (48 $Å^3$), which might create an obstacle to accommodate the large β-ionone ring of retinaldehyde molecule (Table 4.5 and Fig. 4.10). Something also observed in *C. savignyi* ALDH1b (Sobreira et al., 2011). At the position 459 has a Val (105 $Å^3$) conserved with chordates. Though at position 303 have a Cys (86 $Å^3$), which is typical of ALDH2s suggesting that the protein is associated with other catalytic activity since it might process smaller aldehydes such as HsALDH2 (Moore et al., 1998; Sobreira et al., 2011). LpALDH1b have a Phe (135 $Å^3$) at the position

124 that is a larger amino acid compared to Gly (48 $Å^3$) that interfere with the β-ionone ring of retinaldehyde accommodation in the enzyme SEC. It also displays a Val (105 $Å^3$) at position 303 and an Ile at the position 459 (124 $Å^3$) typical of ALDH1 from chordates (Sobreira et al., 2011). Both *P. dumerilii* ALDH1b and ALDH1c have a Phe124 (135 $Å^3$), a Thr303 (93 $Å^3$) and Cys303 (86 $Å^3$), respectively; and a Val459 (105 $Å^3$). Since these two enzymes hold a large Phe124 (135 $Å^3$) and a Cys303 (86 $Å^3$) it might suggest that *P. duimerilii* ALDH1s enzyme do not have the proper substrate channel to accommodate RAL but instead it can be implicated in processing other aldehydes (Sobreira et al., 2011). *P. dumerilii* and *P. depressa* ALDH2 coding sequences display the typical ALDH2 chordate signature with bulky Leu124 (124 $Å^3$), Cys303 (86 $Å^3$) and Phe459 (135 $Å^3$) at the SEC mouth, bottom and neck, respectively indicating a possible role of aldehyde detoxification, a function that can be phylogenetically conserved (Sobreira et al., 2011).

Recent studies demonstrate the presence of all-*trans*-, 9-*cis*-, 13-*cis*-RA, retinol and retinyl ester endogenous levels in mollusks species, including *P. depressa* and *P. vulgata* proposing that a fully elaborated retinoid system might be present and active (Gesto et al., 2012, 2013, 2016). In addition, *in vivo* intramuscular injection of all-*trans*-RAL on *Osilinus lineatus* and *Nucella lapillus* specimens leads to increase of RA isomers tissue endogenous levels (Gesto et al., 2012, 2013). Taken together these findings suggest that a metabolic pathway for RA synthesis through RAL oxidation could exist in mollusks. However, isolated *P. depressa* ALDH1-like enzymes were not capable of using neither of the provided RAL isomers as a substrate in the *in vitro* assay system possibly due to their SEC signature amino acid residues (Sobreira et al., 2011). For *L. gigantea* three ALDH1-like isoforms were genome predicted and have a putative large SEC (Albalat and Cañestro, 2009; Sobreira et al., 2011). Though, the three putative ALDH-like sequences from *L. gigantea* do not clearly point in this direction. Perhaps, only LgALDH1a or LgALDH1c could be expected to accommodate the β-ionone moiety of RAL in the SEC since typical small Gly (48 $Å^3$) at position 124 are present. In LgALDH1c a typical Cys303 ALDH2 is also present in SEC bottom suggesting that might not be well adapted for RA synthesis, whereas in LgALDH1a Ala303 is present (Sobreira et al., 2011). A functional characterization is missing. Another unknown enzyme might exist and be involved in retinoid metabolism capable of using RAL and convert into RA.

In annelids endogenous retinoid content profile were not demonstrated, but metabolic players have been genome predicted in *C. teleta* (Albalat and Cañestro, 2009; Theodosiou et al., 2010). The polychaeta *C. teleta* has three predicted ALDH1 (a, b and c), but all might not be implicated in RAL oxidation given the SEC residues signature and size (Sobreira et al., 2011; Table 4.5). In the amphioxus *B. floridae* endogenous levels of all-*trans*-RAL, all-*trans*-, 9-*cis* and 13-*cis*-RA were detected (Dalfó et al., 2002). Also the

cephalochordate has six ALDH1s in is genome but only two of which were predicted to be able to accommodate RAL in the SEC and used to RA production (Cañestro et al., 2006; Sobreira et al., 2011). In the tunicates *C. intestinalis* and *C. savignyi*, ALDH1-like enzymes capable of processing RAL presence were also shown (Sobreira et al., 2011). These data indicates that chordate invertebrates might show a conserved ALDH1-like function with vertebrates.

Moreover, signaling cascades for mediating retinoid dependent physiological process are present in lophotrochozoa. Both annelids and mollusks retain on its genome retinoid signaling modules, RAR and RXR (Campo-Payasa et al., 2008). For instance, *N. lapillus*, *P. vulgata* and *Thais clavigera* have both retinoid receptors. Yet, only RXR-dependent signaling pathway is *in vitro* responsive to RA isomers (see Chapter 6 and 8; Castro et al., 2007; Urushitani et al, 2011, 2013; Gutierrez-Mazariegos et al., 2014). More recently, an orthologue of RXR and RAR for *P. dumerilii* were isolated, and both were *in vitro* able to active target gene transcription by RA isomers (Chapter 7; Gutierrez-Mazariegos et al., 2014). Evidences clearly predict that those animals must retain an active functional mechanism for RA isomers biosynthesis. Hence, these findings raise a fundamental question: in the case of mollusks and annelids, and perhaps other invertebrate *taxa*, what is the main metabolic route and enzyme(s) family involved in RA synthesis?

An alternative RA metabolic synthesis pathway that do not requires RAL as an intermediate precursor has been proposed (Simões-Costa et al., 2008). This alternative route for RA production involves the asymmetrical cleavage of pro-vitamin A carotenoid β-carotene (βc) by β-carotene 9′, 10′-dioxygenase (BCO-II) (Kiefer et al., 2001). BCO-II cleaves βc at the 9', 10' carbon double bond to yield β-apocarotenals and β-ionone, and then through a stepwise process involving enzymes whose identity remains indeterminate; β-apocarotenal is converted to β-apocarotenoic acid that is further oxidized to RA (Kiefer et al., 2001). A β-carotene like enzyme was genome predicted in *L. gigantea* and *C. teleta*, but were not isolated or functional characterized. Recently we have isolated for *P. dumerilii* a β-carotene oxygenase enzyme-like that preliminary tests (Chapter 2) points to be a BCO-II, indicating a possible alternative metabolic pathway for RA biosynthesis that only requires βc as precursors and not RAL. In the absence of an ALDH1-like enzyme, BCO-II route might be the key synthesis pathway for vitamin A biological active forms in lophotrochozoans, at least for annelids.

For vertebrates it was also shown that members of the cytochrome P450 family also catalyze the irreversible oxidations of retinaldehyde isomers to RA (Duester, 1996; Albalat, 2009). Then, revealing the existence of another alternative pathway that does not required ALDH1s as an intermediated enzyme for RA biosynthesis (Duester, 1996;

Albalat, 2009). However, the biological importance of this pathway remains to be elucidated (Albalat, 2009). Thus, these finding raises the question if a similar pathway is present on invertebrates in the absence of the traditional ones.

It has been proposed that ALDH1 evolutionary origin resulted from an ancestral ALDH1/2, more structurally related to current ALDH2s, in which size SEC shifts might have occurred after gene duplication early in metazoan evolution and later given rise to an enzyme harboring a large SEC capable of accommodating bulkier molecules such as RAL (Sobreira et al., 2011). An evolutionary scenario supported by the assumption that RA cascade evolved from enzymes implicated in detoxification and that pushes back into a more evolutionary basal origin than traditionally anticipated (Yoshida et al., 1998; Campo-Payasa et al., 2008; Simões-Costa et al., 2008; Albalat, 2009; Albalat and Cañestro, 2009; Sobreira et al., 2011). Compiling evidences also indicates that during the course of evolution ALDH1 enzymes emerged in metazoan long before the onset of bilateral symmetry undergoing in many lineages gene duplication and might have gained the ability to metabolize RAL into RA (Albalat, 2009; Albalat and Cañestro, 2009; Sobreira et al., 2011). In this work we provided evidences for the presence of ALDH1s members in lophotrochozoans species that supports that these enzymes emerged during the evolution before protostome–deuterostome split. Although, the isolated enzymes seem not be implicated in RA synthesis, the protein SEC revealed a residue signature that suggest a role similar to vertebrates ALDH2. At least along the chordate clade the ALDH1 involvement in the second RA biosynthesis oxidative step seems to be conserved through evolution (Sobreira et al., 2011).

The biological significance for the existence of so many ALDH1s in invertebrates is still uncertain. For the majority of them a functional characterization is missing, and then is unclear if it is involved in RA metabolism or in mechanism related to processing small aldehydes for detoxification purposes. Although we can speculate that this multiple number of ALDHs in organisms might reflect distinct metabolic requirement on different cells for RA metabolism or in the oxidative pathway of aldehyde detoxification. Clearly more studies are necessary to elucidate the specific roles of ALDH1s in invertebrates.

4.6 Conclusion

We provide here the first isolation and *in vitro* functional characterization of ALDH1 gene orthologues from two lophotrochozoan *taxa*, the mollusk *P. depressa* and the annelid *P. dumerilii*. Multiple sequence alignment analysis revealed that isolated ALDH1-like proteins for both species had in their SEC signature key amino acid residues typical of ALDH2 instead of an ALDH1, suggestive of a role in processing small aldehydes and not an involvement in RA biosynthesis by irreversible oxidation of RAL. Hence, our functional

characterization studies confirm that the isolated enzymes are not involved in annelids and mollusks RAL irreversible oxidation as no RA could be detected. Further studies needs to be conducted in other invertebrate species either from lophotrochozoan as well as other phyla in order to get further evidences to help clarifying the ALDH1 involvement in RA synthesis and its evolutionary origin.

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CHAPTER 5

5 Retinoid level dynamics during gonad recycling in the limpet *Patella vulgata*

5.1 Abstract

Germ cell commitment and meiosis initiation are among the multitude of physiological roles of retinoic acid (RA) in vertebrates. Acting via receptor-mediated transcription, RA induces the expression of meiotic factors, triggering meiosis. Contrasting with vertebrates, invertebrate RA metabolism is scarcely understood. Still, some physiological processes appear to be conserved. Here we set to evaluate the role of retinoids in the gonad maturation process of the marine gastropod *Patella vulgata*. We found that retinoid concentration in gonadal tissue, namely RA, varies between breeding and resting specimens, with maxima attained in the latter. Additionally, we isolated and quantified the expression of both the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) in gonads. In view of the stability of retinoid receptor expression, we suggest that the balance of RA levels operate through the enzymatic control of synthetic and catabolic processes. Overall, the reported data are supportive for a developmental role of RA during gonadal maturation in *P. vulgata*, which should be addressed in other protostome lineages.

5.2 Introduction

Retinoids are vitamin A (retinol)-derived lipophilic molecules that include a biologically active metabolite, retinoic acid (RA). Vertebrate RA signaling contributes to an array of physiological processes in embryo and adult tissues: from development and growth to tissue maintenance, vision and reproduction (Clagett-Dame and DeLuca, 2002; Blomhoff and Blomhoff, 2006; Maden, 2007; Theodosiou et al., 2010; André et al., 2014). RA was also suggested to play a key role in sex-dependent germ-cell fate in mammals, birds, teleosts and anurans, acting as a molecular cue required for meiosis initiation (Bowles et al., 2006; Bowles and Koopman, 2007; Smith et al., 2008; Bowles et al., 2009; Piprek et al., 2013; Rodriguez-Mari et al., 2013). Regardless of sex-determining mechanisms, meiotic progression differs between sexes. For instance, in mammals, egg-producing germ cells initiate meiosis during embryonic development whereas in males meiosis is halted until birth (Bowles et al., 2006). In the teleost *Danio rerio*, presenting no apparent heteromorphic sex chromosomes and a bipotential default ovary, a similar pattern was observed: upon sexual differentiation meiotic schedule is dimorphic (Traut and Winking, 2001; Rodriguez-Mari et al., 2013). To dictate meiotic arrest or progression, RA was suggested to regulate the expression of genes coding for several proteins of the meiotic machinery (Bowles et al., 2006). In fact, vertebrate RA signals through two nuclear

receptors, mediating the transcription of target genes: the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) (Theodosiou et al., 2010). RAR and RXR form a heterodimeric nuclear receptor complex, with RXR serving as obligate partner to RAR (Theodosiou et al., 2010; André et al., 2014). Two RA isomers, all-*trans*-RA and 9-*cis*-RA, serve as high affinity ligands for RARs whereas only 9-*cis*-RA binds with high affinity to RXRs (Blomhoff and Blomhoff, 2006).

Previously believed to be a vertebrate innovation, several molecular components of retinoid signaling and metabolism have now been found in other bilaterian lineages (Albalat and Cañestro, 2009; Theodosiou et al., 2010; André et al., 2014). Regarding retinoid active molecules, polar RA isomers have been identified in different tissues of several invertebrate species (Albalat, 2009; Theodosiou et al., 2010; André et al., 2014). Nonpolar retinoids, such as retinol (ROL) or retinyl esters (REs) have been reported mainly in the gonad (Theodosiou et al., 2010; André et al., 2014). In vertebrates, REs and ROL are typical storage and transport forms which undergo de-esterification and oxidation, respectively, serving as sequential precursors in the biosynthesis of RA (Theodosiou et al., 2010; André et al., 2014). Additionally, esterified retinol, mostly in the form of retinyl palmitate (RP), participates in the homeostatic regulation of retinoid levels, modulating their availability (Gesto et al., 2012a; André et al., 2014). Further RA oxidation leads to the inactivation of the signaling molecule (Theodosiou et al., 2010). Although invertebrate retinoid physiology is poorly understood, several lines of evidence put forward some evolutionary conserved processes (Dmetrichuk et al., 2008; Albalat and Cañestro, 2009). For instance, similarly to vertebrate RA, retinoids drove neuronal outgrowth in the gastropod *Lymnaea stagnalis* (Maden, 2007; Dmetrichuk et al., 2008). In gastropods, several reports also suggest a role of RA signaling in the development of reproductive structures in different species: *Thais clavigera*, *Nucella lapillus* and *Ilyanassa obsoleta* (Nishikawa et al., 2004; Castro et al., 2007; Sternberg et al., 2008). Recent studies have also described significant retinoid content in male gonads of *Osilinus lineatus* and *Patella depressa*, suggestive of a role in testis development and/or reproductive processes (Gesto et al., 2012a; Gesto et al., 2013). This hypothesis is further corroborated by the observed positive correlation between nonpolar retinoid concentration in the digestive gland/gonad complex and male gonadal maturation stage in *O. lineatus* (Gesto et al., 2012a). However, for a yet unknown reason, storage retinoids are apparently absent in some gastropod species (Gesto et al., 2013).

The genetic machinery related to retinoid function has also been documented outside vertebrates. Genes coding for RXR are generally present in bilaterians, while RAR genes though present in lophotrochozoans (e.g. annelids and mollusks) are absent in ecdysozoans (e.g. insects and nematodes) (Albalat and Cañestro, 2009; Theodosiou et

al., 2010; André et al., 2014). Regarding mollusks, functional RXR orthologues were reported in various species such as *T. clavigera*, *Biomphalaria glabrata*, *N. lapillus*, *L. stagnalis* and *I. obsoleta* (Nishikawa et al., 2004; Bouton et al., 2005; Castro et al., 2007; Sternberg et al., 2008; Carter et al., 2010); to date, RAR was only isolated from *N. lapillus*, *T. clavigera* and *L. stagnalis* (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014; Carter et al., 2015). However, while RXRs ligands appear highly conserved across phyla, *N. lapillus* and *T. clavigera* RAR were shown to be unresponsive to retinoids (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014).

Given the prominent role of retinoids in gametogenesis across vertebrate phyla, we aimed at addressing the involvement of the retinoid system, that is retinoid metabolism and signaling, during gonad maturation in an invertebrate group, the gastropods. For the present study, we selected the limpet *Patella vulgata* (Linn., 1758) based on two reasons. First, this species was expected to have a complex retinoid system, with both polar and nonpolar retinoids, since both were previously detected in a related species, *Patella depressa* (Gesto et al., 2013). Secondly, unlike other limpets, *P. vulgata* has a highly synchronized reproductive cycle, with well-defined breeding and resting periods, facilitating the collection of homogeneous specimens, in terms of size and reproductive stage (Ribeiro et al., 2009). After gamete discharge, gonads from both males and females regress to an immature-like or resting spent stage (neuter animals). During the inter-spawning intervals a novel cycle of gamete maturation is resumed (Orton et al., 1956). Thus, we have assessed the gonadal retinoid content in breeding and resting specimens of *P. vulgata*. Additionally, the genes coding for *P. vulgata* RAR and RXR retinoid receptors were cloned and their expression quantified in gonads at different reproductive stages.

5.3 Materials and methods

5.3.1 Animal sampling

A total of 63 limpets (mean shell length: 29.7 ± 3.27 mm; mean body mass without shell: 1714.3 ± 673 mg) were sampled in Apúlia, Portugal, in July 2010. All specimens were in the resting condition (neuter gonad, stage 0 according to Orton et al., 1956), in accordance to the breeding cycle of Portuguese *P. vulgata* described by Ribeiro et al. (2009). In October 2010, 18 more limpets (mean shell length: 31.1 ± 2.9 mm; mean body mass without shell: 2074.8 ± 806.1 mg), 10 mature females (full gonad, stage 4-5) and 8 mature males (full gonad, stages 4-5, Orton et al., 1956), were collected at the same place. After capture, animals were transported to the laboratory and the gonad was immediately sampled and stored at -80℃. Due to the small size of the neuter gonads (6.5

± 2.5 mg), they were pooled together (4 pools of 10-17 gonads). All experiments complied with European Guidelines for the correct use of laboratory animals. Before sacrifice and tissue sampling, animals were always sedated in a 7% magnesium chloride solution for 5 min.

5.3.2 Analysis of retinoid content

Extraction of retinoids from tissues was carried out as previously described (Gesto et al., 2012a). HPLC analysis of free ROL, total ROL (free + esterified) and Retinyl palmitate (RP) was carried out according to Gesto et al. (2012b). For RA quantification, three pools of each group were submitted to AS Vitas (Oslo, Norway), to be analyzed by LC/MS/MS (Gundersen et al., 2007): two gonad pools for mature females and males and pools of 10 to 28 gonads for neuter animals. Statistical analysis was performed using the software SigmaPlot 11.0. After testing ANOVA assumptions, RA levels were analyzed through a one-way ANOVA followed by the Tukey post hoc test.

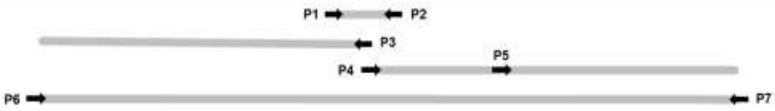
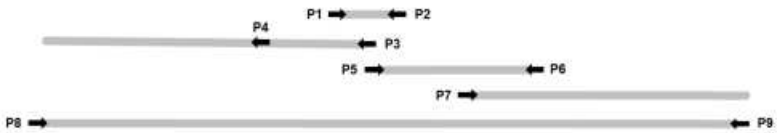
5.3.3 Tissue RNA extraction

Total RNA extraction from limpet gonads was performed with a combination of methods. First, tissues were homogenized with TRIzol reagent® (Invitrogen) and nucleic acids extracted with chloroform, according to the manufacturer's instructions. The resulting aqueous phase was used to isolate total RNA using the Illustra RNAspin Mini RNA Isolation Kit animal tissues protocol (GE Healthcare) with on-column DNase I digestion. RNA quality was assessed by electrophoresis and its concentration determined by fluorescence (Fluoroskan Ascent, Labsystems) using the Quant-iT RiboGreen RNA Assay Kit (Invitrogen). First-strand cDNA was synthesized from 350 ng of total RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions.

5.3.4 pvRAR and pvRXR isolation

P. vulgata RAR and RXR orthologues were isolated using a combination of PCR-based approaches. First, degenerate PCR primers were designed from conserved regions of both nuclear receptors (Table 5.1). To extend the initial sequence RACE-ready cDNA was synthesized using the SMARTer™ RACE cDNA Amplification Kit (Clontech) following the manufacturer instructions. RACE PCR primers were designed using the preliminary sequences and subsequent partial fragments were obtained combining RACE, nested and degenerate PCR approaches (Table 5.1). Finally, the full coding sequences were amplified and cloned (Table 5.1). Purified fragments and whole coding sequences were verified by automated sequencing (Stabvida and GATC).

Table 5.1: Primers used for gene isolation and gene expression quantification.

Gene	Name	5'→3' Sequence	Application
RXR	P1	GGCAGCGGGTGAAGGARAARGGNA	Degenerate PCR
	P2	CAGCCCSGCTCGGTCTTRTCCATNTT	Degenerate PCR
	P3	CCGCTAGTTCTGCCTCCAGGATTTG	RACE PCR
	P4	CCTGGAGGCAGAACTAGCGGTTGAA	Hemi-nested RACE PCR
	P5	CAGAAGTAGTGCCCACCAAGCAGGA	Hemi-nested RACE PCR
	P6	ATGGATCGTTCAGAGATGGAT	Full lenght PCR
	P7	CTACGTCTGTGGGCTTGGGTT	Full lenght PCR
	P8	AATCCTGGAGGCAGAACTAGC	qRT-PCR
	P9	GGTAATTCCGTGAAATGCGG	qRT-PCR
			
RAR	P1	ACGACAAGTCCTCCGGCTAYCAYTAYGG	Degenerate PCR
	P2	ACTTCTGCAGCCGGCARWAYTGRCANC	Degenerate PCR
	P3	GTACGGCTTCCCGTGACATTCCTG	Hemi-nested RACE PCR
	P4	ATGCTCCTCCGAAAGAAACCCTTGC	Hemi-nested RACE PCR
	P5	TCGGTGAGGAACGACAGGAACAAGA	Degenerate PCR
	P6	CGGCCTTCAGCAGGGTDATYTGRTC	Degenerate PCR
	P7	GCGGTGAGAAGAATGGCAGCAAGTT	RACE PCR
	P8	ATGAACCCGAACATGGGAAAT	Full lenght PCR
	P9	TTATGGTATACAGACGTTCTC	Full lenght PCR
	P10	GAGTTCATGCGAAGGATGTAAGG	qRT-PCR
	P11	CGGCAGTATTGGCACCTATTCC	qRT-PCR
			

5.3.5 Phylogenetic analysis

RAR, RXR and HNF4 (Hepatocyte Nuclear Factor 4) sequences from vertebrate and invertebrate species were retrieved from the Ensembl, NCBI databases, and JGI Genome Portal (http://genome.jgi.doe.gov/), via tBLASTn and BLASTp searches (Table 5.2). HNF4 NR was chosen as root given its basal position in the NR gene family and distant relation to both RAR and RXR (Bridgham et al., 2010). Amino acid sequences were aligned with MAFFT alignment software (Katoh and Toh, 2010) using default parameters and visualized and edited in Geneious® v7.1.7. The alignment was stripped from columns containing gaps resulting in an alignment with 37 sequences and 305 amino acid positions. Maximum Likelihood phylogenetic analysis, with 1000 bootstrap replicates was performed using the MEGA 6 software (Tamura et al., 2013). The amino acid substitution model (LG + G) used for phylogenetic analysis was inferred using ProtTest 3.4 (Darriba et al., 2011).

Table 5.2: List of sequences used for phylogenetic analysis.

Species	Abbreviation	Gene	Accession Number	Species	Abbreviation	Gene	Accession Number
Aplysia californica	Ac	RAR	XP_005093176	*Lymnaea stagnalis*	Ls	RAR	ADF43963.1
		RXR	XP_005110810			RXR	AAW34268.1
Ciona intestinalis	Ci	RAR	NP_001071806	*Mus musculus*	Mm	HNF4α	NP_032287
		RXR	NP_001071809			RARα	NP_001170773
Danio rerio	Dr	RARαa	NP_571481			RXRβ	NP_001276690
		RARγa	NP_571414			RARγ	NP_035374
		RXRαa	NP_001155023			RXRα	NP_035435
		RXRβa	NP_571350			RXRβ	NP_001192143
		RXRγa	NP_571292			RXRγ	NP_033133
Homo sapiens	Hs	HNF4α	NP_849180	*Nucella lapillus*	Nl	RAR	AIB06349
		RARα	NP_000955			RXRa	ABS70715
		RXRβ	NP_000956	*Patella vulgata*	Pv	RAR	KT883914
		RARγ	NP_000957			RXR	KT883915
		RXRα	NP_002948	*Xenopus tropicalis*	Xt	RARα	NP_001164665
		RXRβ	NP_001257330			RXRβ	XP_002935274
		RXRγ	NP_008848			RARγ	F7BHG8
Lottia gigantea	Lg	RAR	JGI ID 142734			RXRα	F6VXM9
		RXR	JGI ID 162352			RXRβ	NP_001015937
						RXRγ	F6ZPK4

5.3.6 Real-time PCR assays

The expression of RAR and RXR in the gonads of the sampled animals was determined by real-time PCR assay. The cDNA samples were diluted 1:100 and 2 µL of each cDNA was added to a reaction mixture containing 1x iQ SYBR Green supermix (BIO-RAD) and primers, to a final concentration of 500 nM, in a final volume of 10 µL. Primers sequences are summarized in Table 5.1. Amplicon sizes were 126 and 153 bp for RAR and RXR, respectively. “No template” and “no RT” controls were included in each reaction 96-well plate, and samples were run in duplicate in a Mastercycler EP Realplex Gradient S (Eppendorf) real-time PCR system (Epperdorf). PCR parameters were as follows: initial denaturation at 95℃ for 3 minutes , followed by 40 cycles of denaturation at 95℃ for 15 s, annealing for 30 s at 60℃ and elong ation at 72℃ for 30 s and a final step of elongation at 72℃ for 1 min. A melting curve wa s generated for every run to confirm the specificity of the assays. Given that no suitable reference gene was found, relative gene expression was calculated using the ΔC_T method and results normalized with $2^{-\Delta CT}$, using neuter animals as calibrator (Livak and Schmittgen, 2001). Real-time amplification efficiency was calculated using five five-fold serial dilutions. The obtained efficiencies 1.02 (R^2 = 0.98) and 1.12 (R^2 = 0.96) for RAR and RXR, respectively.

5.4 Results

5.4.1 Retinoid content in *P. vulgata* gonad

ROL and RP were detected in testis and ovary of mature males and females, and in the undifferentiated gonad of neuter animals (Fig. 5.1). The levels of RP and total

nonpolar retinoids were significantly higher in males and neuters than in females yet, no significant differences in non-esterified ROL content were found.

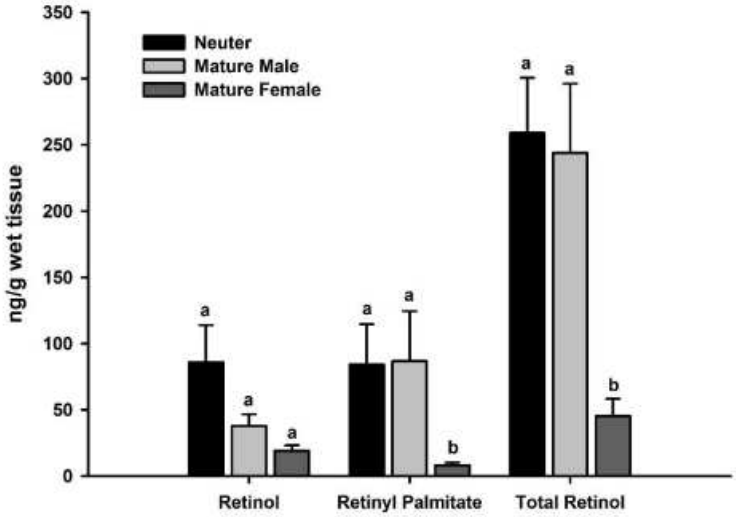


Figure 5.1: Nonpolar retinoid content in the gonad of *Patella vulgata*. Free retinol, retinyl palmitate and total retinol (free + esterified) levels in the gonad of neuter animals and mature males and females. Values are shown as mean ± SEM (n = 4-6). Different letters indicate significant differences among groups ($p < 0.05$, one-way ANOVA followed by the Tukey post hoc test).

The levels of polar, active retinoid forms (i.e. RA isomers), quantified from pools of gonads, were two orders of magnitude lower than those of the nonpolar retinoids (Fig. 5.2). All-*trans*- and 9-*cis*-RA isomers were detected in all groups, whereas 13-*cis*-RA was only detected in mature males. The levels (per unit of gonad mass) of both all-*trans* and 9-*cis*-RA were significantly higher in neuter animals than in mature males. It should be noted that, regarding females, a statistical sample size was only obtained for total RA quantification. Still, total RA quantification yielded similar results, a significantly higher concentration of RA in neuter animals than in mature males or females.

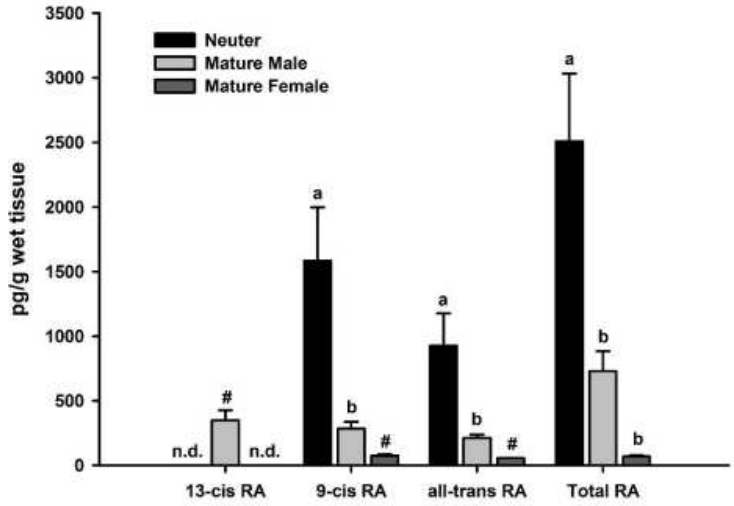


Figure 5.2: Polar retinoid content in the gonad of *Patella vulgata*. Retinoic acid isomers and total retinoic acid (sum of isomers) in the gonad of neuter animals and mature males and females. Values are shown as mean ±S EM (n = 3; # denotes n = 1-2, not included in the statistical analysis). Different letters indicate significant differences among groups ($p < 0.05$, one-way ANOVA followed by the Tukey post hoc test). n.d.: not determined.

5.4.2 Cloning and phylogenetic analysis of pvRAR and pvRXR

Figure 5.3 and Fig. 5.4 depict sequence alignments with ligand binding pocket residues, in close contact with ligands, highlighted. Regarding pvRXR, residues relevant for ligand binding (Egea et al., 2000), are fully conserved (Fig. 5.3). In contrast, pvRAR exhibits 16 out of the reported set of 24 residues suggested to be in contact with the ligand (Fig. 5.4) (Renaud et al., 1995). A similar pattern was reported for *N. lapillus* RAR (Gutierrez-Mazariegos et al., 2014). We next performed molecular phylogenetic analysis to examine the relationships between the isolated receptors and RARs and RXRs from other invertebrate and vertebrate species. Isolated sequences robustly group with other RAR and RXR sequences respectively denoting their orthology (Fig. 5.5).

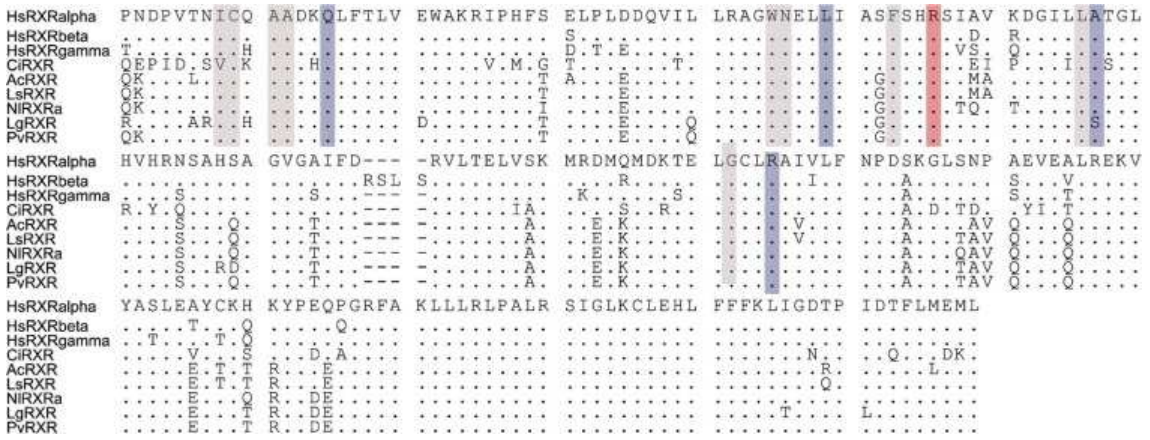


Figure 5.3: Partial alignment of the Ligand Binding Domain of RXR. Residues interacting with 9-*cis*-retinoic acid, retrieved from the crystallographic structure of human RXR alpha, PDB 1FBY, are highlighted. Blue indicates hydrogen bonds, direct or mediated by water molecules, between the substrate and pocket residues. The arginine, in red, forms an ionic interaction with the ligand. In grey, hydrophobic and Van der Waals interactions.

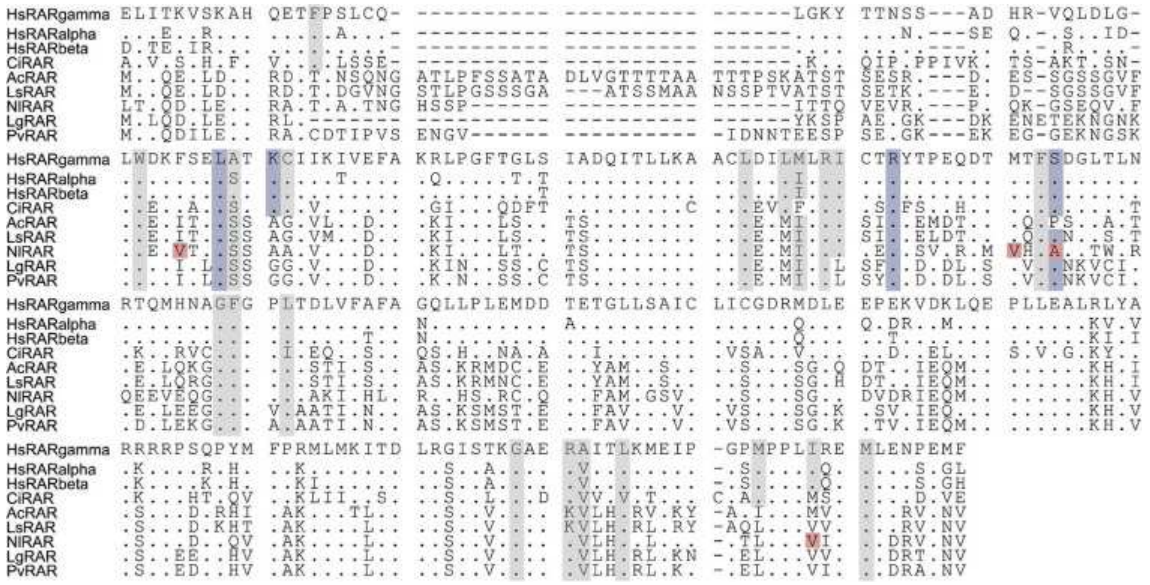


Figure 5.4: Partial alignment of the Ligand Binding Domain of RAR. Human RAR gamma residues interacting with all-*trans* retinoic acid, retrieved from the crystallographic structure PDB 2LDB, are highlighted. Direct or indirect hydrogen bonds, between the substrate and pocket residues, are highlighted in blue. In red, *N. lapillus* residues previously mutated in gain-of-function assays (Gutierrez-Mazariegos et al., 2014). In black, hydrophobic and Van der Waals interactions.

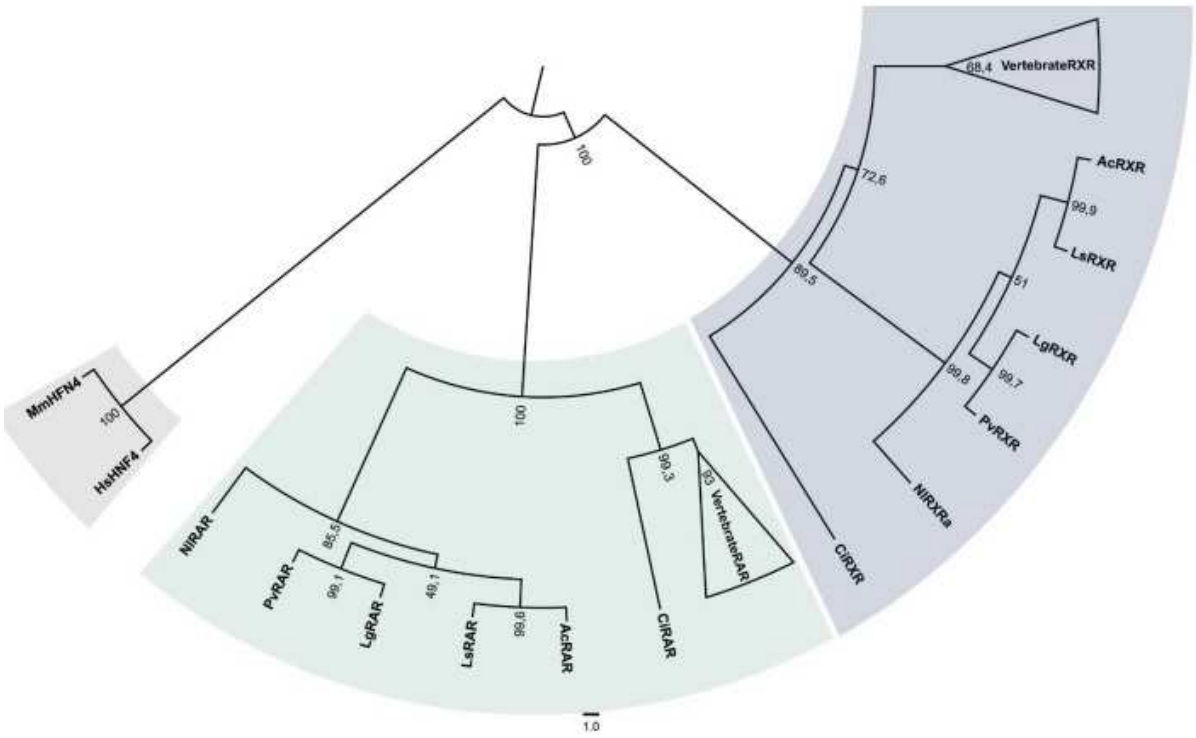


Figure 5.5: Maximum likelihood phylogenetic tree. Vertebrate and invertebrate RAR and RXR sequences were aligned and positions containing gaps and missing data were eliminated. Accession numbers for all sequences are provided in Table 5.2. Node values represent percentages of 1000 bootstrap samples of data. The HNF4 receptor was used as an outgroup to root the tree.

5.4.3 Expression of pvRAR and pvRXR

To determine if the fluctuations in RA concentration were reflected in nuclear receptor expression, we quantitatively assessed the expression profiles of RAR and RXR in the gonads of the sampled specimens. No significant differences in the expression of either nuclear receptor among mature males, mature females or neuter animals, was found (Fig. 5.6).

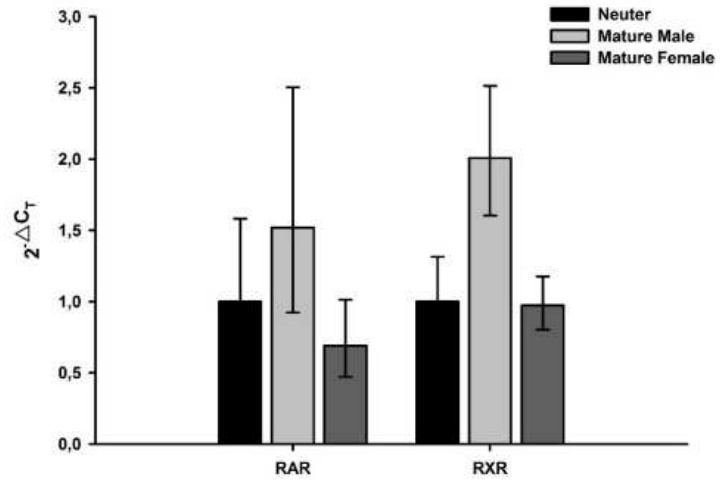


Figure 5.6: RAR and RXR expression in the gonad of *Patella vulgata*. Fold variation of RAR and RXR expression in the gonad of neuter animals, mature males and females. Relative expression was calculated using the ΔC_T method, with neuter animals used as calibrator, and normalized using $2^{-\Delta CT}$. Values are shown as mean ± maximum/minimum fold change (n = 5-7).

5.5 Discussion

In vertebrates, the levels of active retinoids are tightly controlled by retinoid homeostatic mechanisms, since as potent morphogens; both deficiency and excess of RA can be deleterious (Theodosiou et al., 2010). Despite the presence of active retinoid molecules in non-vertebrate species (Dmetrichuk et al., 2008; Gesto et al., 2012a; Gesto et al., 2013), the ability to strictly control their levels outside vertebrates is unknown. This is particularly relevant, since some invertebrate lineages lack some of the key vertebrate components known to participate in retinoid homeostasis, such as storage through ROL esterification into RE (Gesto et al., 2013). Still, there are evidences supporting the conservation of some RA functions between vertebrates and invertebrates (André et al., 2014). In this work, we aimed to address the involvement of the retinoid system in the male and female gonad development of a marine gastropod, *P. vulgata*.

First, we compared the gonadal retinoid content in *P. vulgata* among neuter animals, mature males and mature females. Both polar and nonpolar retinoids were analyzed to provide a snapshot of the signaling status and available retinoid stores, respectively. *P. vulgata* are protandrous hermaphrodites which firstly mature as males and may later transform into females (Le Quesne and Hawkins, 2006). Usually, females outnumber males in animals of larger size. A sex ratio of 50% females – 50% males has been reported for a size class of around 40 mm shell length (Orton et al., 1956). In this study, mature animals (mean size: 31 ± 3 mm shell length) had a sex ratio of 56% females

– 44% males, and the ratio in the neuter animals (mean size: 30 ± 3 mm shell length) was assumed identical.

To compare between maturation stages, data was normalized by gonad mass and retinoid concentration within the gonadal tissue was calculated. The concentration of total retinoids and RP in the neuter gonad is similar to that observed in mature males and is lower in females, suggestive of residual retinoid stores in the latter. The concentration of free ROL in neuter animal seems higher, yet not statistically significant, than in mature animals.

In *O. lineatus* males, we have previously observed that nonpolar retinoid content, both free and esterified, increased with the development of the gonad and decreased during spawning (Gesto et al., 2012a). Regarding females, neither free nor esterified ROL could be detected (Gesto et al., 2012a). Thus, *P. vulgata* and *O. lineatus* retinoid stores, in the form of RE, seem to fluctuate differently in males during gonadal maturation. In the case of *O. lineatus*, retinoids were quantified in the gonad-digestive gland complex. Although the digestive gland is known to contain low levels of retinoids, the gonad/digestive gland mass balance could be at least partially responsible for the observed pattern throughout the maturation stages (Gesto et al., 2012a).

Regarding the active polar retinoids, the concentration of 9-*cis* and all-*trans*-RA isomers is higher in neuter animals than in mature males. Similarly, total RA was higher in neuter animals. 13-*cis*-RA, on the other hand was only detected in mature males. Although still debated, 13-*cis*-RA was suggested to act as an indirect ligand following isomerization to all-*trans*-RA or 9-*cis*-RA (Blaner, 2001). Thus, this isomer could serve as precursor contributing to the signaling pool. However, in vertebrates, the physiological roles attributed to 13-*cis*-RA are not restricted to transcription-dependent processes and include hydroxysteroid metabolism regulation and signal transduction pathways (Blaner, 2001). Interestingly, mature male gonads maintain a higher concentration of total RA isomers than female ovaries. The differences between males and females could reflect different progression schedules during gametogenesis. In fact, when fully ripe, female mature gonads were shown to contain uniform populations of large oocytes whereas male could contain earlier developmental stages in addition to mature gametes (Orton et al., 1956). In addition, our results suggest a positive correlation between increased RA and RP concentrations, in agreement with the role of esterification in retinoid supply and homeostatic maintenance.

Next, we isolated orthologues of the genes coding for the RAR and RXR receptors, part of the genetic machinery involved in cellular retinoid signaling. Vertebrate RAR and RXR are RA-responsive nuclear receptors, operating as heterodimeric partners or, regarding RXR, as homodimers (Theodosiou et al., 2010; André et al., 2014). In mollusks,

several RXR orthologues were isolated and shown to bind 9-*cis*-RA (*T. clavigera* and *N. lapillus*) and mediate ligand-induced gene transcription *in vitro* (*B. glabrata*) (Nishikawa et al., 2004; Bouton et al., 2005; Castro et al., 2007). However, the two molluscan RAR orthologues reported to date appears unresponsive to RA, a feature that seems to be reversed by a minimal set of mutations (Gutierrez-Mazariegos et al., 2014). The obtained sequences suggest RXR binding ability is conserved, because key residues in the binding cavity are unchanged (Fig. 5.3). Similarly to the dogwhelk RAR, the limpet orthologue presumably lacks the ability to bind RAs (Fig. 5.4). Still, one cannot discard a possible role of the RAR/RXR heterodimer, with RXR as ligand-induced partner. The subsequent gene expression analysis revealed no differences in receptor levels between groups (neuter, mature males and mature females); suggesting that the putative receptor-mediated RA signaling is modulated by ligand titers, with RA possibly acting upon a relatively stable pool of receptors.

In vertebrates, the initiation of meiosis during gametogenesis is induced by RA, which triggers the expression of meiotic factors (i.e. stimulated by retinoic acid Gene 8 in mammals) (Bowles et al., 2006; Bowles and Koopman, 2007; Anderson et al., 2008). Our data supports a similar role for RA during gamete development in the gastropod *P. vulgata*. After gamete release, RA increase in neuters animals is concomitant with a novel maturation cycle (Fig. 5.7). In parallel, retinoid storage, possibly required to control RA titers, is also triggered. Mature animals, on the other hand, exhibit lower RA concentrations and, in the case of females, residual retinoid stores.

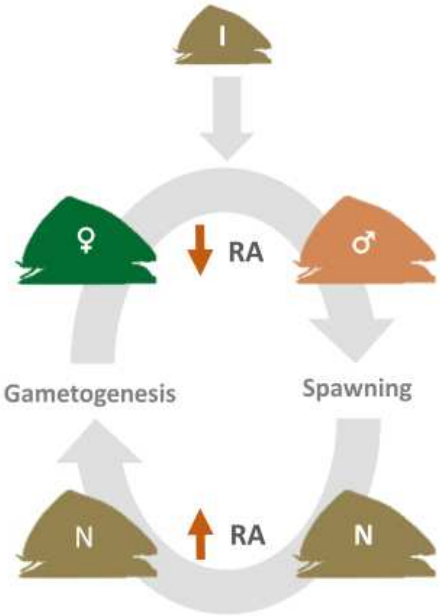


Figure 5.7: *Patella vulgata* gonad recycling. *Patella vulgata* present synchronized breeding and resting periods. In the resting stage, following spawning, animals exhibit immature-like gonads (neuter animals). During this period, a novel gamete production cycle is resumed (circular arrows). RA concentration also fluctuates between breeding and resting periods, decreasing in the former and increasing in the latter (arrows): suggestive of a role in gamete production. I – immature *P. vulgata*; N- neuter or immature-like *P. vulgata*; ♀ - mature female *P. vulgata*; ♂- mature male *P. vulgata*.

During vertebrate gametogenesis, RA balance and, consequently, signaling was shown to be controlled by catabolism and synthesis pathways (Bowles et al., 2006; Bowles and Koopman, 2007; Smith et al., 2008; Bowles et al., 2009; Piprek et al., 2013, Rodriguez-Mari et al., 2013). To arrest meiosis, the expression of a retinoic acid-degrading enzyme, CYP26, which metabolizes RA into inactive forms, is up-regulated. Conversely, a down-regulation of *Cyp26* expression is observed upon meiotic initiation. This suggests the establishment of a RA response pattern induced by enzymatic catabolism (Bowles et al., 2006). Despite an apparently stable nuclear receptor pool, ligand availability, possibly affecting ligand-mediated signaling, varies along gonad maturation in the gastropod *P. vulgata*. A similar enzymatic switch to that observed in vertebrates could be responsible for this fluctuation. However, the genetic machinery involved in retinoid metabolism, notably a CYP26 orthologue, is yet to be isolated and characterized in any gastropod species. Still, genome predicted orthologues suggests the existence of several retinoid metabolic modules, including a CYP26, in the patellogastropod *Lottia gigantea* (Albalat and Cañestro, 2009; Theodosiou et al., 2010).

5.6 Conclusion

Overall, our data support a role of RA-signaling during gonad maturation in *P. vulgata*. RA signaling seems to be controlled by ligand availability, since retinoid receptor expression is apparently stable during the reproductive cycle. The precise role of RA during *P. vulgata* gametogenesis, as well as the mechanisms involved in RA availability in this species, should be the aim of future studies.

5.7 Acknowledgments

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CHAPTER 6

6 The Retinoic acid receptor (RAR) in mollusks: function, evolution and endocrine disruption insights

6.1 Abstract

Retinoid signaling cascade evolution is far from being completely understood. To date studies of retinoid signaling pathways are missing for most invertebrate *taxa*. In order to acquire new insights regarding the evolution of retinoid signaling pathways in mollusks, in the present work we characterized the ability of retinoic acid receptors (RAR) as a monomer and in heterodimer with retinoid X receptor (RXR) to activate gene transcription in species belonging to different classes. For this purpose an *in vitro* luciferase reporter gene transactivation assay approach was used. In general, none of the tested mollusk RARs as a monomer or in heterodimer with RXR was able to activate target gene transcription by retinoids. In contrast a gene transcription repression response was observed for some species.

In the present work, we also addressed the impact of endocrine disruption chemical (EDCs), known to interact with vertebrates retinoid signaling, on mollusk RARs. We demonstrate that no target gene transcription activation was observed, which is most likely associated with the fact that the receptor is unable to activate gene transcription in the presence of putative ligands. Interestingly, both the human and mollusks RAR/RXR heterodimer show a gene transcription repression in the presence of tributyltin (TBT). The biological significant of such observation deserver further studies, as well as the ability of TBT to bind to RAR alone or within the heterodimeric complex.

Taken together, our results show that, even though mollusks retained the retinoid signaling machinery, the signaling pathway might have a different role from those of other lophotrochozoans and chordates *taxa*. Furthermore, our data suggests a conserved response between mollusk and mammalian RAR/RXR heterodimer mediating gene transcription repression with RXR displaying a major role. Mammalian RAR/RXR heterodimer is also able to repress target gene transcription by RXR ligands, in the absence of RAR ligands.

6.2 Introduction

Retinoic acid (RA) is the major natural active metabolite of vitamin A (or retinol) and is essential for proper regulation of crucial biological processes such as reproduction, embryonic development and immune system regulation (Maden and Hind, 2003). The pleiotropic functions of RA are mediated through binding of its isomers, all-*trans*- and 9-*cis*-RA, to retinoic acid receptors (RARs). RARs are members of the superfamily of nuclear hormone receptors (NRs) that function as transcription factors controlling the

expression of specific gene subsets subsequently to ligand-dependent binding (Rochette-Egly and Germain, 2009). Similar to others NRs, RARs have a well-defined domain structure consisting of six regions: an N-terminal A/B domain that contains an transactivation function 1 (AF-1), a central DNA-binding domain (DBD; region C), a flexible hinge named region D, a ligand-binding domain (LBD; region E) with an activation function 2 (AF-2), and a C-terminal region F of unknown function (Aranda and Pascual, 2001). The LBD is a multifunctional region, capable of dimerization, ligand binding, and interaction with transcriptional corepressors and coactivators (Germain et al., 2003). The RARs activities requires obligate heterodimerization with the retinoid X receptors (RXRs) to efficiently bind to specific RA responsive elements (RAREs) in the regulatory regions of target genes mediating changes in their expression (Kurokawa et al., 1995; Chambon, 2005). In the heterodimer, RXR has been reported in mammals to act as a “silent” partner being only activated by binding of all-*trans*-RA or 9-*cis*-RA to RAR (Kurokawa et al., 1994; Valcárcel et al., 1994; Kurokawa et al., 1995). RXR is also involved in RA signaling pathways and its putative natural ligand is 9-*cis*-RA, but other ligands have also been suggested such as arachidonic acid, docosahexaenoic acid and oleic acid (Allenby et al., 1993). RXR also has the ability to form heterodimers with other NRs and therefore is involved in other signaling cascades apart from retinoids (Aranda and Pascual, 2001; Szanto et al., 2004). The RAR/RXR heterodimers can act either as transcriptional repressors or activators. Traditionally, the silencing of transcriptional activity occurs in the absence of RA, where RAR/RXR is bounded to RAREs associated to a protein complex with enzymatic activities which maintain chromatin in a condensed and repressed state (Germain et al., 2003; Rochette-Egly and Germain, 2009). Once RA binds to RAR, a conformational change occurs in the heterodimers leading to corepressors release and coactivators complex recruitment. Additionally, a different enzymatic activity is involved, including histone acetyl and methyl transferases, and DNA-dependent ATPases that alters the chromatin state and subsequently initiates transcription of target genes (Germain et al., 2003; Rochette-Egly and Germain, 2009).

Until recently, the RA-signaling pathways were believed to be a vertebrate novelty (Bertrand et al., 2004; Cañestro et al., 2006; Campo-Paysaa et al., 2008; Albalat and Cañestro, 2009). This was supported by the absence of RA signaling modules in a limited number of invertebrate *taxa* with their genome sequenced (Albalat and Cañestro, 2009). However, genome sequencing of numerous invertebrate non- and model species belonging to different phyla provided evidence suggesting a more ancestral origin than originally proposed regarding both the metabolic and signaling modules (see Fig. 6.1) (Escriva et al., 2006; Campo-Paysaa et al., 2008; Albalat, 2009; Albalat and Cañestro, 2009). Therefore, it has been proposed that RAR has arisen during evolution before

protostome/deuterostome split, being already present in the genome of the last common bilaterian ancestor (Campo-Paysaa et al., 2008; Bridgham et al., 2010). However, it appears that during the evolution specific losses in certain metazoan groups and species have occurred. No RAR orthologues were found in ecdysozoan and appendicularian urochordate species (Albalat and Cañestro, 2009; Gutierrez-Mazariegos et al., 2014b). In contrast to RAR, RXR is highly conserved from cnidarian to vertebrate species indicating that they have emerged during evolution before the onset of bilateral symmetry (Thornton, 2003; Simões-Costa et al., 2008).

Regardless of the evidences pointing to the presence RAR and RXR orthologues in invertebrate genomes, gene isolation and functional characterization as well as information concerning RA role is scarce and the evolutionary origin of the retinoid pathway itself remains elusive (Gutierrez-Mazariegos et al., 2014b; Fig. 6.1). Most studies have focused mostly in classical model chordate invertebrate organisms (e.g. Hisata et al., 1998; Kamimura et al., 2000; Escriva et al., 2002, 2006). Like in vertebrates, chordate invertebrates RARs also have the ability to form heterodimers with RXR, binding and activated gene transcription in the presence of RA (Hisata et al., 1998; Escriva et al., 2002, 2006; Rinkevich et al., 2007). Although some biological effects of RA have been reported in protostomes, particularly mollusks, limited understanding of RA metabolic and signaling modules is available (Créton et al., 1993; Gesto et al., 2012; Coelho et al., 2012; Urushitani et al., 2011; Gesto et al., 2013; Urushitani et al., 2013; Carter et al., 2015). Evidences suggest that RA signaling pathways are present and active. Treatment with all-*trans*-RA in embryos at gastrulation stage from three mollusks species *Lymnaea stagnalis*, *Physa fontinalis* and *Bithynia tentaculata*, leads to deformities on eyes, shell malformations and growth arrest (Créton et al., 1993). In addition, treatment with the vertebrate RARβ-selective antagonist LE135 produced defects in both eye and shell formation in *L. stagnalis* embryos at the same level as all-*trans*-RA (Carter et al., 2015). Endogenous all-*trans* and 9-*cis*-RA were detected in the central nervous system and the hemolymph of the *L. stagnalis*. Treatment of cultured neurons from *L. stagnalis* with all-*trans*-RA induces neurite outgrowth and growth cone turning (Dmetrichuk et al., 2006, 2008). Recent studies have also described the presence of non- and polar retinoid content in the gonads of gastropod species indicative of a possible role on the organ development and/or reproduction (Gesto et al., 2012, 2013, 2016). These data clearly points to the involvement of RA signaling in mollusks mediated through a RAR-dependent pathway. A growing number of evidences report that in mollusks the RA signaling pathways are also vulnerable to endocrine disruptor chemicals (EDCs). The best known example, suggests the involvement of RXR-dependent signaling pathway in imposex development in marine female gastropods exposed to organotin compounds (OTs), tributyltin (TBT) and

triphenyltin (TPT) (Nishikawa et al., 2004; Castro et al., 2007). Injections of female gastropods with 9-*cis*-RA leads to imposex induction like TBT and/or TPT on the mollusks *Thais clavigera* (Nishikawa et al., 2004) and/or *Nucella lapillus* (Castro et al., 2007). Yet, it is still unclear if RXR acts as a homodimer or heterodimer with other member of NRs (Santos et al., 2012). A recent study tries to clarify the mechanism behind imposex induction by isolating and functional characterizing a RAR orthologue in *T. clavigera* (Urushitani et al., 2013). The study showed that *T. clavigera* RAR can form a heterodimer with *T. clavigera* RXR isoforms but is not activated by RA, other retinoids, TBT and TPT, suggesting that the signaling pathways for imposex induction are not mediated through RAR/RXR and that the RAR of mollusks lost the ability to bind its natural ligand (Urushitani et al., 2013). More recently, in *N. lapillus*, a RAR orthologue was isolated and functional characterized (Gutierrez-Mazariegos et al., 2014a). It was shown that RAR specifically bind to DNA response elements organized in direct repeats as a heterodimer with RXR. However, like *T. clavigera*, *N. lapillus* RAR does not bind RA or any other retinoid tested (Gutierrez-Mazariegos et al., 2014a). *N. lapillus* RAR is also unable to activate the transcription of reporter genes and to recruit coactivators in response to retinoid presence (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014a). Three-dimensional modeling of the LBD of *N. lapillus* RAR reveals an overall structure that is similar to vertebrate RARs (Gutierrez-Mazariegos et al., 2014a). However, the LBD of the mollusk receptor display substitution of some key amino acid residues proposed to be crucial to interact with all-*trans*-RA, that consequently might led to an overall decrease in the strength of the interaction with the molecule (Gutierrez-Mazariegos et al., 2014a). Altogether data suggest that, in mollusks, RAR has lost its affinity for retinoids. In contrast with annelids, the sister phylum of mollusks, this ability seems conserved. In *Platynereis dumerilii* RAR bind to and is activated by all-*trans*-RA (Gutierrez-Mazariegos et al., 2014a). In lophotrochozoans, while the ability to form heterodimer with RXR remains conserved, the ability to bind and activate gene transcription in retinoid presence has been lost in mollusks. These data raises some fundamental questions: Is the RAR-dependent signaling loss evolutionary conserved among mollusks classes? During the evolution of mollusks, when was RAR signaling lost? If mollusks RAR can form heterodimer with RXR, how the heterodimer behaves in the presence of retinoids? Is the RAR/RXR unresponsiveness to retinoids in common to all mollusks clades? Another interesting issue is related to the possibility for the existence of anthropogenic chemicals, the so called EDCs, with potential to disrupt mollusks RAR dependent signaling. Studies have shown that some anthropogenic compounds that occur in the environment, have the ability to bind *Homo sapiens* RAR isotypes and activate transcription reporter genes (Lemaire et al., 2005; Kamata et al., 2008). Since RAR in mollusks might have lost the

ability to bind and be activated by retinoids, we questioned if mollusk RAR can be modulated by EDCs known to be able to bind and active human RAR isomers.

In the present work we aimed at answering the above questions in order to get new insights regarding RAR-signaling pathways evolution in mollusks. Therefore, in the present study we selected four mollusks species taking in consideration its phylogenic position: *Acanthochitona crinita*, (class Polyplacophora) *Crassostrea gigas* (class Bivalvia), *N. lapillus* (class Gastropoda) and *Patella vulgata* (class Gastropoda). We characterized mollusk RAR and RXR orthologues as monomer and in heterodimer using an *in vitro* luciferase reporter assay approach in the presence of putative natural ligands, synthetic agonist and antagonists, and TBT. We aimed to evaluate if some of the compound with similar structure to retinoids may be a ligand capable of activating the receptor. Our results suggest that RAR is unresponsiveness to retinoids and it seems to be a conserved feature in mollusks. TBT leads to an interesting response in the heterodimer leading to a transcription repression. Also for *N. lapillus* we evaluated the RAR ability to activate transcription in the presence of EDCs known to be able to active target gene transcription of mammalian RARs. Similarly to other tested ligands, no transcription activation has been observed.

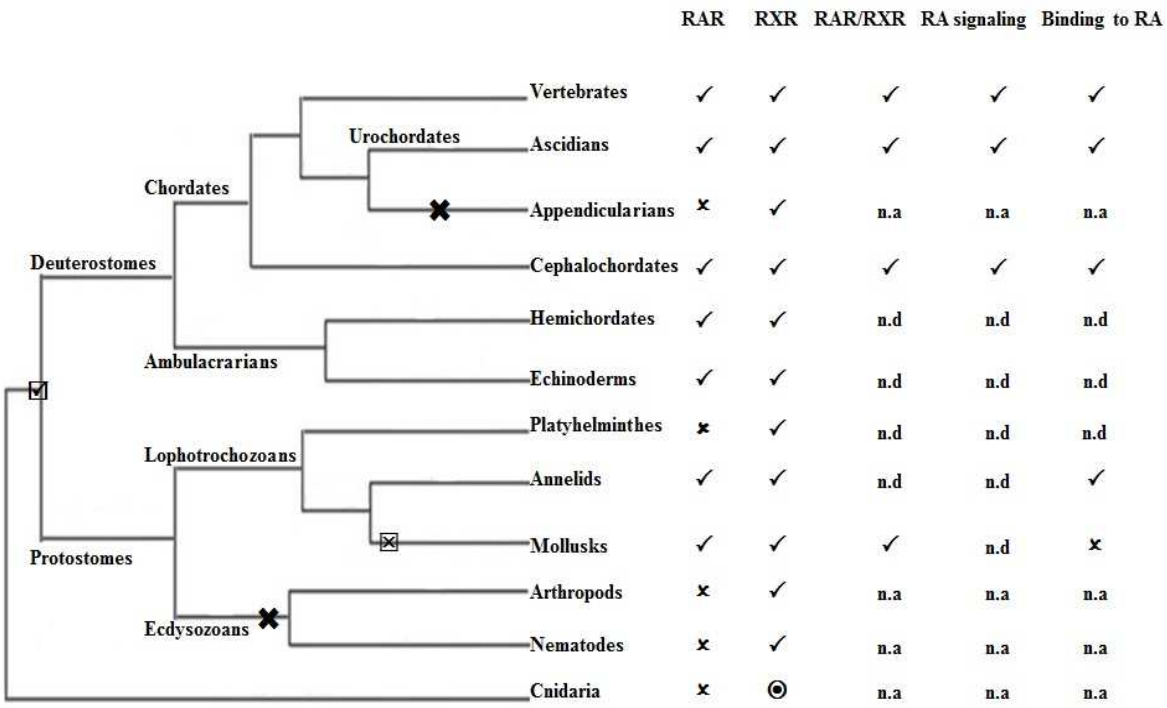


Figure 6.1: Proposed evolutionary history of the RAR ability to bind and respond to retinoids in Metazoans. RAR might have already been present in the last common ancestor of all bilateral metazoans. Yet, it seems that during evolution some loss might have occurred given RAR absence from ecdysozoan and cnidarian genomes, and also RAR has been secondarily lost in an appendicularian tunicate lineage. The ability to binding to retinoids has been suggested to be already present in the last common bilateral ancestral with reported specific ability loss so far described to have occurred at the mollusk phyla (Bertrand et al., 2004; Gutierrez-Mazariegos et al., 2014a, b). (☑) RAR ligand binding activity gain of function, (☒) RAR ligand binding loss of function, (✖) RAR orthologue is missing from genome, (⦿) not all species from the group has the gene present, (×) absent of retinoid receptors and/ or receptor binding ability loss; (✓) presence of retinoid receptors and/ or presence of RA signaling, (n.a) not applied, (n.d) not determined.

6.3 Material and methods

6.3.1 Compounds

All-*trans*-RA, 9-*cis*-RA, TBT, Endrin, Dieldrin, Naphthalene, 4-n-nonyphenol and sterile Dimethyl sulfoxide (DMSO) were purchase from Sigma-Aldrich. 4- [(E)-2- (5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl- 2-naphthalenyl) -1-propenyl] benzoic acid (TTNPB) a RAR isotypes agonist, 4- (7,8,9,10-Tetrahydro-5,7,7,10,10 -pentamethyl- 5H-benzo [e]naphtha [2,3-b] [1,4]diazepin-13-yl) benzoic acid (LE135) selective RARβ antagonist and selective RARβ/γ antagonist 4-[6-[(2-Methoxyethoxy) methoxy] -7-tricyclo [3.3.1.13,7] dec-1-yl-2-naphthalenyl) benzoic acid (CD2665), RXR synergist 4- (7,8,9,10- Tetrahydro- 7,7,10,10-tetramethylbenzo[b]naphtho[2,3-f] [1,4]thiazepin-12-yl-benzoic acid (HX630) were

purchase from Tocris Bioscience, 4-oxo-all-*trans*-retinoic acid (4-oxo-RA) purchase from TLC PharmaChem Inc., and 4-n-Heptylphenol from Alfa Aesar.

6.3.2 Selected mollusk species

In the present study we choose to functional characterized RAR orthologues from four mollusk species: *N. lapillus* (Linnaeus, 1758; Family Muricidae, clade Caenogastropoda) and *P. vulgata* (Linnaeus, 1758; family Patellidae, clade Patellogastropoda) both from gastropod class, *A. crinita* (Pennant, 1777; family Acanthochitonidae) from polyplacophora class and *C. gigas* (Thunberg, 1793; family Ostreidae) from bivalves. We start with the isolation of a RAR orthologue in A*. crinita* (described next). For *N. lapillus* and *P. vulgata* a RAR orthologue was previously isolated (Gutierrez-Mazariegos et al., 2014a; Chapter 5), whereas for *C. gigas* we used a gene sequence already published in National Center for Biotechnology Information (NCBI; XM_011444997.1; Zhang et al., 2012; Vogeler et al., 2014). Next, we performed a functional characterization of mollusk RAR as a monomer and as heterodimer with RXR using an *in vitro* luciferase reporter assay. We started by evaluating if all-*trans*-, 9-*cis*- and 4-oxo-RA were able to induce target gene transcription activation in *P. vulgata*, *C. gigas* and *A. crinit*a RARs. For gastropod *L. stagnalis* has been reported that some agonist and antagonist of RAR cause *in vivo* malformation of embryos (Carter et al., 2015). Then, we decide to evaluate if synthetic agonist or antagonist are able to transactivate RAR signaling dependent pathway in gastropods in order to try to gather molecular data that supports the observed *in vivo* results. Next, for *N. lapillus*, *P. vulgata* and *C. gigas* we evaluated the ability of RARs and RXRs as monomers and in heterodimer, to induce target gene transcription activation in response to RXR synthetic agonist, all-*trans*-, 4-oxo-RA and TBT. We also performed a gain of RAR function and a loss of RXR functions for *N. lapillus* and/or human in order to get additional data to support the obtain results with RAR as heterodimers in the presence of retinoids and TBT. We do not tested *A. crinita* RAR in heterodimer due the fact, that until the present date we have not been able to isolate the T-box and LBD of a RXR orthologue.

Finally we evaluated the ability of *N. lapillus* RAR to be transactivated with other EDCs. We only selected *N. lapillus* because this has been used as a sentinel species in ecotoxicological, particularly in relation with OTs contamination, and one of the most studied marine mollusks species (Castro et al., 2007; Coelho et al., 2012; Gesto et al., 2013; Gutierrez-Mazariegos et al., 2014a).

6.3.3 RNA extraction

Total RNA from adult mollusk gonads were extracted using a combination of methods. Initially, tissues were homogenized with TRIzol reagent® (Invitrogen) and nucleic acids extracted with chloroform, according to the manufacturer's instructions. The resulting aqueous phase was used to isolate the total RNA using the Kit illustra RNAspin Mini RNA Isolation, GE Healthcare (animal tissues protocol), with on column DNAse I digestion, starting from the ethanol step. The cDNA synthesis was performed with the iScript™cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions, using one microgram of total RNA.

6.3.4 RAR isolation in *A. crinita*

A. crinita RAR orthologue was isolated using a combination of PCR-based approaches (Fig. 6.2). In order to obtain a first sequence, a pair of degenerate oligonucleotide primers was designed based on the DNA-binding domain of RAR from annelid *Capitella teleta*, and two mollusk species, *Lottia gigantea* and *Lymnaea stagnalis* (Table 6.1 and Fig. 6.2).

Table 6.1: Primers used for RAR gene isolation in the *A. crinita*.

Name	5'-3'Sequnece	Application
P1	ACGACAAGTCCTCCGGCtaycaytaygg	Degenerated PCR
P2	ACTTCTGCAGCCGGCarwaytgrcanc	Degenerated PCR
P3	AACTGTCCGATTAACAAAGTTACA	Degenerated PCR
P4	CGGCCTTCAGCAGGgtdatytgrtc	Degenerated PCR
P5	TCCTCCGGCTATCACTACGGGGTTA	Degenerated PCR
P6	TGGACCGCAGGTCGgtnaryttca	Degenerated PCR
P7	CGGCTTCCTGCTTTGCCTTTTTCT	RACE PCR
P8	TGACGGAGCTCTCATCCAAGGGAAT	Nested RACE PCR
P9	GAGGACCCTGAGAGGATAGAACAGATGCAG	Nested RACE PCR
P10	AAAGTCCTACATCTGCGGCTGGAA	RACE + degenerated PCR
P11	CACGTTCTCCTGCCGGtcnarcatytc	RACE + degenerated PCR
P12	ATGGAGATGCCGCCTGTAA	Full-length PCR
P13	CTATGGAATGCAAACATTCTCC	Full-length PCR

Then, to extend the initial sequence a Rapid amplification of cDNA ends (RACE) - ready cDNA was synthesized using the SMARTer™ RACE cDNA amplification Kit (Clontech Laboratories Inc) following the manufacturer instructions. The 5'- and 3'-ends of the *A. crinita* RAR were amplified by RACE. Gene-specific RACE primers were designed using the preliminary degenerated sequences and subsequent partial fragments were

obtained combining RACE, Nested and degenerate PCR approaches (Table 6.1 and Fig. 6.2). Combination of the specific forward oligonucleotide primers with degenerated reverse primers in PCR reaction using cDNA as a template were also performed to help obtaining gene full-length coding sequence (Table 6.1 and Fig. 6.2). The reverse degenerated primers were design on conserved regions of the LBD using sequences from mollusk species. Finally, the full-length transcript encoding the open reading frame was PCR amplified with using a primer pair specific for the 5'- first methionine and 3'- stop codon (Table 6.1). All resultant amplification products during cDNA coding protein isolation and sequence verification were subcloned into pGEM-Easy vector (Promega) and their sequences were confirmed by sequencing analysis (GATC). All the PCR amplification reactions were undertaken using Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) following the manufactures instruction.

The amino acid sequences and molecular weight of *A. crinita* RAR were deduced using the ExPASy software (http://web.expasy.org/translate/; Artimo et al., 2012).

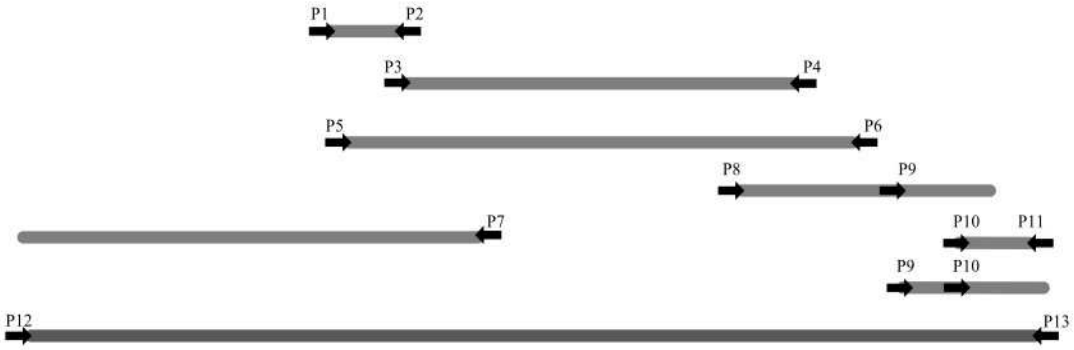


Figure 6.2: Schematic representation of the oligonucleotide primers strategy followed to isolate the complete cDNA coding sequence of a RAR orthologue in *A. crinita.*

6.3.5 Phylogenetic analysis

Retinoic acid receptor protein sequence alignment was generated with Clustal Omega from European Molecular Biology Laboratory web tool (http://www.ebi.ac.uk/Tools/msa/clustalo/; Sievers et al., 2011). Conserved parts of the amino acid sequences relevant for interaction with all-*trans*-RA were highlighted according to previous work from Renaud et al. (1995), Hisata et al. (1998), Escriva et al. (2006), Gutierrez-Mazariegos et al. (2014a) and Gesto et al. (2016).

Using MEGA 5 software (Tamura et al., 2011) a Neighbor-joining phylogenetic tree was computed (Saitou and Nei, 1987), corrected by a Poisson distribution of amino acid substitutions for evolutionary distance determination (Zuckerkandl and Pauling, 1965). A

thousand repetitions were run for bootstrap support (Felsenstein, 1985). Nuclear receptors protein or nucleotide sequence accession numbers used for phylogenetic tree or/and alignment are as follows: *Homo sapiens* (Hs) RARα AAD05222.1, HsRARβ BAH02279.1, HsRARγ NP_000957.1, HsRXRα NP_002948.1, HsRXRβ NP_001257330.1, HsRXRγ AAA80681.1, HsThRα BAH02277.1, HsTRβ NP_001239563.1, *Mus musculus* (Mm) RARα NP_001170773.1, MmRARβ NP_001276690.1, MmRARγ NP_035374.3, *Danio rerio* (Dr) RARαa Q90271, DrRARαb Q7ZTI3, DrRARγa Q91392, DrRARγb A2T928, *Polyandrocarpa misakiensis* (Pm) RAR BAM66777.1, *Ciona intestinalis* (Ci) RAR NP_001072037.1, *Branchiostoma floridae* (Bf) RAR AAM46149.1, *C. teleta* (Ct) RAR JGI_168520, *Biomphalaria glabrata* (Bg) RXR AAL86461.1, *L. gigantea* RAR XP_009050765.1, *T. clavigera* (Tc) RAR BAN82614.1, *N. Lapillus* (Nl) RAR AIB06349.1, NlRXRa ABS70715.1, *C. gigas* (Cg) RAR XM_011444997.1, CgRXR XP_011434498.1, *L. stagnalis* (Ls) RAR ADF43963.1. Sequences were retrived from GenBank (http://www.ncbi.nlm.nih.gov/genbank/; Geer et al., 2010; Benson et al., 2013).

6.3.6 Construction of plasmid vectors

The RAR T-box + LBD were amplified by PCR with Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) following the manufactures instruction. The PCR product was gel-purified, enzymatically restricted digested for subcloned into pBIND and/or pACT expression vector (Table 6.2). Then all cloned DNA sequences were verified by sequencing analysis (GATC) before transfection to COS-1 cells to confirm frame and absence of amino acid substitution and depletion. Retinoid receptors nucleotide sequence accession numbers used are as follows: HsRARγ NM_000966.5, HsRXRα NM_002957.5, NlRAR KJ410131.1, NlRXRa EU024473.1, CgRAR XM_011444997.1, CgRXR XM_011436196.1. Sequences were retrived from GenBank (Geer et al., 2010; Benson et al., 2013). *P. vulgata* retinoid receptors sequence used is the one already presented in the previous Chapter 5 (Gesto et al., 2016) of this thesis.

Table 6.2: List of primers used to amplify the NRs T-box + LBD sequence to be subcloned into pBIND or pACT expression vector

Specie and NR	Sequence	Restriction enzymes used
*N. lapillus*RAR	F: CATG**GGATCC**GTAAGCTGGCCCAGATG	BamHI
	R: GCATG**TCTAGA**TCACGGGATGCAGACGTTC	XbaI
*N. lapillus*RXRa	F: ACTG**TCTAGA**AAGAGAGAGGCCGTGCAGGAG	XbaI
	R:TATA**GGTACC**AACCCAAGACACAACTGACGATGG	KpnI
*H. sapiens*RARγ	F: CATG**GGATCC**TAGAGGTGAAGGAAGAAGGG	BamHI
	R: GCATG**TCTAGA**TCAGGCTGGGGACTTCAG	XbaI
*H. sapiens*RXRα	F: AATT**TCTAGA**GCCGTGCAGGAGGAGCGGCA	XbaI
	R: AATT**GGTACC**AGTCATTTGGTGCGGCGCCT	KpnI
*A. crinita*RAR	F: ATAT**TCTAGA**AAGGCAAAGCAGGAAGCCG	XbaI
	R: ATCG**GGTACC**CTATGGAATGCAAACATTCTCC	KpnI
*P. vulgata*RAR	F: ACTG**TCTAGA**AAATCAAAACCAGAAAGT	Xbai
	R: TATA**GGTACC**TTATGGTATACAGACGTT	kpni
*P. vulgata*RXR	F: GTCA**GGATCC**AAAGGCAGAGAACTAAGGAAAA	BamHI
	R: TATA**GGTACC**AACAGCTACGTCTGTGGGCT	KpnI
*C. gigas*RAR	F: CATG**GGATCC**GTAAGCCAAAGCTAGAGAAC	BamHI
	R: TATA**GGTACC**TCAAGGAATACACACATT	KpnI
*C. gigas*RXR	F: CATG**GGATCC**GTAAGAGAGAAGCTGTACAA	BamHI
	R: TATA**GGTACC**TCAAGTGCTGCTTGGGGA	KpnI

For the *N. lapillus* RAR gain of function a double mutation of two LBD residues, Ala230Ser and Val353Ile, were achieved. For and human RXRα and *N. lapillus* RXRa mutation of Cys432Ala and Cys409Ala, respectively, were also made for loss of ability to interact with TBT. The mutant protein synthesis was performed by Nzytech.

6.3.7 Cell culture conditions

COS-1 (monkey kidney tissue) cells were maintained Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) with phenol red and supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). The cells were cultured at 37℃ in a humidified atmosphere containing 5% CO_2.

6.3.8 COS-1 cells transfections for *in vitro* luciferase reporter gene transactivation assays

The day before transfection, cells were seeded on 24-well culture plates at a density of 2 x 10^5 cells per well and cultured in DMEM (PAN-Biotech) supplemented with 10% fetal bovine serum (Invitrogen, Thermo Fisher), and 1% penicillin/streptomycin (Invitrogen, Thermo Fisher). To studying RAR or RXR activity as monomer, after 24 h, the cells were transfected with 0.5 µg pBIND NR-LBD constructs and 1 µg of the pGL4.31

luciferase reporter vector, for a final transfection volume of 50 µL using 2 µL Lipofectamine® 2000 Transfection Reagent (Invitrogen, Thermo Fisher) and Opti-MEM reduced serum medium (Gibco, Thermo Fisher) according to manufacturers' instruction. For RAR/RXR heterodimer the cells were transfected with 0.5 µg pBIND NR1-LBD constructs, 0.75 µg of pACT NR2-LBD constructs and 0.5 µg of pGL4.31 luciferase reporter vector in a final transfection volume of 50 µL using 2 µL Lipofectamine® 2000 Transfection Reagent (Invitrogen, Thermo Fisher) and Opti-MEM reduced serum medium (Gibco, Thermo Fisher) also following to manufacturers' instruction. After 5 h of incubation at 37℃ in a humidified atmosphere containing 5% of CO_2, transfected cells were washed once with Phosphate Buffer Saline (PBS; PAA Biotech) to eliminate Opti-MEM traces and cells were exposed to the test ligands provided in 1 mL phenol red-free DMEM supplemented with 10% dextran-coated charcoal-treated serum (Invitrogen, Thermo Fisher) and 1% penicillin/streptomycin (Invitrogen, Thermo Fisher) and left for incubation for 24 h at 37℃ in a humidified atmosphere containing 5% of CO_2. All chemicals stock solutions were prepared in DMSO, and the concentration in the culture medium did not exceed 0.1%. DMSO was used as a vehicle control. After 24 h, the cells were washed once with PBS to eliminate phenol DMEM traces and 100 µL of Passive Lysis Buffer (Promega) was added to lysate the cells at 37ºC for 15 min with gentle agitation (90 rpm), and store at -80ºC no more than a week, until data reading. All the experiments were carried out at least in triplicate independent experiments for each condition.

6.3.9 Dual Luciferase Assays

Firefly and Renilla luciferase activities were determined on 20 µL of cell lysate using the Dual luciferase assay system kit (Promega) according to manufacturer's instructions. Luminescence was measured with a Synergy HT Multi-Mode Microplate reader (BioTek) after injecting 50 µL of LARII reagent (fire fly luciferase substrate, pGL4.31) followed by addition of 50 µL of Stop and Glo reagent (Renilla luciferase substrate, pBIND). To determine transfection efficiency firefly luciferase activity was normalized using the Renilla luciferase activity. Transactivation is detected when a substrate increases reporter gene transcription, and thus luminescent activity (luciferase).

6.3.10 Statistical analysis

All data from transactivation assays were obtained from the same numbers of replicated experiments carried out independently at least 3 times. Statistical analysis was performed using the software IBM Spss version 22.1 software provided by the University of Porto. After verifying that ANOVA assumptions failed or data were not normal distributed we applied Kruskal–Wallis one-way ANOVA Test K for independent samples to

the raw data. In all cases, means were considered significant different at $p < 0.05$. All data are reported as means ± Standard Error (StE).

6.4 Results and discussion

Initially an *in silico* phylogenetic analysis revealed that a RAR from the limpet *L. gigantea* has a high degree of amino acid residues conservation with human RAR and invertebrate chordates (Campo-Paysaa et al., 2008). Considering such findings it was postulated that mollusks RAR orthologue may be putative *bona fide* RAR capable to bind and respond to retinoids (Campo-Paysaa et al., 2008). Although orthology does not necessary means functional conservation (Hsu et al., 1999), and a functional characterization for the mollusks RAR was missing. Recently, a RAR orthologue has been isolated for two mollusk gastropod species *T. clavigera* and *N. lapillus* (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014a). Although both gastropods RAR revealed high sequence similarity to human RARs a functional characterization showed that the receptors failed to bind retinoids and activate target gene transcription (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014a). Findings suggested that during evolution, RAR orthologue from mollusks seems to have lost the ability to bind and activate gene transcription in response to retinoids (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014a; Fig. 6.1). Though, the mollusk species where such observation was made belong to the gastropoda class. It was unknown if RAR retinoid binding ability and transactivation response might have been lost in all mollusk classes. Considering such findings we decided to investigate if the loss of RAR ability to transactivate target gene transcription in response to retinoids might have occurred and is conserved along all the mollusk phyla or if it is a clade, class or species specific. Another interesting aspect is the fact that the mollusk RAR is able to heterodimerized with RXR, but the ability of the RAR/RXR heterodimer to respond to retinoids and other compounds was not explored before (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014a). Hence, since a loss of retinoid signaling mediated by RAR has occurred how the RAR/RXR might behave in the presence of retinoids has also been addressed here.

Aculifera is a clade of mollusks that includes the class polyplacophora and is the sister clade of Conchifera that comprises the gastropod and bivalve classes (Fig. 6.3). These two clades had its evolutionary origin from the last common mollusks ancestral (Kocot et al., 2011). Among them, the polyplacophora is the most basal and closely related group to the mollusks ancestor (Ruppert et al., 2004; Kocot et al., 2011). For the present study, we have selected mollusks from different class: gastropods, *N. lapillus* (Caenogastropoda) and *P. vulgata* (Patellogastropoda), from bivalves *C. gigas* and from polyplacophora the *A. crinita* (Fig. 6.3). We used *P. vulgata* sequence already isolated

and presented in this thesis (Chapter 5). *C. gigas* RAR was retrieved from the NCBI (RAR isoform sequence 9) and *N. lapillus* RAR, for *A. crinita* was in the present study isolated as described next.

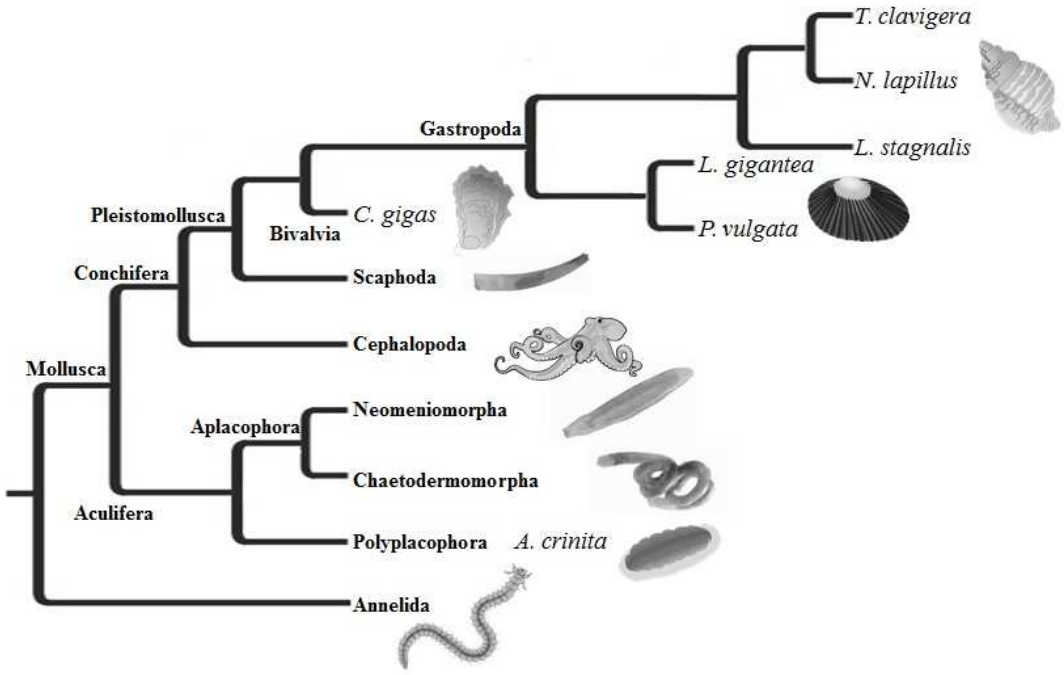


Figure 6.3: Mollusks evolutionary history based on Kocot et al. (2011). Some of the used animal illustrations were retrieved and modified from Kocot et al., 2011 and IAN Symbol Libraries (http:// ian.umces.edu/symbols/).

6.4.1 Cloning a RAR orthologue in the polyplacophora *A. crinita*

We initially isolated a cDNA encoding for a RAR-like from the *A. crinita* that we designated as AcRAR. To isolate the AcRAR we designed a pair of degenerated primers to match motifs in the DNA-binding domain (DBD) of RAR from other mollusks, *L. gigantea* and *L. stagnalis*, from and an annelid specie *C. teleta*. Fragments amplified with these primers were cloned and the sequences determined. On the basis of the obtain sequence we have performed RACE PCR strategies in order to get the full-length cDNA encoding sequence of AcRAR. Then a full-length cDNA encoding sequence of 432 amino acid residues, with a predicted molecular weight of 48.45 was isolated (Fig. 6.4).

```
atggagatgccgcctgtaacggacaactacggtaacatgggtcagacggccttcgccaac
 M  E  M  P  P  V  T  D  N  Y  G  N  M  G  Q  T  A  F  A  N
tcggaggcctacgacaactacgtctacaccatgacctacctctcacaacagaacgaccag
 S  E  A  Y  D  N  Y  V  Y  T  M  T  Y  L  S  Q  Q  N  D  Q
cggcgtatgtacgagcaggggatgtacggcatagatttacagagcccgacgacgctgtcc
 R  R  M  Y  E  Q  G  M  Y  G  I  D  L  Q  S  P  T  T  L  S
gagccgaaccagtcgccgtctcctcccccaccacctagggtctacaaaccgtgtgtggtt
 E  P  N  Q  S  P  S  P  P  P  P  P  R  V  Y  K  P  C  V  V
tgtaacgacaaatcttccggcttccattacggggttagctcatgcgaaggctgtaagggt
 C  N  D  K  S  S  G  F  H  Y  G  V  S  S  C  E  G  C  K  G
tttttccgaagaagcgtccagaaaaatatgcaatacacatgtcacaaagacaagaactgt
 F  F  R  R  S  V  Q  K  N  M  Q  Y  T  C  H  K  D  K  N  C
ccgattaacaaagttacacggaaccgctgccagtcgtgtaggctccagaagtgcttctcc
 P  I  N  K  V  T  R  N  R  C  Q  S  C  R  L  Q  K  C  F  S
gctgggatgtctaaagaagctgtacgaaatgaccgcaataagaagaaaaaggcaaagcag
 A  G  M  S  K  E  A  V  R  N  D  R  N  K  K  K  K  A  K  Q
gaagccgcagcgacaagttgtgggtcagcctcctcgacgtcgtcaacgtcgtccagtcag
 E  A  A  A  T  S  C  G  S  A  S  S  T  S  S  T  S  S  S  Q
tgcgaggacctgacacccaccgacacctcactcatacaggacatcctggaggctcaccag
 C  E  D  L  T  P  T  D  T  S  L  I  Q  D  I  L  E  A  H  Q
acgacgttcccgacgggcagcatgtcgctagaggagctcgaggaggataggaaaatggtg
 T  T  F  P  T  G  S  M  S  L  E  E  L  E  E  D  R  K  M  V
gaaacgttaaaaaagccggacggtcccagtgcaatgttatgggagcgagtgacggagctc
 E  T  L  K  K  P  D  G  P  S  A  M  L  W  E  R  V  T  E  L
tcatccaagggaattatcaaaatcgtggaattttccaagaagattccagggtttatgtcc
 S  S  K  G  I  I  K  I  V  E  F  S  K  K  I  P  G  F  M  S
ttaaccacttccgaccaaataacgctgttaaaagcagcatgtttagaaatcatgatttta
 L  T  T  S  D  Q  I  T  L  L  K  A  A  C  L  E  I  M  I  L
cgactgtgcttgcggtacacgttagagaaagacgtaatgttgttttcaaacgggctggag
 R  L  C  L  R  Y  T  L  E  K  D  V  M  L  F  S  N  G  L  E
ttgacgcggaatgaactacaagccggcggatttgggtcactcacggatacgatatttaac
 L  T  R  N  E  L  Q  A  G  G  F  G  S  L  T  D  T  I  F  N
tttgcagcttccttaaaaagaatggatgtagacgagtccgagttcgctattctctcagct
 F  A  A  S  L  K  R  M  D  V  D  E  S  E  F  A  I  L  S  A
atttgtttaataagtggagatcggtcaggtttggaggaccctgagaggatagagcagatg
 I  C  L  I  S  G  D  R  S  G  L  E  D  P  E  R  I  E  Q  M
caagagcccgtcctggaggcactcaaacactacatccggtcgcggcgacccaaccagccg
 Q  E  P  V  L  E  A  L  K  H  Y  I  R  S  R  R  P  N  Q  P
catatcttcgccaaactcctaatgaaactcactgatttaaggagcctgagtgtaaaaggt
 H  I  F  A  K  L  L  M  K  L  T  D  L  R  S  L  S  V  K  G
gctgaaaaagtcctgcatctgcggctggaaatgcctggggatttacctcccttgattatt
 A  E  K  V  L  H  L  R  L  E  M  P  G  D  L  P  P  L  I  I
gaaatgttggacagagcggagaatgtttgcattccatag
 E  M  L  D  R  A  E  N  V  C  I  P  -
```

Figure 6.4: Complete *A. crinita* RAR orthologue gene cDNA, including the deduced amino acid sequence.

6.4.2 Phylogenetic and sequence analysis

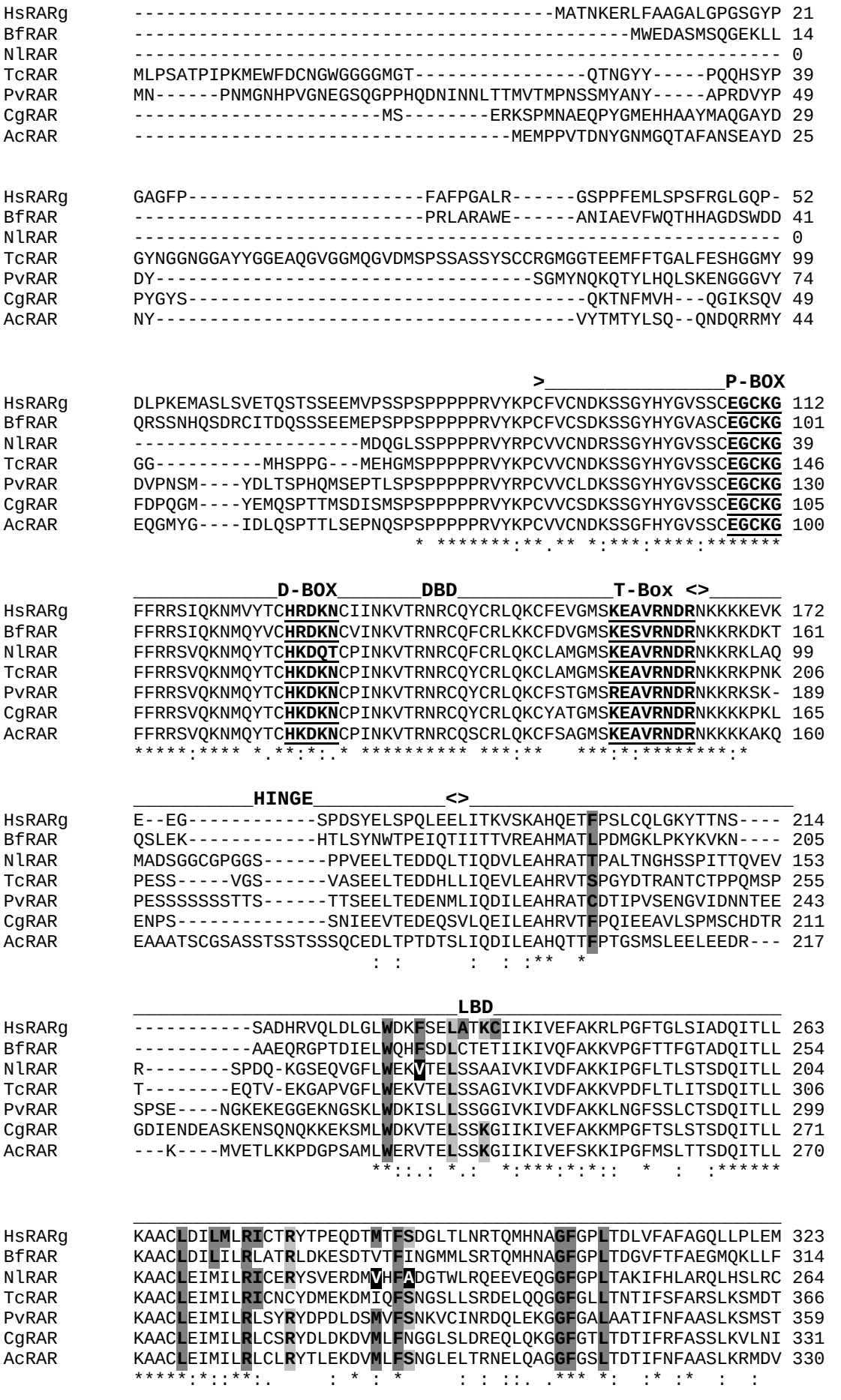
A comparison with other RARs protein sequences from other mollusks and chordates species showed that AcRAR mostly closely homology resemble to the annotated *C. gigas* RAR isoform 9 (65% of identity) and to human RARβ (57%; NP_001277195.1). *A. crinita* RAR shares higher DBD sequence homology to other

mollusks RAR and chordates (85-93%; Table 6.3). Regarding the LBD higher sequence similarity exists between other mollusks species (62-67%; Table 6.3).

Table 6.3: Amino acid percentage (%) identity between RAR from mollusks, *H. sapiens* and *B. floridae* RAR isoforms conserved main domain, the DBD and the LBD. Percentage determine by sequence alignment in NCBI protein blastp tool.

	Nl		Cg		Pv		Ac	
	DBD	**LBD**	**DBD**	**LBD**	**DBD**	**LBD**	**DBD**	**LBD**
HsRARα	81%	53%	86%	49%	86%	55%	85%	51%
HsRARβ	81	50%	85%	51%	86%	56%	85%	52%
HsRARγ	85%	50%	89%	58%	89%	53%	89%	49%
BfRAR	81%	49%	85%	50%	84%	48%	84%	46%
TcRA	95%	68%	96%	62%	93%	66%	93%	67%
NlRAR			91%	60%	88%	63%	89%	62%
CgRAR	91%	60%			95%	65%	92%	69%
PvRAR	88%	63%	95%	65%			93%	64%
AcRAR	89%	62%	92%	69%	93%	64%		

In figure 6.5 is represented the sequence alignment of RAR from the selected mollusk species together to human RARγ and *B. floridae* RAR. Amino acid sequence alignment comparisons revealed that the selected study mollusk RAR orthologues had the entire main features domain typical of the RAR family, with highly conserved residues in the P-, D- and T-box, that belongs to the DBD (Fig. 6.5). The P-box is important for determining the specificity of DNA binding, while D- and T-box are both crucial to the heterodimerezation interaction of RAR and RXR on the DNA (Perlmann et al.,1993; Zechel et al., 1994a, b); T-box also plays an important role in RXR self heterodimerezation to DNA (Zechel et al., 1994b). Within the DBD mollusks RAR P- and T-box sequence amino acid residues are fully conserved compared to human RARγ. In the case of the D-box only NlRAR has a single amino acid residues substitution (Fig. 6.5).


HsRARg
BfRAR
NlRAR
TcRAR
PvRAR
CgRAR
AcRAR
P-BOX
D-BOX
DBD
T-Box
HINGE
LBD

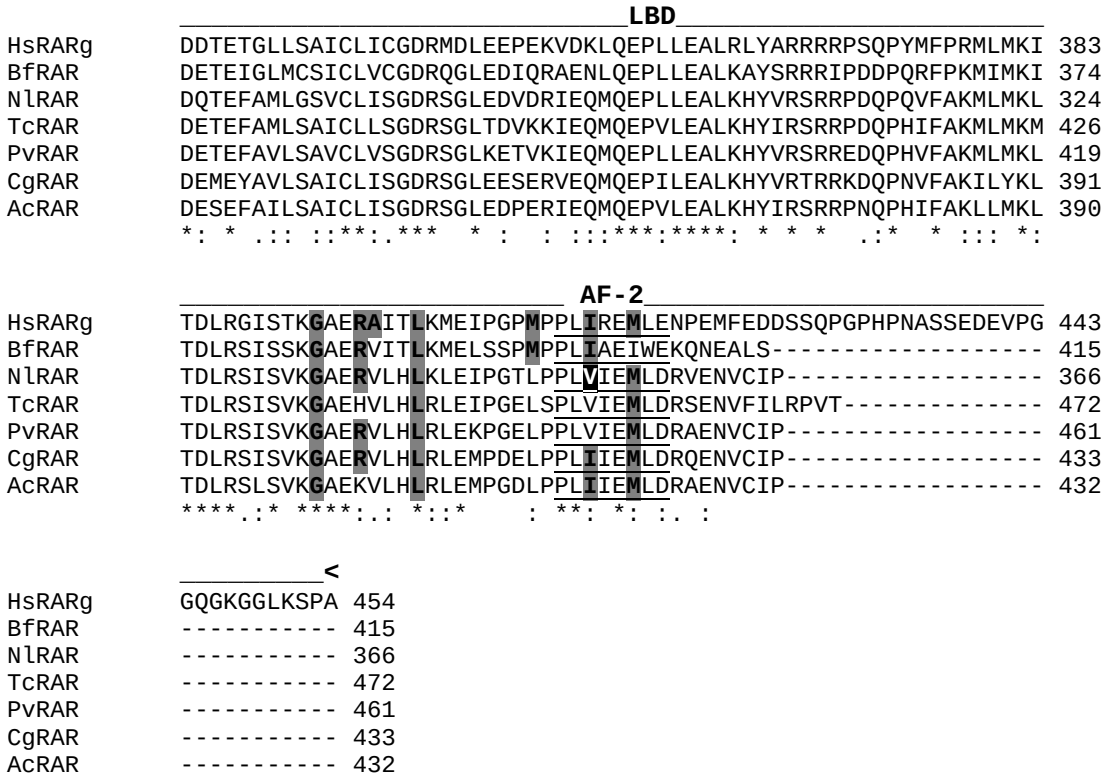


Figure 6.5: Alignment of RAR protein sequence from human, *B. floridea* and mollusks. Key amino acid residues that interact with all-*trans* RA in the human RARγ ligand-binding pocket are highlighted: direct or indirect hydrogen bonds, between the substrate and ligand binding pocket residues, are highlighted in grey and bold letters; in bold and dark grey shading amino acid implicated for hydrophobic and Van der Waals interactions. Amino acids that have been mutated in NlRAR for gain of function are shown bold white letters highlighted in black. Underline the P-, D- and T-boxes; bold underlined transactivation function AF-2 (*) - shows identical residues; (:) shows one conservative amino acid substitution; and (.) shows two conservative amino acid substitutions. Domain inferred from: Renaud et al., (1995), Hiasata et al. (1998), Escriva et al. (2006), Gutierrez-Mazariegos et al. (2014a) and Gesto et al. (2016). *H. sapiens* (Hs), *B. floridae* (Bf), *C. teleta* (Ct), *L. gigantea*, *T. clavigera* (Tc), *N. Lapillus* (Nl), *P. vulgata* (Pv), *C. gigas* (Cg), *L. stagnalis* (Ls), *A. crinita* (Ac).

Overall the LBD of mollusks RAR shares 60-68% sequence homology between them compared to the LBD from chordates (46-58%; Table 6.3). Previous crystallographic analysis revealed that 25 amino acid residues within human RARγ LBD are putatively involved in all-*trans*-RA direct contact (Renaud et al., 1995); these amino acids are highlighted in the alignment (Fig. 6.5) and presented in table 6.4. The *A. crinita*, *N. lapillus*, *P. vulgata* RARs have 15 out of the 25 residues key amino-acid residues of human RARγ, whereas *C. gigas* has 16 (Fig. 6.5 and Table 6.4).

Table 6.4: Putative 25 amino acid residues within the ligand binding pocket of RAR that interact with the all-*trans*-RA. The number is according to the *H. sapiens* RARγ sequence.

	227	230		234	236				272	278				289					397		405				
Hsγ	W	F	L	A	K	C	L	L	M	R	I	R	M	F	S	G	F	L	G	R	A	L	M	I	M
Hsα	W	F	L	S	K	C	L	L	I	R	I	R	M	F	S	G	F	L	G	R	V	L	M	I	M
Hsβ	W	F	L	A	K	C	L	L	I	R	I	R	M	F	S	G	F	L	G	R	V	L	M	I	M
Bf	W	F	L	C	E	T	L	L	I	R	L	R	M	F	I	G	F	L	G	R	V	L	M	I	I
Pm	W	F	L	S	K	S	L	L	F	R	I	R	M	F	S	G	F	M	G	R	A	I	M	M	M
Nl	W	V	L	S	A	A	L	M	I	R	I	R	V	F	A	G	F	L	G	R	V	L	L	V	M
Tc	W	V	L	S	A	G	L	M	I	R	I	C	I	F	S	G	F	L	G	H	V	L	L	V	M
Pv	W	I	L	S	G	G	L	M	I	R	L	R	M	F	S	G	F	L	G	R	V	L	L	V	M
Cg	W	V	L	S	K	G	L	M	I	R	L	R	M	F	N	G	F	L	G	R	V	L	L	I	M
Ac	W	V	L	S	K	G	L	M	I	R	L	R	M	F	S	G	F	L	G	K	V	L	L	I	M

In human RARγ all-*trans*-RA molecule enters the LBD and it forms a stable hydrogen bond between its carboxyl group and the Lys236, Arg278, and Ser289 amino acid residues (amino acid positions according to human RARγ; Renaud et al., 1995). In *A. crinita* RAR LBD the amino acids reported to form a hydrogen bond with all-*trans*-RA molecule are conserved with respect to human RARγ. The RARs LBD of the other mollusks show at least one substitution, the Arg278 being the only conserved in all studied species (Fig. 6.5 and Table 6.4). Amino acids residues Met272 and Ala234 interact with the isoprene tail and Ala397 with the β-ionone ring of all-*trans*-RA (Renaud et al., 1995). The mollusk RAR LBD does not show this residue signatures conserved but instead resembles that from the human RARα and β Met272Ile, Ala234Ser and Ala397Val (Table 6.4). In addition, other three amino acid residues were also shown to be crucial for RAR binding capability and function in response to all-*trans*-RA. Mutations in human RARγ LBD at residues Phe230, Ala397 and Met405 lead to impairment of both ligand binding and transactivation ability (Renaud et al., 1995). At these positions, none of the amino acids are conserved in mollusks; Phe230 is substituted by a Val or Ile, and Met405 is replaced by Leu (Table 6.4). Instead of Ala234 mollusks RARγ show a switch by Ser similar to the human RARα and β. Recently, a gain of function assays was performed in which double mutation at RAR LBD residues in *N. lapillus*: Ala242Ser and Val365Ile or Ala242Ser and Val239Met lead to gain of transactivation of response to 9-*cis*-RA and all-*trans*-RA (Gutierrez-Mazariegos et al., 2014a). These mutated amino acids to such positions are conserved in *A. crinita* RAR (Fig. 6.5 and Table 6.4). Taking in consideration amino residues conservation at the LBD we hypothesized that at least *A. crinita* RAR would be responsive to retinoids.

A very well resolved Neighbor-join phylogenetic tree was constructed with MEGA 5 software program using full-length amino acid sequences of RAR from selected species; sequences from RXR and thyroid hormone receptor (ThR) related nuclear receptors were used to root the tree (Fig. 6.6). The phylogenetic analysis showed that mollusks were strongly clustered together and with other lophotrochozoa the annelid *C. teleta*. Interesting *A. crinita* RAR cluster close within *C. gigas* indicating high sequence similarities (Fig. 6.6).

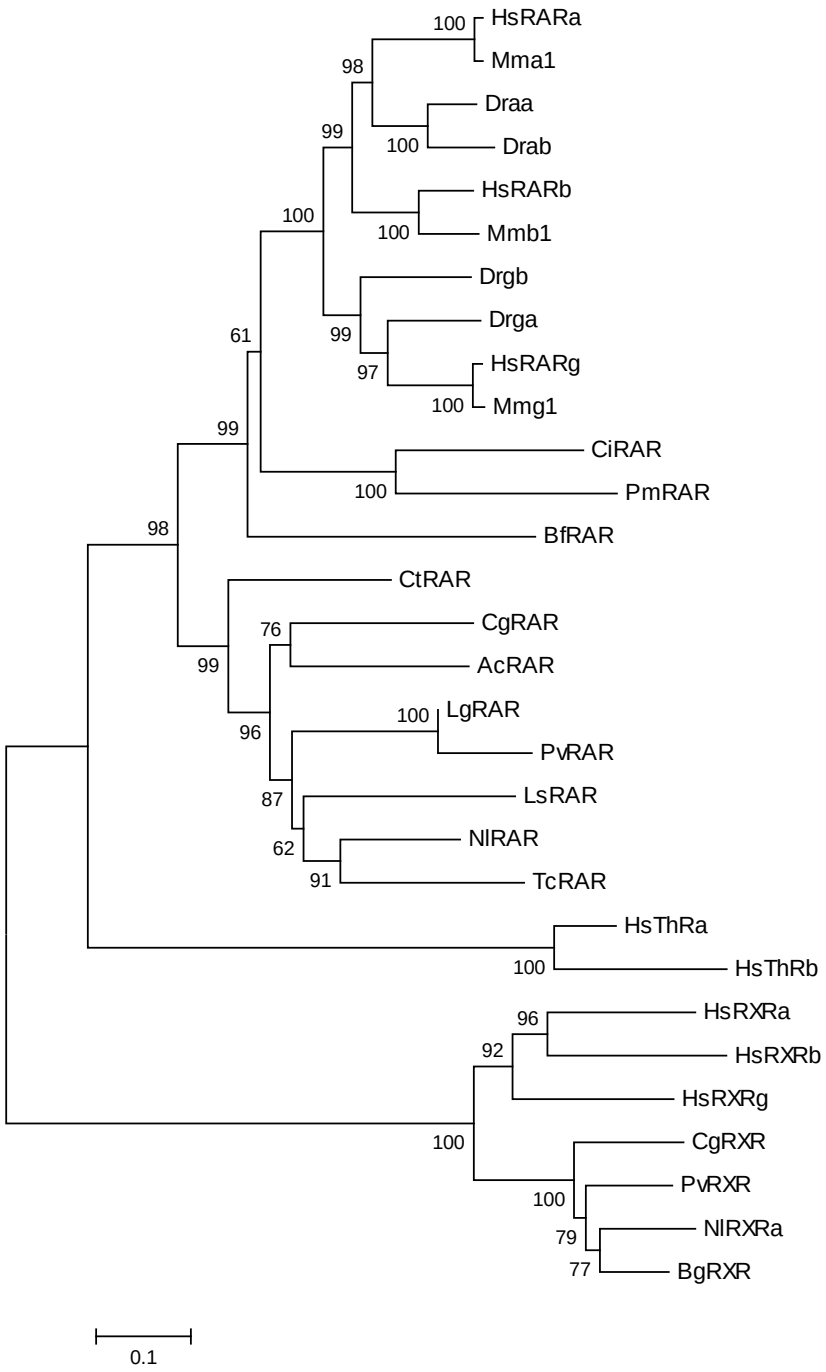


Figure 6.6: Phylogenetic tree of Retinoic acid receptor. The trees were constructed by the Neighbor-joining method using the amino acid sequences of RAR rotated using RXR and Thyroid hormone nuclear receptors. The percentages of replicate trees in which the associated *taxa* clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 30 amino acid sequences. *H. sapiens* (Hs), *M. musculus* (Mm), *Danio rerio* (Dr), *Polyandrocarpa misakiensis* (Pm), *Ciona intestinalis* (Ci), *B. floridae* (Bf), *C. teleta* (Ct), *Biomphalaria glabrata* (Bg), *L. gigantea*, *T. clavigera* (Tc), *N. Lapillus* (Nl), *P. vulgata* (Pv), *C. gigas* (Cg), *L. stagnalis* (Ls), *A. crinita* (Ac).

6.4.3 Transcriptional activity of mollusks RAR expressed in mammalian COS-1 cells lines in the presence of retinoid and rexinoids

To study and characterize the *P. vulgata*, *C. gigas* and *A. crinita* RAR ability to induce transcription of luciferase reporter gene in response to retinoids we established a reporter mammalian COS-1 cell lines *in vitro* assays. The COS-1 cells were transiently transfected with mollusks RAR LBD genes fused with a pBIND expression vector that holds the GAL4-DBD, and pGL4.31 luciferase reporter vector that contains the GAL4 binding site fused to the protein of luciferase reporter, to assess whether putative ligands could activate the RAR-mediated signaling transcription (Fig. 6.7). We selected to test RA isomers 9-*cis*- and all-*trans*-RA considered as the main natural high affinity ligands of human RARs (Allenby et al., 1993). Previous studies reported that in mammals several metabolites from catabolism of all-*trans*-RA are biological active. For instances, 4-oxo-RA seems to be a highly active modulator in embryogenesis (Pijnappel et al., 1993). Also 4-oxo-RA, 4-hydroxy-all-*trans*-RA, and 5,6-epoxy-all-*trans*-RA has the ability to inhibit the growth of several breast cancer cell lines (van der Leede et al., 1997; van Heusden et al., 1998). In addition 4-oxo-RA, 4-hydroxy-all-*trans*-RA, 5, 6-epoxy-all-*trans*-RA and 18-hydroxy-all-*trans*-RA are capable to bind and to induce transcriptional activity mediated by RARs isoforms (Idres et al., 2002). Considering the multiple biological activities of all-*trans*-RA oxidative metabolites, we were interested to further understand if any of these metabolites could work as a mollusk RAR ligand. We therefore assessed 4-oxo-RA metabolite ability to transactivate the reporter gene.

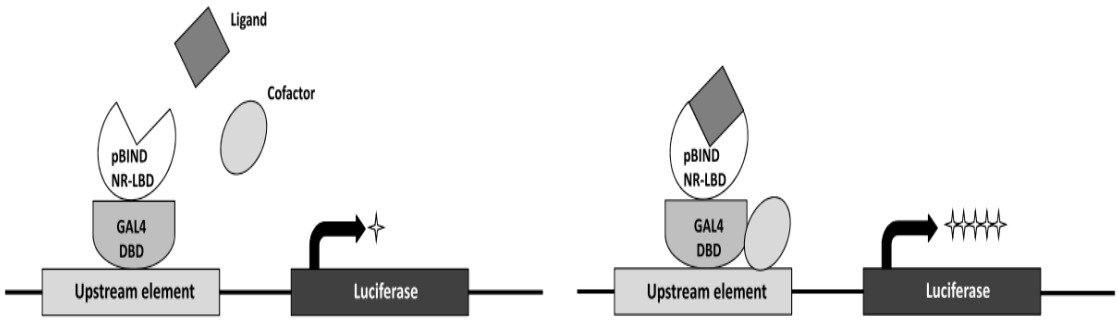


Figure 6.7: Schematic representation of the ability of pBIND/NR-LBD constructs to activate luciferase reporter gene transcription. Chimeric receptors protein is constructed by fusing GAL4 DNA-binding domain to the LBD of NR (pBIND expression vector). These chimeric proteins can bind to a GAL4 upstream activation responsive element sequence in the promoter region of a luciferase reporter construct but can only activate transcription after ligand binding to the receptor. The chimeric proteins also require the presence of a cofactor (coactivators or corepressors) that differ according to the cell line used.

Figure 6.8 displays the results from the reporter gene transactivation activity response for AcRAR, PvRAR and CgRAR under exposure to RA isomers and 4-oxo-RA. The CgRAR was unresponsive to retinoids, no gene luciferase transcription activation or repression occurred. In contrast, for PvRAR a slight significant transactivation ~1.2 fold induction in the presence of 9-*cis*-RA was observed (Fig. 6.8). Interestingly, for AcRAR exposed to some retinoids tested a significantly transactivation repression was observed.

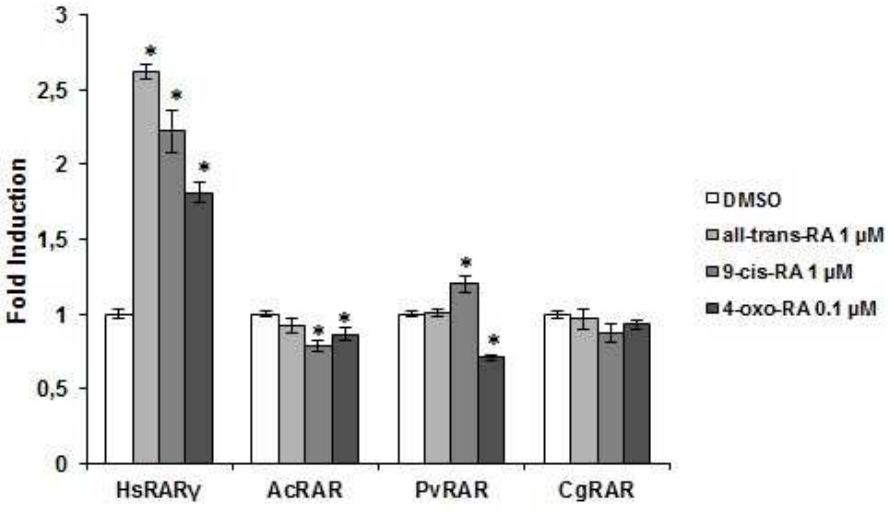


Figure 6.8: Fold induction of luciferase reporter gene activity via HsRARγ-LBD, AcRAR-LBD, PvRAR-LBD and CgRAR-LBD. All results are expressed as average fold activation after normalization to Renilla (means ± S.E.). Significant differences from vehicle control DMSO (*) were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples ($P < 0.05$).

A recent study revealed that *L. stagnalis* embryos when exposed to the vertebrate RARβ selective antagonist LE135, develop defects in both eye and shell formation (Carter et al., 2015), similar to defects reported by retinoic acid application (Créton et al., 1993). Since none of the putative natural retinoid are capable of induce reporter gene transactivation mediated by RAR, we next addressed if any of the synthetic putative agonists and antagonist might be able to do so in gastropod species. We have tested transcriptional gene activation in the presence of selective vertebrate RARβ antagonists LE135 and RARβ/γ selective antagonist CD2665, and one RARα/β/γ selective agonist TTNPB for the RAR only from the gastropods *P. vulgata* and *N. lapillus*. None of the tested synthetic compounds were able to induce gene transcriptional activity. A slight significant transactivation was obtained for NlRAR exposed to LE135 and PvRAR exposure to CD2665 (Fig. 6.9). Although the statistical analysis revealed a significant different compared to the control DMSO, when we test a 10 µM concentration of this

synthetic compounds no transactivation increase response has been observed (data not shown). Our results suggested that in the case of *L. stagnalis* the embryo reported effects might be due to all-*trans*-RA and LE135 interaction with other unknown signaling pathway or RXR-mediated signaling pathway.

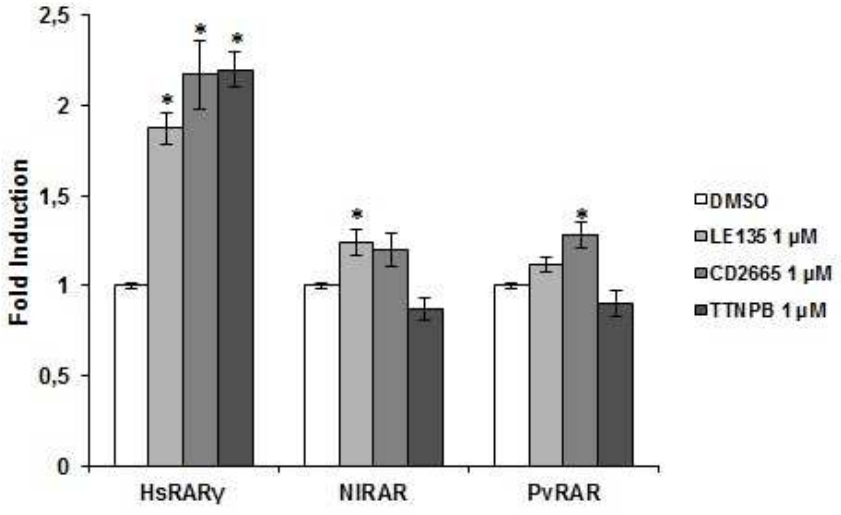


Figure 6.9: Fold induction of luciferase activity via NlRAR-LBD and PvRAR-LBD. All results are expressed as average fold activation after normalization to Renilla (means ± S.E.). Significant differences from vehicle control DMSO (*) were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples ($P < 0.05$).

These findings suggest that the loss of the ability for gene transactivation mediated by RAR in response to retinoids can be conserved through the entire phyla. Our results are in agreement and supports previously postulated hypothesis from Gutierrez-Mazariegos et al. (2014a): The evidences indicate that the ability of RAR to bind retinoids was already present in the last common ancestral of all lophotrochozoans, and might have been lost during evolution in mollusks after spilt from the annelid phyla (Fig. 6.1). Loss of response to retinoids might be due to the fact that mollusks RAR LBD during evolution suffers key amino acid residues substitution. Although we cannot exclude the fact that probably it is not only the 25 identified amino acids that determine the ability for binding, interaction of all the LBD amino acid residues might also be important to accommodate ligands.

Another interesting finding demonstrated that human RARα binds to retinoids and is capable of dissociated from corepressor and binding to a steroid receptor coactivator-one (SRC-1) nuclear receptor two (NR2) coactivator (Gutierrez-Mazariegos et al., 2014a). In contrast *N. lapillus* RAR does not binds retinoids and is incapable to binding SRC-1 NR2 coactivator peptide (Gutierrez-Mazariegos et al., 2014a). On the other hand the

NlRAR LBD interacts efficiently with corepressor-derived peptides and none of the tested retinoids is able to dissociate such interaction, proposing that the receptor might act as a constitutive repressor (Gutierrez-Mazariegos et al., 2014a). This might explain our target gene transactivation repression and lack of response to stimulation by retinoids for mollusks RAR.

For human RARγ, our positive control, the results are in agreement with previous findings that report its ability to bind and activate gene transcription by 9-*cis*-RA, all-*trans*-RA, 4-oxo-RA, LE135 and TTNPB (Li et al., 1999; Idres et al., 2002; Lemaire et al., 2005). In contrast to what was expected, the selective CD2665 antagonist of RARγ was capable to induce transcriptional activation (Bernard et al., 1992).

6.4.4 Transcriptional activity of mollusks RAR/RXR heterodimer expressed in mammalian COS-1 cells lines in the presence of rexinoids

In vertebrates RAR requires heterodimerization to RXR for proper ligation to cognate DNA on RA responsive element (RARE) and transcriptional function activity (Chambon, 2005). For mollusks, it has been shown that *T. clavigera* and *N. lapillus* RAR is capable of heterodimerization with RXR (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014a). For other mollusks species where RAR and RXR were isolated there is no reports if the heterodimer is capable of interacting with putative ligands neither if in their presence is able to induce gene transcriptional activation (Gutierrez-Mazariegos et al., 2014a; Carter et al., 2015). In the present study we addressed this issue. To assess the possible ability of mollusks RAR/RXR heterodimer to activate target gene transcription in response to selected ligands, we performed a mammalian two-hybrid assay. COS-1 cells were transiently transfected with constructs of RAR LBD fused with a pBIND expression vector that holds the GAL4 DBD and the LBD of RXR fused in the pACT expression vector that holds a VP16 activation domain and pGL4.31 luciferase reporter vector (Fig. 6.10).

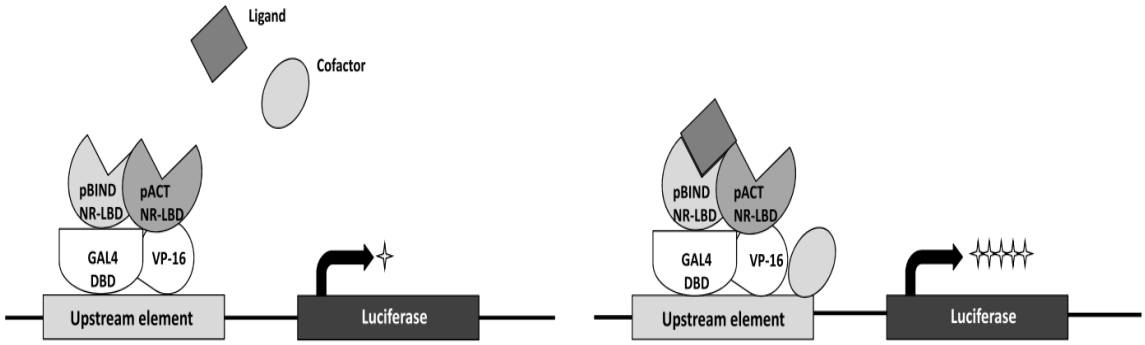


Figure 6.10: Schematic representation of the protein-protein interaction assay (NR heterodimer complexes). Protein-protein interaction is detected when a transcriptional activator (GAL4) and its enhancer (VP16) promote gene transcription (Luciferase). A chimeric heterodimer construct is constituted by a chimeric protein constructed by fusing GAL4 DNA-binding domain to the LBD of a NR (pBIND expression vector) that interacts with other chimeric receptor constructed by fusing the LBD other NR to a upstream VP16 activation domain (pACT expression vector); GAL4 binding domain interacts with VP-16 domain whereas both NR LBD interact with each other. Interaction between the two chimeric proteins fusion constructs, results in an increase in luciferase expression over the controls. The chimeric heterodimer binds to a GAL4 upstream activation responsive element sequence in the promoter region of a luciferase reporter construct that is activate by a ligand to the NR, requiring also the presence of a cofactor (coactivators or corepressors).

We first confirmed that in our *in vitro* assay system the RAR LBD and RXR LBD are capable of heterodimerize. Indeed, for the selected mollusk RAR species the nuclear receptor heterodimerizes with RXR in a very efficient way since significantly enhanced luciferase expression in transfected COS-1 cells was observed in the presence of solvent control DMSO (Fig. 6.11).

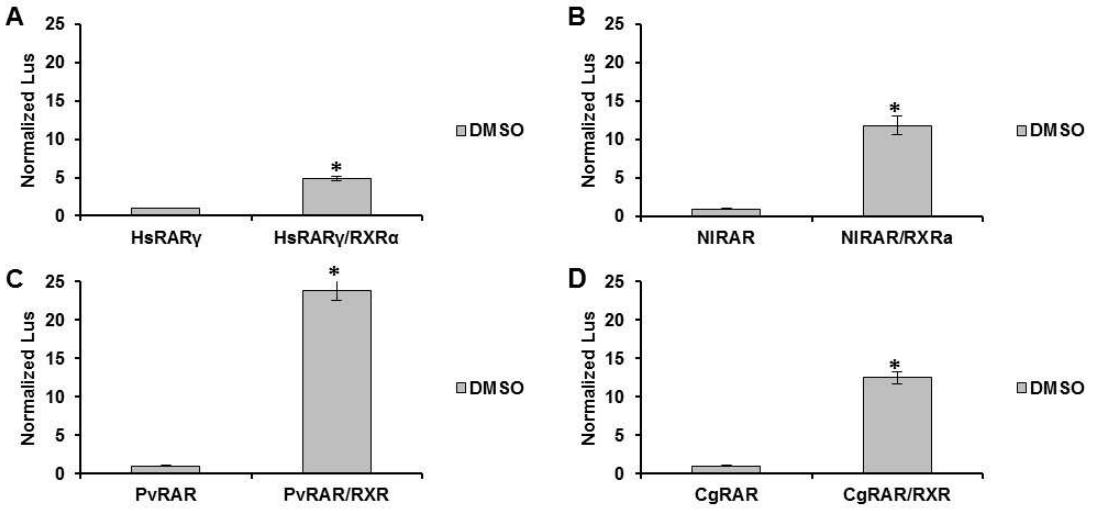


Figure 6.11: Analysis of the potential of the mollusks and human RAR LBD to form a heterodimer with the LBD of the partner RXR by means of a mammalian two-hybrid assay in COS-1 cells. The results are expressed as average fold activation after normalization to Renilla (means ± S.E.). Significant differences (*) were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples ($P < 0.05$). Letters represent the results for a) human RARγ/RXRα, b) NlRAR/RXRa, c) PvRAR/RXR and d) for CgRAR/RXR.

Next, we exposed COS-1 cells transfected with RAR LBD, RXR LBD and RAR/RXR constructs for the human and the mollusks species to a single concentration of all-*trans*-RA, 4-oxo-RA, TBT and a RXR selective agonist HX630. Tributyltin is an organotin compound considered one of the most toxic anthropogenic substances introduced into the aquatic environment (Gumy et al., 2008). The best known adverse health effect on aquatic wildlife is imposex development in gastropods (Lima et al., 2011, 2015). In addition to imposex, adverse effects have been also reported in other taxonomic groups. For instance, TBT also affects *C. gigas*, inducing changes in shell thickening and leads to perturbation at the level of reproduction mainly fertility, offspring mortality, decrease in larval and juveniles growth (Héral et al., 1989). TBT is capable to interfere with lipid homeostasis by modulation of PPARγ/RXRα signaling pathways in mammals and fish (Santos et al., 2012; Lyssimachou et al., 2015). At environmentally relevant levels TBT reduced the hatchability and caused morphological abnormalities including dorsal curvature, twisted tails and pericardial edema in *Sebastiscus marmoratus* (Zhang et al., 2011); and significantly decreased fecundity and altered zebrafish sex ratio (Lima et al., 2015). For the present study, TBT was selected for the transactivation assay both with RAR and RXR (as positive control) alone, and in heterodimer. In the case of *T. clavigera* RAR no transactivation or repression has been reported (Urushitani et al., 2013). Although, RAR heterodimerized with RXR the ability of the heterodimer mediate reporter gene transactivation in the presence of TBT has not been evaluated (Urushitani et al.,

2013). HX630, known to be a selective RXR agonist was also tested taking in consideration its selectiveness and the fact that a recent study reported its ability to induce imposex in gastropods (Umemiya et al., 1997a; Stange et al., 2012).

Human RARγ and RARγ/RXRα heterodimer significant transactivated in the presence of all-*trans*-RA (Fig. 6.12A). In agreement with previous reports for *N. lapillus* RAR, all-*trans*-RA did not induce a gene reporter transcriptional response (Fig. 6.12A; Gutierrez-Mazariegos et al., 2014a). For all mollusks RAR/RXR heterodimer a significant repression in the transactivation assay was observed in the presence of all-*trans*-RA (Fig. 6.12B). For mollusks obtained results might be due to the receptor repressor constitutive function (Gutierrez-Mazariegos et al., 2014a). Human RXRα and mollusks RXR transactivated in the presence of all-*trans*-RA (Fig. 6.12C). Previous studies reported that mammalian RXRs only bind with high affinity to 9-*cis*-RA (Heyman et al., 1992; Levin et al., 1992; Allenby et al., 1993). RXRα has little binding affinity for all-*trans*-RA, however it can be activated by this molecule (Levin et al., 1992). Yet, it has been demonstrated that the RXRα activation by all-*trans*-RA is due to its conversion to an isomer with higher binding affinity. Indeed, it that has reported that 9-*cis*-RA is a RXR ligand up to 40-fold more potent than all-*trans*-RA and binds with high affinity (Heyman et al., 1992). All-*trans*-RA can isomerize to 9-*cis*-RA, which directly binds and activates RXRα (Levin et al., 1992). So, we suggest that isomerization of all-*trans*-RA to 9-*cis*-RA might have occurred during the exposure assay leading to the observed results.

No transcription activation was obtained for *N. lapillus* RXRa and *C. gigas* RAR when exposed to 4-oxo-RA (Fig. 6.12A and C). Significant repression in the transactivation assay was observed for *N. lapillus* RAR and RAR/RXRa heterodimer, *C. gigas* RAR/RXR heterodimer and RXR, and in the case of *P. vulgata* RAR, RXR and RAR/RXR heterodimer. As expected, 4-oxo-RA induced human RARγ/RXRα heterodimer gene transcription activation, whereas RXRα alone did not (Fig. 6.12C). Some evidences suggest that the RXRs probably do not influence the response of luciferase activity induction, because 4-oxo-RA binds weakly to RXRα (Pijnappel et al., 1993). This data confirms that in the case of human RARγ/RXRα, RARγ is the receptor activated by the ligand and that mediates the gene transcription regulation. Evidences also explain the lack of transactivation response and repression of mollusk RXR in the absence of ligand.

Human RXRα (the positive control) transactivation activity was significantly induced in the presence of TBT, known to be a high affinity ligand (Nishikawa et al., 2004; Kanayama et al., 2005; Hiromori et al., 2015). The results are in agreement with previous work that report gene transactivation (Kanayama et al., 2005; Hiromori et al., 2015). We report here that similar to the *T. clavigera* and *N. lapillus*, *C. gigas* and *P. vulgata* RXR are also responsive to TBT with significant reporter gene transactivation (Urushitani et al.,

2011). For *N. lapillus* RXR these results further supports the evidences that RXR-mediated signaling pathways are implicated in imposex development induced by TBT (Castro et al., 2007). In the case of *C. gigas* and *P. vulgata* the biological implication of the observed response still needs to be addressed. For example, in the case of *C. gigas* might be involved in the reported negative effects in development and reproduction (Héral et al., 1989). Interestingly, our data show that a significant repression in the transactivation assay occurred for RAR/RXR heterodimer of all the mollusks and also for human RARγ/RXRα in the presence of TBT (Fig. 6.12B). For *P. vulgata* a slight RAR transactivation was observed (average of 1.3 fold induction), whereas for human RARγ and for the other tested mollusk RARs, a significant repression was observed (Fig. 6.12A). A CoA-BAP systems approach carried out to evaluate ligand-dependent interaction between human RARs and coactivator transcriptional intermediary factor 2 (TIF2) revealed no effects after TBT exposure (Kanayama et al., 2005). Though, Yonezawa et al. (2007) suggested that TBT has the ability to interact with a RAR-dependent pathway by binding to RXR. TBT inhibited osteoclast differentiation at concentration range of 3-30 nM. Treatment with a Ro41-5253, a RAR-specific antagonist, restored the TBT inhibition effect on osteoclastogenesis. The results of the present study for human and mollusks RARs suggest that TBT is able to interact with RAR and/ or the cell line environmental, i.e., co-factors (coactivators and corepressors) leading to the transcriptional repression response. Additional studies are required to clarify the exact mechanism underlying this result.

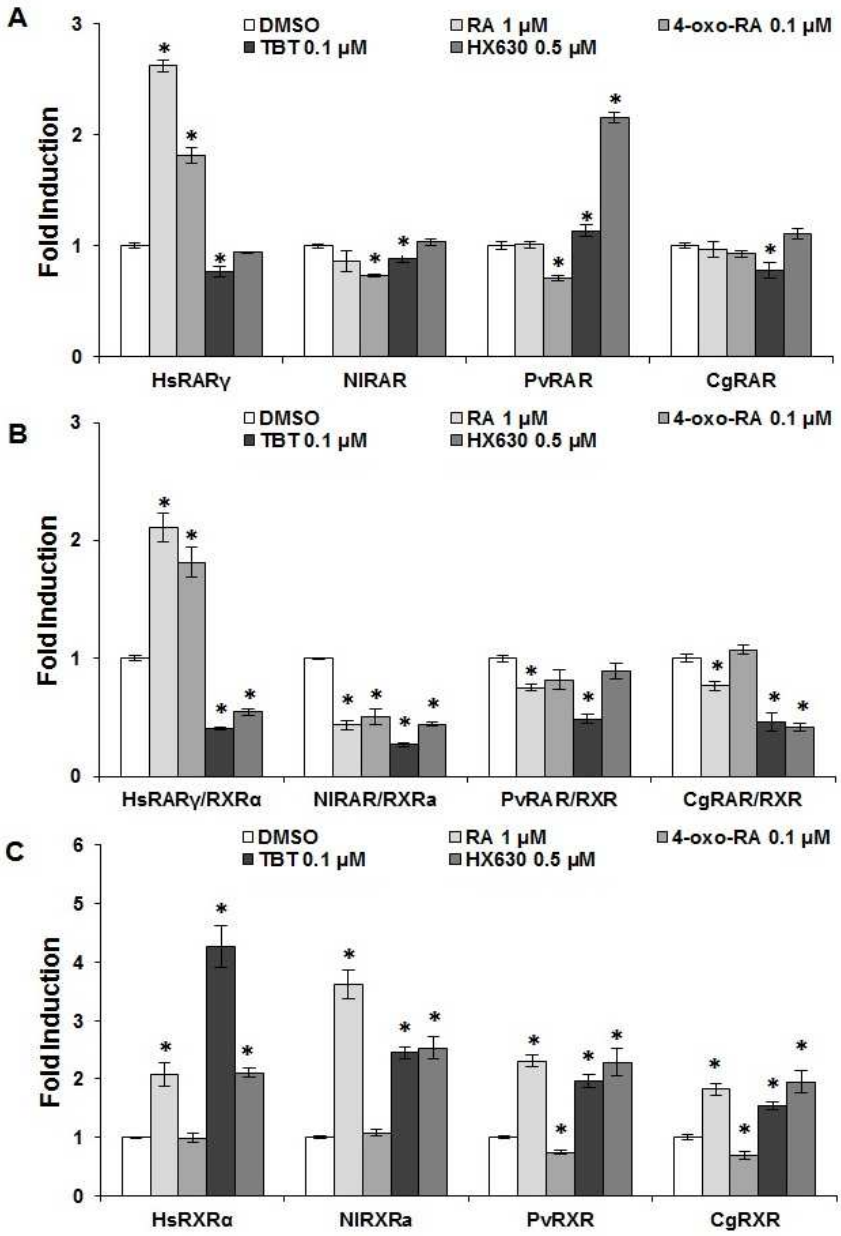


Figure 6.12: Reporter gene transactivation activity response in COS-1 cells expressing Human, *N. lapillus*, *P. vulgata* and *C. gigas* RAR, RAR/RXR or RXR after 24 h exposure to a single concentration of all-*trans*-RA, 4-oxo-RA, HX630 and TBT. The results are expressed as average fold activation after normalization to Renilla (means ± S.E.). Significant differences from vehicle control DMSO (*) were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples ($P < 0.05$). A) Displays results from RARs response to selected compounds; B) Presents data from RAR/RXR whereas in C) the transactivation activation of RXRs is showed.

For human, *N. lapillus* and *C. gigas* RARs no transactivation response was observed after exposure to HX630. Interestingly a significant repression was observed in the transactivation assays when RAR/RXR was tested in heterodimer (Fig. 6.12A). An interesting observation relates with the significant transcriptional activation of the

luciferase reporter gene observed for PvRAR in the presence of HX630. However, PvRAR/RXR was unresponsive to HX630 (Fig. 6.12A and B). The literature suggests that HX630 is a specific and selective RXR agonist having a minor affinity for RAR at least in mammals. HX630 has been reported to be a weak RAR-antagonistic and a RXR-agonistic and able to interact with both receptors in the heterodimeric complex RAR-RXR (Umemiya et al., 1997a, b). The results regarding the repression response in the transactivation assays suggest that similar to TBT, HX630 not only has the ability to bind RXR, but also interacts with the RAR (Umemiya et al., 1997a, b). For PvRAR/RXR, we hypothesize that HX630 competition for binding to RXR might have occurred leading to no transactivation or repression. Regarding RXR from human control and mollusks a significant transactivation in response to HX630 was observed with similar fold induction. NlRXRa is able to activate the transcription of the luciferase reporter in the presence of HX630, as expected data is in agreement with studies that report that NlRXR-mediated signaling is implicated in imposex development induced by those compounds (Stange et al., 2012).

The roles of the individual receptors within non-permissive heterodimers are still under debate. Initially, it has been proposed that RXR was unable to bind its ligands in the heterodimer RAR/RXR. RAR heterodimer formation with RXR enhances the binding affinity and the specificity of the interactions and binding to the receptors cognate DNA in RARE (Glass, 1994; Kurokawa et al., 1995; Chambon, 2005). Within the heterodimer, in mammalian models, RXR seems to act as a “silent” partner (Kurokawa et al., 1994; Valcárcel et al., 1994; Kurokawa et al., 1995). It was later demonstrated that RXR is in fact able of ligand binding and coactivator interaction leading to a conformational change in the presence of RARE (Kersten et al., 1996; Minucci et al., 1997; Germain et al., 2002; Lammi et al., 2008). In the heterodimer RAR and RXR binding to its ligand proceeds independently (Kersten et al., 1996). Yet, RXR is unable to release corepressors in response to its ligands in heterodimer, prohibit coactivator access and fails to activate transcription (Minucci et al., 1997; Germain et al., 2002). The corepressors released and transcription activation just happens upon ligand binding to RAR (Germain et al., 2002). Furthermore, heterodimerization with RAR has also been suggested to enhance the interaction between RXR and corepressors (Lammi et al., 2008). Cells treatment with a pan RXR agonist, SR11237 leads to increase interaction between corepressor SMRT and the RAR/RXR heterodimer in the absence of RAR ligand (Lammi et al., 2008). In other words, RXR in heterodimer with RAR seems to operate as a repressor when in the presence of its own ligands. It has also been reported that co-existence of ligands for both receptors can lead to synergistic activation of transcription within the heterodimer due to binding to both RAR and RXR (Minucci et al., 1997). Moreover, some studies demonstrate

that mammalian RARs is also able of repressing transcription when in heterodimer with RXR, in the presence of its own ligands by corepressor interaction (Kurokawa et al., 1995; DiRenzo et al., 1997). In mammalian cells, this repression of transactivation activity is dependent on the polarity of binding of RAR/RXR heterodimers to specific RAREs (Kurokawa et al., 1995; DiRenzo et al., 1997). The RAR/RXR heterodimer can binds to the downstream half-site of the RAREs consists of direct repeats spaced by five base pairs (DR5), releasing corepressors and recruiting coactivators upon ligand binding, activate transcription (Kurokawa et al., 1995). The RAR/RXR heterodimer can also bind to the upstream half-site RAREs consisting of direct repeats spaced by 1 base par (DR1). In this last case, RAR/RXR heterodimers remains associated with corepressor even in the presence of RAR ligands, resulting in a constitutive repression (Kurokawa et al., 1995; DiRenzo et al., 1997). All together these findings suggest that mollusks transactivation repression results for cells transfected with chimer heterodimers seems to be an ancient behavior that is conserved between mollusks and mammals. In the case of human and mollusks RAR/RXR heterodimer exposure to TBT and HX630 suggest that both compounds effectively bind to the RXRs; since no ligand is expected to bind to RAR the corepressors may not be release and that leads to a gene target transactivation repression. RAR enhance in this case the interaction between RXR and corepressors. In addition, in the case of mollusks RAR, findings suggest that lost the ability to bind retinoids and that the receptor *per se* might be a constitutive transcription factor repressor (Gutierrez-Mazariegos et al., 2014a) and that functional feature might also contribute to the observed results.

6.4.5 Transcriptional activity of mollusks RAR/RXR heterodimer with NlRAR double mutation for gain of function, and HsRXRα and NlRXRa loss of ability to bind TBT

To further understand the transcription repression results obtained for NlRAR/RXRa in the presence of all-*trans*-RA, we decided to perform a RAR double mutation (see Fig. 6.13) according to Gutierrez-Mazariegos et al. (2014a) for the gain of function/response to RA isomers.

```
                                  230
NlRAR_Wild_type    ICERYSVERDMVHFADGTWLRQEEVEQGGFGPLTAKIFHLARQLHSLRCDQTEFAMLGSV
NlRAR_Mutant       ICERYSVERDMVHFSDGTWLRQEEVEQGGFGPLTAKIFHLARQLHSLRCDQTEFAMLGSV

NlRAR_Wild_type    CLISGDRSGLEDVDRIEQMQEPLLEALKHYVRSRRPDQPQVFAKMLMKLTDLRSISVKGA
NlRAR_Mutant       CLISGDRSGLEDVDRIEQMQEPLLEALKHYVRSRRPDQPQVFAKMLMKLTDLRSISVKGA
                                      353
NlRAR_Wild_type    ERVLHLKLEIPGTLPPLVIEMLDRVENVCIP
NlRAR_Mutant       ERVLHLKLEIPGTLPPLIIEMLDRVENVCIP
```

Figure 6.13: Partial alignment of RAR amino acid showing the double mutation for the gain of function assay.

Figure 6.14 displays the confirmation the double NlRAR LBD mutated efficiency to heterodimerizes with the heterodimeric partner RXR, a significantly enhanced luciferase expression in transfected COS-1 cells occurred. Next, we analyzed the NlRAR double mutated and the heterodimer transactivation response to all-*trans*-RA. With the double mutation for an all-*trans*-RA concentration of 10 µM a significant transactivation of luciferase reported gene was obtained (Fig. 6.15) which is in agreement with previous reports (Gutierrez-Mazariegos et al., 2014a). Interestingly, the heterodimer was unresponsive to all-t*rans*-RA (Fig. 6.15).

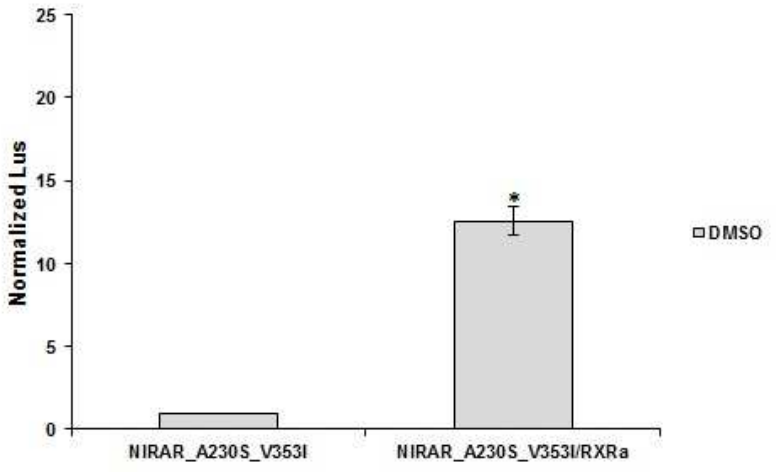


Figure 6.14: Analysis of the potential of the *N. lapillus* RAR LBD double mutated in A230S and V353I to form a heterodimer with the LBD of the partner RXRa by means of a mammalian two-hybrid assay in COS-1 cells. The results are expressed as average fold activation after normalization to Renilla (means ± S.E.; at least n = 3). Significant differences were obtained using Kruskal–Wallis one-way ANOVA Test K for independent samples (*; $P < 0.05$).

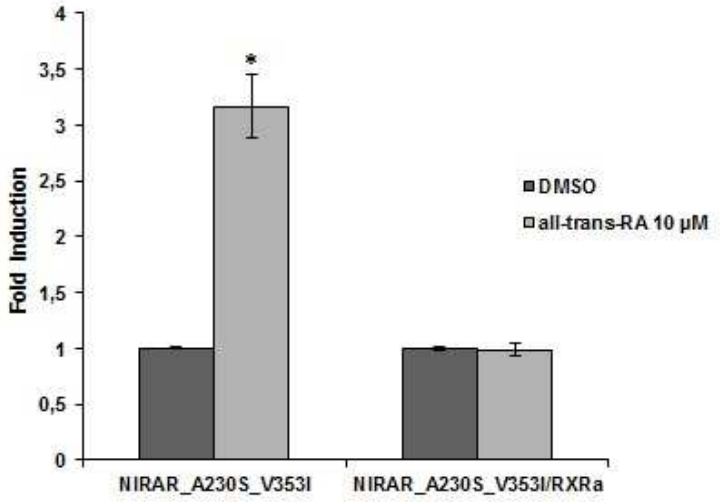


Figure 6.15: Transactivation activity of mutant NlRAR and mutant NlRAR/RXRa constructs fused with GAL4-DBD in response to all-*tans*-RA at 10 µM. The results are expressed as average fold activation after normalization to Renilla (means ± S.E.; n = 3). Significant differences were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples (*; P < 0.05).

Human RXRα and NlRXRa mutation of the key cysteine residue identified to be crucial for LBD binding and interaction with TBT was carried out (Fig. 6.16). The aim was to make the protein unable to bind to TBT and further understand if in the heterodimer RAR is in fact capable to interact with the organotin compound.

```
HsRXRα        KGLSNPAEVEALREKVYASLEAYCKHKYPEQPGRFAKLLLRLPALRSIGLKCLEHLFFFK
HsRXRα_C432A  KGLSNPAEVEALREKVYASLEAYCKHKYPEQPGRFAKLLLRLPALRSIGLKALEHLFFFK
NlRXRa        KGLQAVQEVEQLREKVYASLEEYCKQRYPDEPGRFAKLLLRLPALRSIGLKCLEHLFFFK
NlRXRa_C409A  KGLQAVQEVEQLREKVYASLEEYCKQRYPDEPGRFAKLLLRLPALRSIGLKALEHLFFFK
```

Figure 6.16: Partial RXR amino acid sequence alignment displaying the mutation for the key cysteine involved on TBT ligation and interaction for the loss of function assay.

The cysteine mutation did not affect the heterodimer formation for both human RXRα and NlRXRa mutants as a significant increase of luciferase expression in transfected COS-1 cells occurred (Fig. 6.17). Next, we analyzed how the heterodimer and monomer responded in the presence of TBT. The RXR mutant alone for both human and *N. lapillus* was not responsive to TBT, although we observed a significant target gene transcription activation repression (Fig. 6.18). Furthermore, also for the heterodimer a target gene transcription activation repression was observed in the presence of TBT but smaller than the one observed for the heterodimer with the wild type receptor, which supports our hypothesis. Though further studies must be made to verify if RAR and

corepressors are or not capable to interact with TBT. Also further studies to address the fact that mollusks RAR might interact with corepressors should be performed.

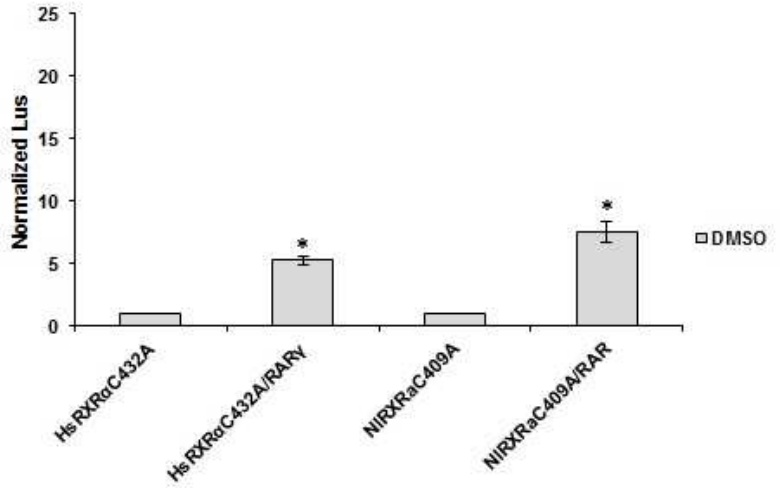


Figure 6.17: Analysis of the potential of the HsRXRαC432A and NlRXRaC409A RAR LBD mutated to form a heterodimer to respective RAR LBD partner by means of a mammalian two-hybrid assay in COS-1 cells. The results are expressed as average fold activation after normalization to Renilla (means ± S.E.; n = 3). Significant differences were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples (*; P < 0.05).

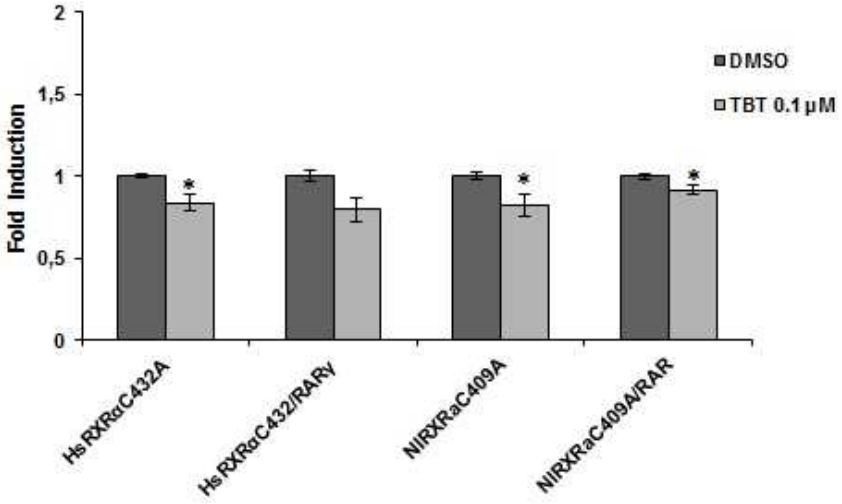


Figure 6.18: Transactivation activity of mutant HsRXRα and NlRXRa and respective heterodimers constructs fused in response to TBT. The data are expressed as average fold activation after normalization to Renilla (means ± S.E.; n = 4). Significant differences were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples (*; P < 0.05).

Genome research studies predict the presence of retinoid metabolic and signaling key players in mollusks (Campo-Paysaa et al., 2008; Theodosiou et al., 2010). Gastropods species have endogenous retinoids including, retinyl ester, retinol, 9-*cis*-RA, 13-*cis*-RA and all-*trans*-RA present in their tissues mainly in gonads (Dmetrichuk et al., 2006, 2008; Gesto et al., 2012, 2013, 2016). In addition it has been demonstrated that *N. lapillus* and *Osilinus lineatus* are capable to metabolize retinoid precursors into RA isomers (Gesto et al., 2012, 2013). These findings clearly indicate the presence of an active pathway for the biosynthesis of RA in mollusks. In support of this, some biological effects of retinoids have been described and considered to be conserved between mollusks and vertebrates (Créton et al., 1993; Campo-Paysaa et al., 2008; Gesto et al., 2016). For instance, treatments with RA induce neurite outgrowth and growth cone turning in neurons, and also improves viability neurons in culture of *L. stagnalis* (Dmetrichuk et al., 2006, 2008). More recently, evidences suggested a role of retinoid signaling pathway on gonads development of *P. vulgata* which could involve a balance of RA levels that seems to be achieved through the enzymatic control of synthetic and catabolic processes (Gesto et al., 2016). Additionally, previous findings and our current data revealed that mollusks RXR orthologues are functional (Nishikawa et al., 2004; Bouton et al., 2005; Castro et al., 2007; Urushitani et al., 2011; Gutierrez-Mazariegos et al., 2014a; Chapter 8). As a monomer mollusk RAR does not bind retinoids and is unable to activate the transcription of target genes in response to stimulation by retinoid (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014a). Moreover, our data revealed that RAR in heterodimer with RXR leads to repression or no activation of the transcription of reporter genes in response to some ligands. Taken together, data suggests that the biological effects described, so far for mollusks, might be regulated by target gene transcription repression mediated through a RAR/RXR-dependent signaling pathways. The biological implications in mollusks of the gene transactivation repression in the presence of retinoids and synthetic ligands should be addressed in future studies.

6.4.6 Transcriptional activity of *N. lapillus* RAR in the presence of common EDCs

In general for mollusk species little is known about the mechanisms of endocrine disruption of RA signaling pathways mostly because of the lack of knowledge concerning the retinoid system. The only known example of retinoid signaling modulation by ECDs is related to the imposex development through RXR-dependent signaling following organotin exposure. In the past decade, several studies showed that signaling through mammalian RAR can be disrupted by some environmental pollutants (Lemaire et al., 2005; Kamata et al., 2008; Inoue et al., 2010). Given that mammalian RAR can be modulated by environmental pollutants, we tested if synthetic chemicals, known to interact with human

RARs, are able to induce mollusk RAR gene transactivation. Based on previously studies (Lemaire et al., 2005; Kamata et al., 2008), we have selected two organochlorine pesticides, Endrin and Dieldrin, an alkylphenols 4-n-nonylphenol, a monoalkylphenols 4-n-Heptylphenol and a polycyclic aromatic hydrocarbon, Naphthalene. None of the tested EDCs were capable to induced transactivation response of a reporter gene *N. lapillus* RAR, only a significant repression has been observed in the presence of Dieldrin (Fig. 6.19). Yet, for human RARγ positive control only the pesticides, Endrin and Dieldrin, induces a significantly transactivation gene (Fig. 6.19). Contrary to previously reported, human RARγ was not able to induce target gene transcription activation in the presence of 4-n-heptylphenol and 4-n-nonylphenol (kanamata et al., 2008). Only the lack of transactivation for Naphthalene exposure is in accordance with kanamata et al. (2008) work. We hypothesize that this can be explained by differences in the methodological approach. Our results suggest that mollusks are not subject to RAR-mediated endocrine disruption by the same pollutants as the human RAR-dependent signaling pathway. Some of the reporter key amino acid residues known to interact with retinoid substitution in mollusks RAR LBD might also have contributed to absence of target gene activation in presence of selected pollutants.

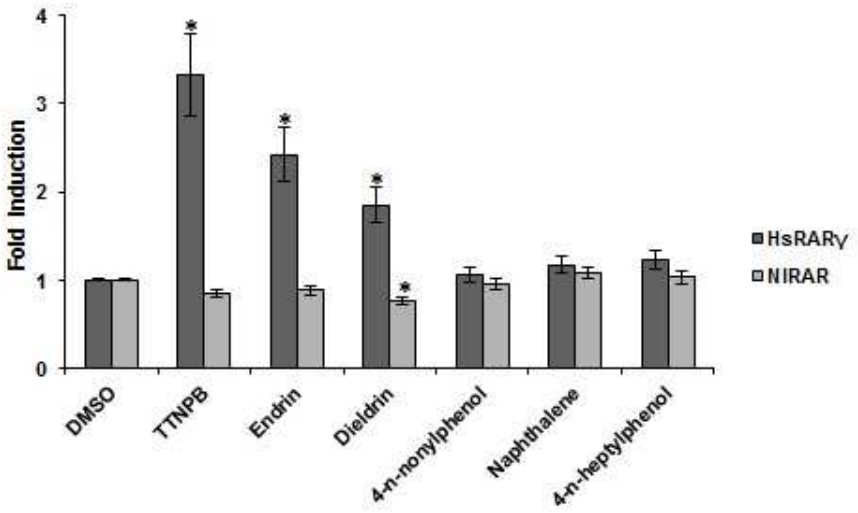


Figure 6.19: Transactivation activity of NlRAR–LBD and HsRARγ-LBD (positive control) constructs fused with GAL4-DBD in response selected EDCs (10 µM) and TTNPB (1 µM). The data are expressed as average fold activation after normalization to Renilla (means ± S.E.; at least n = 3). Significant differences were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples (*; $P < 0.05$).

Given that we observed a significant repression of the luciferase gene transcription for *N. lapillus* RAR in the presence of the pesticide Dieldrin, we next evaluated how the heterodimer and RXR alone might behave (Fig. 6.20). No effect of the organochlorine

pesticide on human RXRα activity was reported by Lemaire et al. (2005). On the contrary, in present study Dieldrin was capable to significant induce RXR-mediated luciferase repression on human and also on *N. lapillus* (Fig. 6.20). Interesting, an induction of *N. lapillus* RAR/RXR and human RARγ/RXRα mediated luciferase transactivation activation activity has been observed, but that reached statistical significance only for the dogwhelk (Fig. 6.20). The precise mechanism underlying Dieldrin interaction with RAR and RXR of both human and *N. lapillus* is unknown. Additional studies must be done to address this issues and to evaluate the biological outcomes.

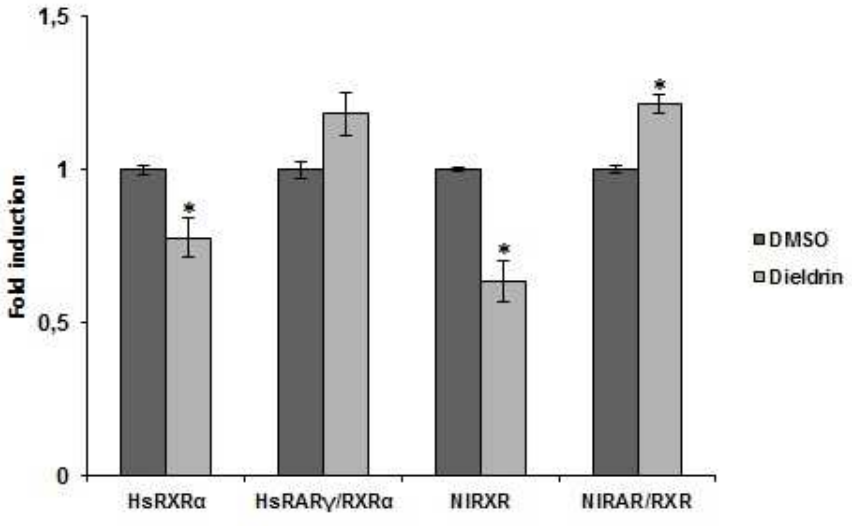


Figure 6.20: Transcriptional activity of Human RXR, RAR/RXR, NlRXRa and NlRAR/RXRa in response of Dieldrin (10 µM). The data are expressed as average fold activation after normalization to Renilla (means ± S.E.; n = 3). Significant differences were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples (*; $P < 0.05$).

6.5 Conclusion

In conclusion, we have shown that mollusks RAR orthologues lost the ability to activate target gene transcription in response to retinoids probably, due to key amino acid substitution on its LBD; instead, for some species a transcription repression has been observed. Furthermore, we have revealed that mollusks RAR conserved the ability to form heterodimer with RXR. Like the vertebrates RXR, the mollusks RXR, is responsive to its putative ligands (all-*trans*-RA, TBT and HX630) and is able to mediated target gene transcription activation. In addition we provided evidences that suggest not only that mollusk RXRs might have a major role on RAR/RXR heterodimer gene transcription regulation, but also that this same role might be ancestral and conserved with vertebrates. Mollusks RAR/RXR is able to induce target gene transcription repression in response to some RXR ligands. Mammalian RAR/RXR heterodimer only activates target gene

transcription in the presence of RAR ligands. A target gene transcription repression might occur when only RXR is bounded to its ligands.

Additionally, we have shown and hypothesize that the lack of ability to activate target gene transcription in response to retinoids also seems to make mollusks RARs unable to interact with most pollutants know to be able to modulate target gene transcription mediated in human RARs. Our data suggested that some LBD amino acid residues proposed to interact with retinoids also are important players in the interaction with EDCs. In addition, our results advocate that mollusks RAR-dependent signaling pathway might not be target of modulation by the same EDCs as human RAR signaling cascade.

6.6 Acknowledgments

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CHAPTER 7

7 Cloning and functional characterization of a retinoid X receptor orthologue in *Platynereis dumerilii*: insights on evolution and toxicology

7.1 Abstract

Retinoic X receptor is a ligand-dependent transcription factor that belongs to the nuclear receptor (NRs) superfamily, and is highly conserved throughout Metazoans. It plays an important role in the gene transcription regulation and is the obligate heterodimeric partner of others NRs. Phylogenetic and structural studies have revealed that throughout metazoan evolution ligand binding and function ability of RXRs seems conserved across most metazoan *taxa*; with the exception described for ecdyozoans whose receptor ligand recognition has been changed independently in some lineages. Yet, RXR isolation and functional characterization is still missing for most *taxa*. The RXR signaling cascade is also a prime target of endocrine disrupting chemicals, especially the organotin compounds tributyltin (TBT) and triphenyltin (TPT) which have high affinity to human RXR orthologues. However, reported effects were only described so far in vertebrates and mollusk species. For other metazoans effects on the modulation of RXR-dependent signaling remain unclear. Though, existing data predicts that modulation by organotins might be conserved across metazoans.

Therefore, in the present work, we reported for the first time the isolation and functional characterization of an annelid species, *Platynereis dumerilii*, RXR orthologue. We performed a functional characterization using an *in vitro* luciferase reporter assay. The results suggest that the RXR ability to be transactivated by its putative natural ligand, 9-*cis*-RA, is conserved between vertebrates and annelids. Next, we demonstrate that TBT and TPT were able to induce target gene transcription activation mediated by *P. dumerilii* RXR orthologue. The findings presented here highlight a clear conserved modulation of RXR-dependent signaling pathways by organotin compounds across metazoan, and strongly indicates that annelids are most likely impacted in the wild by exposure to environmentally relevant concentrations of organotins.

7.2 Introduction

Retinoid X receptors (RXR) are ligand-induced transcription factors that belong to the nuclear receptor superfamily (NRs) (Mangelsdorf et al., 1995; Owen and Zelent, 2000). Similar to other NRs, RXRs have five modular regions classified from A to E (Aranda and Pascual, 2001). The A/B region is a variable N-terminal domain that has a ligand-independent transcriptional activation function domain 1 (AF-1) (Aranda and Pascual, 2001); region C is highly conserved and serves as the DNA-binding domain (DBD), that mediates receptor recognizing and binding to specific response elements,

found in the promotor region of target genes (Dawson and Xia, 2012). The region E, also known as the ligand binding domain (LBD), is moderately conserved and it harbors the ligand binding pocket, a ligand-dependent transcriptional activation function 2 (AF-2) and a dimerization surface (Aranda and Pascual, 2001). Between the DBD and LBD, a less conserved region is located, named D domain, which serves as a flexible hinge allowing RXR to bind to other receptors (Dawson and Xia, 2012). The RXRs are considered to be unique due to is exclusive ability to form homodimer, and heterodimer with other NRs such as retinoid acid receptor (RAR), peroxisome proliferator-activated receptor (PPAR), thyroid hormone receptor (ThR) and vitamin D receptor (VDR) (Kersten et al., 1995; Vivat-Hannah et al., 2003; Szanto et al., 2004; Germain et al., 2006a; Lefebvre et al., 2010). Thus, RXRs are key players in numerous signaling pathways and therefore implicated in distinct biological functions including homeostasis and embryonic development (Mangelsdorf and Evans, 1995; Szanto et al., 2004; Mark and Chambon, 2003; Mark et al., 2006; Philip et al., 2012). RXRs are activated by small hydrophobic compounds, such as 9-*cis*-retinoic acid (9-*cis*-RA), considered its major putative natural ligand (Allenby et al., 1993; de Urquiza et al., 2000; Egea et al., 2002; Dawson and Xia, 2012). Other ligands were also proposed, such as arachidonic acid, docosahexaenoic acid and oleic acid (Dawson and Xia, 2012).

In vertebrates three RXR isotypes are present as a consequence of the two rounds of whole genome duplications that occurred during evolution: RXRα (NR2B1), RXRβ (NR2B2), and RXRγ (NR2B3) (Germain et al., 2006a; Lefebvre et al., 2010). Each isotype exists in several isoforms with tissue-specific distribution and expression patterns, mainly during embryonic development (Germain et al., 2006a; Lefebvre et al., 2010). With the exception of sponges and some cnidarians, RXR seems highly conserved from placozoans to vertebrates (Bridgham et al., 2010). In fact, one or more RXR orthologues have been found in most metazoan *taxa* (Owen and Zelent, 2000; Campo-Paysaa et al., 2008; Bridgham et al., 2010). Moreover, in the majority of *taxa* the ability to bind and response to 9-*cis*-RA seems to be conserved. Regarding cnidarians, a RXR orthologue with the ability to bind 9-*cis*-RA was identified in *Tripedalia cystophora* (Kostrouch et al., 1998). Ability to bind 9-*cis*-RA was also demonstrated for some molluskan RXRs: *Thais clavigera* and *Nucella lapillus* (Nishikawa et al., 2004; Castro et al., 2007). In addition, *Biomphalaria glabrata* and *T. clavigera* RXR was also shown to activate transcription in the presence of 9-*cis*-RA (Bouton et al., 2005; Urushitani et al., 2011). In the particular case of ecdysozoans, it seems that RXR ligand recognition has been altered independently in different lineages (Billas et al., 2001; Clayton et al., 2001; Iwema et al., 2007; Wang and LeBlanc, 2009). For instance, insects express a RXR homologue known as ultraspiracle (USP) that for must *taxa* is unresponsive to RXR ligands. The USP from

Tribolium castaneum does not have a ligand-binding pocket and therefore is not activated by RXR ligands (Iwema et al., 2007). In mecopteridan USPs the ligand-binding pocket is very large and is only occupied by a phospholipid (Billas et al., 2001; Clayton et al., 2001). The UPS LBD of *Locusta migratoria* shows a great similarity to human RXRs than to other USPs insects, and it binds to 9-*cis*-RA with high affinity (Nowickyj et al., 2008). In the case of crustacean RXR orthologue seems to be capable to bind 9-*cis*-RA. For instance, it has been demonstrated that *Daphnia magma* RXR orthologue is able to bind 9-*cis*-RA and methyl farnesoate (crustacean hormone) but is unable to activate transcription (Wang and LeBlanc, 2009). The cephalochordate, *Branchiostoma floridae* RXR LBD exhibits a similar structure to the arthropod RXR/USPs but is able to bind and is activated by RXR ligands, although less efficiently than vertebrates (Tocchini-Valentini et al., 2009).

Given its ubiquitous presence within metazoans and its activation by small lipophilic ligands, RXR is a known prime target of endocrine disrupting chemicals (EDCs) (e.g. Grün et al., 2006; le Maire et al., 2009; Castro and Santos, 2014). Very well-known EDCs of RXR-mediated signaling pathways are the organotin compounds, tributyltin (TBT) and triphenyltin (TPT) (Nishikawa et al., 2004; Kanayama et al., 2005; Grün et al., 2006; Castro et al., 2007; le Maire et al., 2009). Since the mid-1960s, TBT and TPT have been extensively used worldwide in multiple applications, such as biocides in wood preservatives and in antifouling paints for ships, boats and fishing nets (Fent, 1996). In consequence, contamination by such compounds has spread worldwide, particularly through aquatic environments, endangering wildlife (Bettin et al., 1996; Rumengan and Madoka, 2012; Wu et al., 2014). The best known effect of TBT and TPT in wildlife, at very low concentrations (1 ng/L), is the irreversible superimposition of male sexual organs (penis and/or vas deferens) on female gastropods: a feature termed imposex (Smith, 1971; Bryan et al., 1986; Gibbs et al., 1987; Bryan et al., 1988; Horiguchi et al., 1994, 1997). In advanced stages of imposex, reproductive failure may occur and the condition ultimately results in population decline or mass extinction (Matthiessen and Gibbs, 1998; Santos et al., 2000; Santos et al., 2004). Due to the observed effects on aquatic wildlife, their use as antifouling paints has been banned from the 1st of January 2003, and its presence has been forbidden on ship hulls from the 1st of January of 2008 (MEPC, 2001). The mechanism by which TBT and TPT induce imposex has yet to be fully characterized, although strong evidences indicate that it is associated with modulation of the molluscan RXR or RXR/NR heterodimer (Santos et al., 2012; Castro and Santos, 2014). It has been demonstrated that TBT and TPT bind the human RXRs with high affinity (Nishikawa et al., 2004; Kanayama et al., 2005). Moreover, injection of 9-*cis*-RA (1 µg/g wet tissue) into females of the species *T. clavigera*, induced the development of imposex; rock shell RXR orthologue is able to bind organotins and activated reporter gene transcription *in vitro*

(Nishikawa et al., 2004; Urushitani et al., 2011). Experiments performed by Castro et al (2007) gave further support to this hypothesis showing that injection of 9-*cis*-RA (1 µg/g wet tissue), the putative natural ligand, also induces imposex in females of *N. lapillus* to the same degree as TBT. More recently, Stange et al. (2012) reported that the injection of the RXR synthetic agonist HX630 (1 µg/g wet tissue) also leads to imposex development in female *N. lapillus*. In vertebrates, TPT and TBT are also high-affinity ligands for RXRα and PPARγ and stimulate the differentiation of pre-adipocyte 3T3-L1 cells to adipocytes (Kanayama et al., 2005; Santos et al., 2012). Furthermore, nanomolar concentrations of TBT can activate the PPARγ/RXRα heterodimer, primarily through its interaction with RXRα, promoting adipocyte differentiation leading to obesity (Grün et al, 2006; le Maire et al., 2009; Lyssimachou et al., 2015). Additional effects have been reported, in both vertebrate and invertebrate *taxa*, after TBT and TPT exposure. For instance, exposure to TPT causes morphological abnormalities during larvae development in *Danio rerio* (Strmac and Braunbeck, 1999) and impairs gametogenesis of male *Sebastiscus marmoratus* (Sun et al., 2011). During sea urchin *Lytechinus variegatus* embryo-larval development is observed a delay of growth and presence of body deformities after TBT and/or TPT exposure (Perina et al., 2011). Still, for most species the impacted signaling pathways remain unknown (Hagger et al., 2002; Wu et al., 2014). Given the striking RXR homology among metazoans and the available toxicological data, we hypothesize that RXR-dependent modulation by TBT and TPT might be evolutionary conserved throughout metazoans.

RXR orthologue isolation and functional characterization is still missing for major *taxa* including for the mollusk sister group, the annelids (Keay and Thornton, 2009). Hence, in the present study we isolated and characterized a RXR orthologue from a model annelid species, the polychaeta *Platyneresi dumerilii*, using an *in vitro* luciferase reporter assay. Our results suggest that RXR responsiveness to 9-*cis*-RA is conserved between vertebrates and annelids. We also demonstrate that TBT and TPT are able to transcriptionally activate the *P. dumerilii* RXR orthologue. The findings presented here suggest a clear conserved modulation of RXR-dependent signaling pathways by organotin compounds across metazoans.

7.3 Material and Methods

7.3.1 Test compounds

9-*cis*-RA (High performance liquid chromatography grade ≥ 98%), tributyltin chloride (TBT-Cl, 96%), triphenyltin chloride (TPT-Cl, 95%) and sterile-filtered dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich.

7.3.2 *P. dumerilii* RXR gene isolation

For gene isolation, *P. dumerilii* were collected at low tide in June of 2012 in Mindelo (Portugal) rocky shore and placed in seawater from the sampling site for transportation. In the laboratory, the annelids were acclimatized for a day before sacrifice in 500 mL plastic containers filled with natural seawater (salinity 35‰) with aeration. Temperature was set to 18ºC and the light: dark rhythm was 14 h: 10 h. Specimens were anesthetized in a 7% magnesium chloride solution for 10-15 min before sacrifice for tissue sampling.

Total RNA was extracted from body parts of an immature specimen using the Illustra RNAspin Mini RNA Isolation Kit, GE Healthcare (animal tissues protocol), with on column DNAse I digestion. The cDNA synthesis was performed with the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions, using 1 µg of total RNA.

To isolate RXR from *P. dumerilii* a combination of PCR strategies was used (Table 7.1 and Fig. 7.1). Retinoid X receptor amino acid sequences belonging to various invertebrates species were retrieved from Joint Genome Institute (JGI; http://genome.jgi.doe.gov/; Grigoriev et al., 2012), and on the basis of the most conserved sequences regions, several sets of degenerated primers were designed, using the software Consensus-degenerate hybrid–oligonucleotide primers (CODEHOP; http://blocks.fhcrc.org/codehop.html; Rose et al., 2003). One set of primers was found to be successful (Table 7.1).

Table 7.1: List of primers used for gene isolation.

Designation	Sequence	Use
P1	CGGGCCTCCGGCaarcaytaygg	Degenerated Sequence
P2	CAGCCCAGCTCGGTCttrtccatntt	Degenerated Sequence
P3	AGCTGCGATGCACGTGTAATCCTGT	RACE PCR
P4	GACCCTGGGGAATGCATAGAACCAA	RACE PCR nested
P5	CGTATACAGTTGCGAGGGGTGCAAA	RACE PCR
P6	AACGCACAGTTCGCAAGGATCTGAC	RACE PCR
P7	GGGAGTGGGAACCATCTTTGACAGG	RACE Touchdown
P8	TCTCGAAAGAAGCACACATATC	Open reading frame confirmation
P9	AGCATCTCCATGAGGAAAGTAT	Open reading frame confirmation

The polymerase chain reaction (PCR) was performed with 5 µM of each primer, Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific). Cycle parameters included an initial denaturation at 98ºC for 10 seconds (s), followed by 45 cycles of denaturation at 98ºC for 1 s, annealing at 50ºC for 5 s and extension at 72ºC for 15 s/kb, and a final extension step at 72ºC for 1 min. Bands of the appropriate size were isolated (Nzytech pure gel) and sequenced (Stabvida).

The full-length sequence was obtained through 5' and 3' Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE-PCR) using gene specific primers (GSPs) designed using the Primer3 (v.0.4.0) server (Table 7.1 and Fig. 7.1) (http://bioinfo.ut.ee/primer3-0.4.0/; Koressaar and Remm, 2007; Untergrasser et al., 2012). RACE cDNA was synthetized following the SMART™ RACE cDNA amplification kit manufacturer's instructions (Clonetch).

The 5' RACE PCR was initially performed with GSP P3 (Table 7.1 and Fig. 7.1). The RACE PCR reaction mixture contained the Advantage Polymerase Mix (a Titanium ® Taq DNA Polymerase), 5 % (v/v) 5' RACE cDNA (1.25 µL), 17.25 µL sterilized water, 2.5 µL of 10 x advantage buffer, 0.5 µL of dNTP mix (to a final concentration of 0.2 mM), 2.5 µL Universal primer (UP, provided with the SMART™ RACE cDNA amplification kit) and 0.5 µL GSP (0.25 µM) in a total final volume of 25 µL. The PCR profile was as follows: an initial denaturation at 94ºC for 2 min, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 65ºC for 30 s and extension at 72ºC for 1 min/kb, and a final extension step at 72ºC for 3 min. An incomplete 5' fragment was obtained. Then, using the obtained sequence, a new GSP P4 (Table 7.1 and Fig. 7.1) was designed and RACE PCR was performed using the Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) in the following reaction condition: 2 µL of 5' RACE cDNA were used in reaction mixture, together with 1 µL of the GSP to a final concentration of 0.5 µM and 2 µL of Up was used in final reaction volume of 20 µL. The PCR profile was as follows: an initial

denaturation at 98ºC for 10 s, followed by a round of 40 cycles of denaturation at 98ºC for 1 s, annealing at 63ºC for 5 s and extension at 72ºC for 15 s/kb, and a final extension step at 72º for 1 min. Products of the expected size were isolated and purified (Nzytech pure gel) and sequenced (Stabvida).

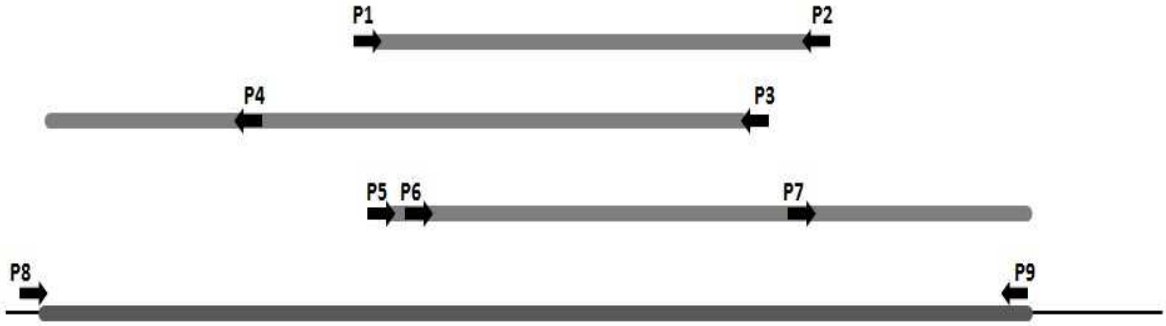

Figure 7.1: Diagram representation of the strategy followed for *P. dumerilii* RXR isolation.

Regarding the 3' RACE PCR was initially performed with GSP P5 followed by a nested PCR using GPS P6 (Table 7.1 and Fig. 7.1) and the Nested Universal primer (provided with the SMART™ RACE cDNA amplification kit), yielding an incomplete 3' fragment. RACE PCR was performed using the Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) in the following reaction condition: 2 µL of template, together with 1 µL of the corresponding specific RACE primer to a final concentration of 0.5 µM and 2 µL of Universal primer was used in final reaction volume of 20 µL. The PCR profile was as follows: an initial denaturation at 98ºC for 10 s, followed by a round of 40 cycles of denaturation at 98ºC for 1 s, annealing at 63ºC for 5 s and extension at 72ºC for 15 s/kb, and a final extension step at 72º for 1 min. Using the obtained sequence, a new GSP P7 was designed and a touchdown RACE PCR was performed using the Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) in the following reaction condition: 2 µL of 3' RACE cDNA were added to the reaction mixture, together with 1 µL of the GSP P7 to a final concentration of 0.5 µM, and 2 µL of Universal primer was used in final reaction volume of 20 µL. The PCR profile was as follows: an initial denaturation at 98ºC for 10 s, followed by a first round of 5 cycles of denaturation at 98ºC for 1 s, annealing at 63ºC for 5 s and extension at 72ºC for 15 s/kb, and a second round of 35 cycles of denaturation at 98ºC for 1 s, annealing at 60ºC for 5 s and extension at 72ºC for 15 s/kb, and a final extension step at 72º for 1 min. Products of the expected size were isolated and purified (Nzytech pure gel). Next, RACE PCR products were ligated with Speedy ligase (Nzytech, Portugal) to the pGEM®-T easy vector (Promega), and transformed in Nzy5α *Escherichia coli* competent cells (Nzytech). Positive colonies were selected for plasmid purification (Nzytech Miniprep Kit) and sequenced by Stabvida.

To amplify the predicted full open reading frame (ORF), specific primers flanking the coding sequences were designed (Table 7.1 and Fig. 7.1). PCR was performed with the Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) using 10 ng of cDNA and each primer to a final concentration of 5 µM in 20 µL total reaction volume. Cycle conditions included an initial denaturation at 98ºC for 10 s, followed by 40 cycles of denaturation at 98ºC for 1 s, annealing at 50ºC for 5 s annealing and extension at 72ºC for 15 s/kb, with a final extension step at 72º for 1 min. PCR products were gel-purified (Nzytech pure gel), ligated with Speedy ligase (Nzytech, Portugal) to the pGEM®-T easy vector (Promega), and transformed into Nzy5α *E. coli* competent cells (Nzytech). Positive colonies were selected for plasmid purification (Nzytech Miniprep Kit) and sequenced by Stabvida.

P. dumerilii RXR amino acid sequence was inferred by Expasy translate tool (http://web.expasy.org/tools/translate/; Artimo et al., 2012).

7.3.3 *P. dumerilii* RXR basal tissue expression

Total RNA was extracted from *P. dumerilii* tissues, including head, upper gut, intestine and gonads with a combination of methods. First, tissues were homogenized with Purezol reagent® (Invitrogen) and nucleic acids extracted with chloroform, according to the manufacturer's instructions. The resulting aqueous phase was used to isolate total RNA using the Illustra RNAspin Mini RNA Isolation Kit animal tissues protocol (GE Healthcare) with on-column DNase I digestion. RNA quality was assessed and its concentration determined by nanodrop plate reader Synergy HT BioTek Gen5™. First-strand cDNA synthesis was performed using the iScript^{TM} cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions. PCR was performed using the Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) with the gene-specific primers reverse 5'-TTCTCTTTGCCCAGTCTACC-3' and forward 5'-GAATCCTGGAGGCTGAGTTA-3' under the following conditions: initial denaturation at 98℃ for 10 s followed by 35 cycles of denaturation at 98℃ for 1 s, annealing at 68℃ for 5 s, and extension at 72℃ for 15 s, and a final ex tension at 72ºC for 1 min. In the reaction mixture primer and cDNA concentrations were 0.5 µM and 10 ng/µL, respectively. The amplification products were resolved on a 1.2% agarose gel.

7.3.4 Phylogenetic analysis

To infer phylogenetic relationships, *P. dumerilii* RXR amino acid sequence was aligned with sequences from other metazoan species. Seventeen amino acid sequences were retrieved from GenBanK (http://www.ncbi.nlm.nih.gov/genbank/; Geer et al., 2010; Benson et al., 2013) and used to construct the phylogenetic tree: *Homo sapiens* RXRα

(HsRXR; BAH02296.1), *Xenopus laevis* RXRα (XlRXR; P51128.1), *D. rerio* RXR (DrRXR; AAC59720.1), *B. floridae* RXR (BfRXR; AF378829_1), *Ciona intestinalis* RXR (CiRXR; NP_001071809.1), *Polyandrocarpa misakiensis* RXR (PmRXR; BAM66778.1), *T. clavigera* isoform 1 (TcRXR; BAJ76722.1), *N. lapillus* RXR isoform a (NlRXR; ABS70715.1), *Lymnaea stagnalis* RXR (LsRXR; AAW34268.1), *B. glabrata* RXR (BgRXR; NP_001298239.1), *D. magna* RXR (DmRXR; ABF74729.1), *L. migratoria* RXR (LmRXR; AAQ55293.1), *Uca pugilator* RXR (UpRXR; AAC32789.3), *Marsupenaeus japonicas* RXR (MjRXR; BAF75376.1), *Drosophila melanogaster* RXR/USP (DrmRXR/UPS; AAF45707.1); *T. castaneum* RXR (TrcRXR/Ups; EFA04649.1) and *Bombyx mori* RXR/UPS (BmRXR/UPS; AAC13750.1). The phylogenetic analysis was conducted using MEGA version 5 (Tamura et al., 2011). A phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) based on the full RXR amino acid sequences. The reliability of the branches was evaluated by bootstrapping with 1000 replicates (Felsenstein, 1985). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965).

A sequence alignment of the RXR from *P. dumerilii*, *N. lapillus* RXRa and, *H. sapiens* RXRα, and *B. glabrata* RXR were performed using Clustal Omega sequence alignment tool (http://www.ebi.ac.uk/Tools; Sievers et al., 2011) with default parameters to infer about conserved key residues. The P-, D- , T-boxes from DBD, the hinge region, LBD and AF-2 transactivation function region as well as putative key residues known to interact with 9-*cis*-RA and TBT were predicted based on previously published works from Egea et al. (2000 and 2002), de Groot et al. (2005), le Maire et al. (2009), Tocchini-Valentini et al. (2009) and Gesto et al. (2016).

7.3.5 Construction of plasmid vectors

P. dumerilii RXR and *H. sapiens* RXRα LBD and hinge regions (D domain) were amplified by PCR using the Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) under the following conditions: initial denaturation at 98℃ for 10 s followed by 35 cycles of denaturation at 98℃ for 1 s, annealing at 50℃ for 5 s, and extension at 72℃ for 15 s, and a final extension at 72ºC for 1 min. The PCR product was gel-purified and digested with XbaI and KpnI restriction enzymes (Table 7.2). The obtained insert was subcloned into the pBIND expression vector (Promega), which includes a yeast GAL4 DNA-binding domain upstream the cloning site, to produce a GAL4 DBD/RXR LBD hybrid protein.

Table 7.2: List of primers used to amplifying the RXR hinge + LBD sequence to be subcloned into pBIND expression vector.

Species and isoform	Sequence	Restriction enzymes used
P. dumerilii RXR	F: GCC**TCTAGA**CAGAGAGTAAAAGAAAAAGGG	XbaI
	R: TAT**GGTACC**TCAGACCGGCGTGGGTGCTTC	KpnI
H. sapiens RXRα	F: AATT**TCTAGA**GCCGTGCAGGAGGAGCGGCA	XbaI
	R: AATT**GGTACC**AGTCATTTGGTGCGGCGCCT	KpnI

7.3.6 Cell culture conditions

COS-1 cells were maintained Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) with phenol red and supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). The cells were cultured at 37℃ in a humidified atmosphere containing 5% CO_2.

7.3.7 COS-1 Cells Transfections for *in vitro* luciferase reporter gene transactivation assays

Cells were seeded on 24-well culture plates at a density of 2 x 10^5 cells per well and cultured in DMEM (PAN-Biotech) supplemented with 10% fetal bovine serum (Invitrogen, Thermo Fisher), and 1% penicillin/streptomycin (Invitrogen, Thermo Fisher). After 24 h, cells were transfected with 0.5 µg pBIND RXR-LBD constructs and 1 µg of the pGL4.31 luciferase reporter vector in a final transfection volume of 50 µL using 2 µL Lipofectamine® 2000 Transfection Reagent (Invitrogen, Thermo Fisher) and Opti-MEM reduced serum medium (Gibco, Thermo Fisher) according to manufacturers' instruction. After 5 h of incubation, cells were washed once with Phosphate Buffer Saline (PBS; PAA Biotech) and exposed to the test ligands in 1 mL phenol red-free DMEM supplemented with 10% dextran-coated charcoal-treated serum (Invitrogen, Thermo Fisher) and 1% penicillin/streptomycin (Invitrogen, Thermo Fisher). After 24 h, the cells were washed once with PBS and incubated with 100 µL of Passive Lysis Buffer (Promega) at 37ºC for 15 min with gentle agitation (90 rpm). DMSO was used as vehicle to prepare the stock solutions and for solvent control. The concentration of DMSO in the culture medium did not exceed 0.1%. Selected test concentrations of 9-*cis*-RA, TBT and TPT were based on previous published works (Kanayama et al., 2005; Gumy et al., 2008; le Marie et al., 2009; Urushitani et al., 2011).

7.3.8 Dual Luciferase Assays

Firefly luciferase (pGL4.31) and Renilla luciferase (pBIND) activities were determined using the Dual luciferase assay system kit (Promega) according to manufacturer's instructions. Luminescence was measured with a Synergy HT Multi-Mode Microplate reader (BioTek). To determine transfection efficiency, Firefly luciferase activity was normalized using the Renilla luciferase activity.

7.3.9 Statistical analysis

The measured transactivation activities did not meet ANOVA assumptions (homogeneity of variances and/or normal distributed data). Hence, we used the non-parametric Kruskal–Wallis one-way ANOVA with Test K for independent samples. All data are reported as means ± Standard Error (StE). In all cases, means were considered significantly different at $P < 0.05$. Statistical analysis was performed using IBM SPSS version 22.1 software, license and software provided by the University of Porto.

7.4 Results

7.4.1 Cloning and phylogenetic analysis of *P. dumerilii* RXR

A full-length ORF of 1398 base pairs (bp) was isolated from *P. dumerilii*, encoding a 466 amino acid protein (Fig. 7.2).

```
atggacaccagagagatggattccatggagtcgagcatggacagcaccatgactccagtg
 M  D  T  R  E  M  D  S  M  E  S  S  M  D  S  T  M  T  P  V
aatagcatgggagggatgtccggacccccggatatgaagccggacctatccacgttgaac
 N  S  M  G  G  M  S  G  P  P  D  M  K  P  D  L  S  T  L  N
agcccctcctcaagtcagcctggccaggcattttcaccctcctccacaagtcctgggttc
 S  P  S  S  S  Q  P  G  Q  A  F  S  P  S  S  T  S  P  G  F
ttcaacatgggccatgtcacttcctcatctcagatgacacctcctaataaccacatgcat
 F  N  M  G  H  V  T  S  S  S  Q  M  T  P  P  N  N  H  M  H
atgcagatgtcaccaggagcaggcccactgcagagccccaccttgaactcagttggttct
 M  Q  M  S  P  G  A  G  P  L  Q  S  P  T  L  N  S  V  G  S
atgcattccccagggtcaatgccctcgccaagcggattgcaatcgccctcatacacgcct
 M  H  S  P  G  S  M  P  S  P  S  G  L  Q  S  P  S  Y  T  P
ctcggtggtcccagttccactatgggctcaccaggaggcatgggaggcatgctcggtcac
 L  G  G  P  S  S  T  M  G  S  P  G  G  M  G  G  M  L  G  H
tccaccaaacacatttgcgccatctgtggcgacaaggcttccggaaagcactatggcgta
 S  T  K  H  I  C  A  I  C  G  D  K  A  S  G  K  H  Y  G  V
tacagttgcgaggggtgcaaaggcttcttcaaacgcacagttcgcaaggatctgacgtac
 Y  S  C  E  G  C  K  G  F  F  K  R  T  V  R  K  D  L  T  Y
gcctgccgagacgacaaacagtgcatgatagacaaaagacagaggaacagatgccagtac
 A  C  R  D  D  K  Q  C  M  I  D  K  R  Q  R  N  R  C  Q  Y
tgccgatacatgaagtgcttggccagtgggatgaagcgggaagctgtccaagaagaacgg
 C  R  Y  M  K  C  L  A  S  G  M  K  R  E  A  V  Q  E  E  R
cagagagtaaaagaaaaaggggatggagaagtggagagctcaagttgcgccaattccgag
 Q  R  V  K  E  K  G  D  G  E  V  E  S  S  S  C  A  N  S  E
atgcccgtggaaagaatcctggaggctgagttagctgtggagcccaagattgacacttat
 M  P  V  E  R  I  L  E  A  E  L  A  V  E  P  K  I  D  T  Y
gtggacacccagagggacactgtgaccaacatatgccaggcggctgacaaacagctcttc
 V  D  T  Q  R  D  T  V  T  N  I  C  Q  A  A  D  K  Q  L  F
actctggtagactgggcaaagagaatccctcacttctcagaactgtctctggacgaccag
 T  L  V  D  W  A  K  R  I  P  H  F  S  E  L  S  L  D  D  Q
gttattcttctaagagcaggttggaacgagctcctaattgctgccttctcccaccgctcc
 V  I  L  L  R  A  G  W  N  E  L  L  I  A  A  F  S  H  R  S
atatccgtgaaggacggcattctcttagcgacaggattacacgtgcatcgcagctcggcc
 I  S  V  K  D  G  I  L  L  A  T  G  L  H  V  H  R  S  S  A
caccaagcgggagtgggaaccatctttgacagggtcctcacggaactcgtggctaaaatg
 H  Q  A  G  V  G  T  I  F  D  R  V  L  T  E  L  V  A  K  M
cgagagatgaagatggacaagaccgaactgggctgtctcagggctatcgtgcttttcaat
 R  E  M  K  M  D  K  T  E  L  G  C  L  R  A  I  V  L  F  N
ccagacgccaagaacttgcagtcggtgcaacaggtggaagcgttgagagagaaagtgtat
 P  D  A  K  N  L  Q  S  V  Q  Q  V  E  A  L  R  E  K  V  Y
gcatccctggaggagtactgtaaaacgcagtaccccgacgaacccggcagattcgccaag
 A  S  L  E  E  Y  C  K  T  Q  Y  P  D  E  P  G  R  F  A  K
ttgctcctccgtctgcccgctctgcgcagcatcggtctcaagtgcctggaacatctcttc
 L  L  L  R  L  P  A  L  R  S  I  G  L  K  C  L  E  H  L  F
tttttcaaattaatcggagaaactcctatcgatactttcctcatggagatgctagaagca
 F  F  K  L  I  G  E  T  P  I  D  T  F  L  M  E  M  L  E  A
cccacgccggtcccttaa
 P  T  P  V  P  -
```

Figure 7.2: Complete *P. dumerilii* Retinoid X receptor gene sequence (nucleotide and the deduced amino acid sequence).

Amino acid sequence alignments revealed that *P. dumerilii* RXR displays the main domains typical of the RXR family, with highly conserved residues in the P-box, D-Box and T-box of the DBD (Fig. 7.3). The P-, D- and T-boxes are DBD short motifs that determine the specificity of DNA-binding and dimerization of the receptor (Zhao et al., 2000; Germain et al., 2003, 2006b).

In the *P. dumerilii* RXR LBD relevant key residues know to interact and bind to 9-*cis*-RA (Egea et al., 2000; Hiromori et al., 2015) as well as organotin compounds (le Maire et al., 2009; Hiromori et al., 2015) are present and fully conserved (Fig. 7.3).

```
HsRXRa      ------MDTKHFLPL--DFSTQVNSSLTSPTGRGSMAAPSL------------HPS--L 37
NlRXRa      MDRS-DMDTLENNGPPGGMPGGMHMGVPVMGGMGGPHQQDIKPDISTLNAPSSTHPGFYP 59
BgRXR       MDRSEGMDTLEN-----SMPSGMSMGMT-MGGHQGHPPPDIKPDISSLTSPTSTHGYYGF 54
PdRXR       ------MDTREMDSMESSMDSTM-TPVNSMGGMSG--PPDMKPDLSTLNSPSSSQPGQAF 51
                     *** .       :   :   :    *  .    .:            :

HsRXRa      GPGIGSPGQLHSPISTLSSPINGMGPPFSVIS--SPMGPHSMSVPTTPTLGFSTGSPQLS 95
NlRXRa      GYGG-------MPSMPSSTQ-------------ASPGGPNMTSPQIHS----------- 87
BgRXR       GPGG-------MPSMASSTQ-------------PSPGPQQMHSPGMHS----------- 82
PdRXR       SPSSTSPGFFNMGHVTSSSQ---MTPPNNHMHMQMSPGAGPLQSPTLNSVGSMHSPGSMP 108
            . .             *:                  *   :  *   :

                                              > ______DBD________P-box
HsRXRa      SPMNPVSSSEDIKPPLGLNGVLKVPAHPSGNMASFTKHICAICGDRSSGKHYGVYSCEGC 155
NlRXRa      PASSLSSPSMMCLSPTGSS-TPGM-----PHSGL-SKHICAICGDRASGKHYRVYSCEGC 140
BgRXR       PTSSMGSPPMLCLSPSGPSPSPGL-----PHSSLHTKHICAICGDRASGKHYGVYSCEGC 137
PdRXR       SPSGLQSPSYTPL--GGPSSTMGSPGGMGGMLGHSTKHICAICGDKASGKHYGVYSCEGC 166
                  *        * .             .  :*********::***** *******

            _____________D-box_____________________________T-box__<______
HsRXRa      KGFFKRTVRKDLTYTCRDNKDCLIDKRQRNRCQYCRYQKCLAMGMKREAVQEERQRGKDR 215
NlRXRa      KGFFKRTVRKDPTYACRDDKNCMIDKRQRNRCQFCRYMKCLAQGMKREAVQEERQRVKEK 200
BgRXR       KGFFKRTVRKDLTYACRDDKNCMIDKRQRNRCQYCRYMKCLSMGMKREAVQEERQRVKEK 197
PdRXR       KGFFKRTVRKDLTYACRDDKQCMIDKRQRNRCQYCRYMKCLASGMKREAVQEERQRVKEK 226
            *********** **:***:*:*:**********:*** ***: ************ *::

            Hinge__>_____________________________________________________
HsRXRa      NENEVESTSSANEDMPVERILEAELAVEPKTETYVEANMGLNPSSPNDPVTNICQAADKQ 275
NlRXRa      GDGEVESTSGANSDMPVEHVLEAEVAVEPKIDTYVDV--------QKDPVTNICQAADKQ 252
BgRXR       GDGEVESTSGANNDMPVEQILEAELAVDPKIDTYIDA--------QKDPVTNICQAADKQ 249
PdRXR       GDGEVESSSCANSEMPVERILEAELAVEPKIDTYVDT--------QRDTVTNICQAADKQ 278
             : ****:* **.:****::****:**:** :**::.         .* ***********

            _____________________________LBD_____________________________
HsRXRa      LFTLVEWAKRIPHFSELPLDDQVILLRAGWNELLIASFSHRSIAVKDGILLATGLHVHRN 335
NlRXRa      LFTLVEWAKRIPHFIELPLEDQVILLRAGWNELLIAGFSHRSTQVTDGILLATGLHVHRS 312
BgRXR       LFTLVEWAKRIPHFTELPLEDQVILLRAGWNELLIAGFSHRSIMAKDGILLATGLHVHRS 309
PdRXR       LFTLVDWAKRIPHFSELSLDDQVILLRAGWNELLIAAFSHRSISVKDGILLATGLHVHRS 338
            *****:******** ** *:****************.*****  ..**********.

            _____________________________________________________________
HsRXRa      SAHSAGVGAIFDRVLTELVSKMRDMQMDKTELGCLRAIVLFNPDSKGLSNPAEVEALREK 395
NlRXRa      SAHQAGVGTIFDRVLTELVAKMREMKMDKTELGCLRAIVLFNPDAKGLQAVQEVEQLREK 372
BgRXR       SAHQAGVGTIFDRVLTELVAKMRDMKMDKTELGCLRAVVLFNPDAKGLTAVQEVEQLREK 369
PdRXR       SAHQAGVGTIFDRVLTELVAKMREMKMDKTELGCLRAIVLFNPDAKNLQSVQQVEALREK 398
            ***.****:**********:***:*:***********:******:* *    :** ****
```

```
                                                                    AF-2
HsRXRa      VYASLEAYCKHKYPEQPGRFAKLLLRLPALRSIGLKCLEHLFFFKLIGDTPIDTFLMEML 455
NlRXRa      VYASLEEYCKQRYPDEPGRFAKLLLRLPALRSIGLKCLEHLFFFKLIGDTPIDTFLMEML 432
BgRXR       VYASLEEYTKSRYPEEPGRFAKLLLRLPALRSIGLKCLEHLFFFKLIGDQPIDTFLMEML 429
PdRXR       VYASLEEYCKTQYPDEPGRFAKLLLRLPALRSIGLKCLEHLFFFKLIGETPIDTFLMEML 458
            ****** * * :**::*********************************: **********

            _________<
HsRXRa      EAPHQMT-- 462
NlRXRa      ESPSTQLAT 441
BgRXR       ENPSPAT-- 436
PdRXR       EAPTPV--- 464
            * *
```

Figure 7.3: Sequence alignment of RXR from *P. dumerilii*, *H. sapiens* RXRα, *N lapillus* RXRa and *B. glabrata* RXR. Key residues interacting with 9-*cis*-RA are highlighted. Hydrogen bonds, direct or mediated by water molecules, between the substrate and pocket residues, are highlighted in grey. In dark grey hydrophobic and Van der Waals interactions. The arginine, in bold underlined, forms an ionic interaction with the ligand. The cysteine residue, suggested to form a covalent bond with TBT, is highlighted in black. Underlined is the P-, D- and T-boxes. The AF-2 transactivation function is in bold italics. (*) - denotes identical residues; (:) - denotes single conservative amino acid substitutions; and (.) denotes two conservative amino acid substitutions. Domains were inferred from de Groot et al. (2005), Castro et al. (2007), Urushitani et al. (2011) and Lv et al. (2013).

The deduced *P. dumerilii* RXR sequence shares a high degree of amino acid conservation with other Lophotrochozoan species and with *H. sapiens*, particularly in the DBDs (C domains; 90 to 95%) and LBDs (E domain; 83 to 89%), and a lower conservation in the A/B domains (33 to 48%; Fig. 7.4A). Next, to examine the relationships between the isolated RXR and other metazoan RXRs, a molecular phylogenetic analysis was performed. The Neighbor-joining tree shows that *P. dumerilii* RXR robustly clustered with other Lophotrochozoan RXR sequences (Fig. 7.4B).

A

	A/B	DBD	D	LBD	Full coding sequence
HsRXRα	33%	90%	54%	83%	65%
NlRXRa	45%	92%	100%	89%	75%
BgRXR	48%	95%	100%	86%	70%

B

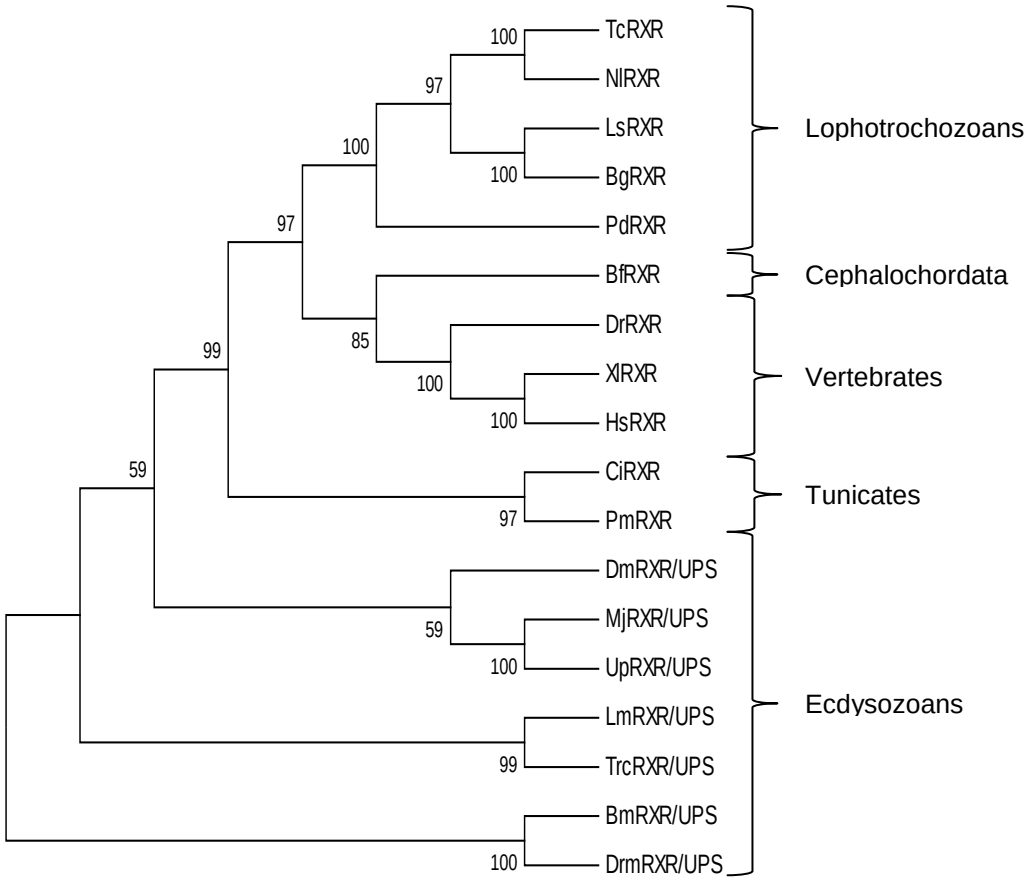

Figure 7.4: Comparison of the deduced amino acid residues of *P. dumerilii* RXR. (A) Deduced amino acid sequences from conserved domains in terms of identity (%) between *P. dumerilii* and other species. Conserved domain identities inferred in NCBI Blastp Tool. (B) Phylogenetic analysis of RXR determined by the Neighbor-Joining method: The percentages of trees (1000 replicates) in which the associated *taxa* clustered together is shown next to the branches. *H. sapiens* RXRα (HsRXR), *X. laevis* RXRα (XlRXR), *D. rerio* RXR (DrRXR), *B. floridae* RXR (BfRXR), *C intestinalis* RXR (CiRXR), *P. misakiensis* RXR (PmRXR), *T. clavigera* RXR (TcRXR), *N. lapillus* RXR (NlRXR), *L. stagnalis* RXR (LsRXR), *B. glabrata* RXR (BgRXR), *P. dumerilii* RXR (PdRXR), *D. melanogaster* RXR/USP (DrmRXR/UPS), *D. magna* RXR (DmRXR), *L. migratoria* RXR (LmRXR), *U. pugilator* RXR (UpRXR), *M. japonicas* RXR (MjRXR), *T. castaneum* RXR (TrcRXR/Ups), and *B. mori* RXR/UPS (BmRXR/UPS).

7.4.2 *P. dumerilii* RXR tissue expression patterns

Transcripts of the *P. dumerilii* RXR are ubiquitously expressed along the body axis of both genders, being detected in all sampled sections (brain, upper gut, intestine and gonads) from both male and females (Fig. 7.5).

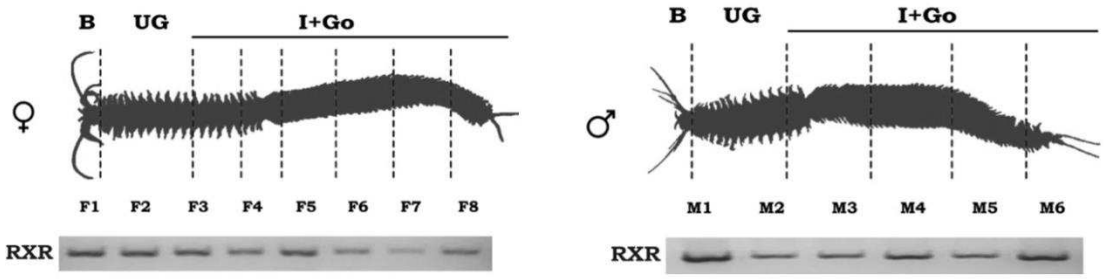

Figure 7.5: Expression patterns RXR in adult females (left panel) and males (right panel) of *P. dumerilii*. RXR is ubiquitously expressed along the body axis. Legend: B – brain; UG – upper gut; I – intestine; Go – gonads (Drawings were modified from Hauenschild and Fischer, 1969).

7.4.3 Transactivation assay

Since the main residues implicated in 9-*cis*-RA and organotin interactions are fully conserved in *P. dumerilii* RXR LBD, we tested whether such ligands were able to activate the hybrid GAL4 DBD/RXR LBD receptor and regulate the transactivation of a reporter gene, in transient transfection assays using mammalian COS-1 cell lines. When 9-*cis*-RA was added to the medium, *P. dumerilii* RXR was able to induce luciferase activity, although for the highest concentration was less efficient when compared to the human RXRα LBD (Fig. 7.6). Regarding the tested organotin compounds, they were also able to activate *the P. dumerilii* RXR and promote the expression of the luciferase reporter gene, with a higher transcriptional activity observed in the presence of TBT when compared to TPT (Fig. 7.7). Also, taking in account the transactivation response in the presence of both organotin compounds, a similar pattern was observed between *P. dumerilii* RXR and human RXRα (Fig. 7.7).

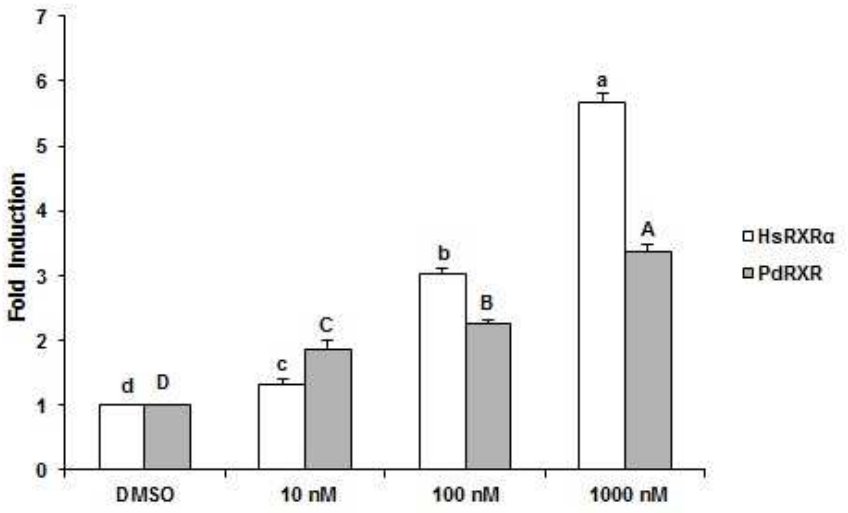

Figure 7.6: Transactivation activity of luciferase reporter gene mediated by GAL4 DBD-RXR LBD fusion constructs in COS-1 cells in the presence of 9-*cis*-RA. Data represent means ± S.E. (n ≥ 3). The results are expressed as average fold activation after normalization with Renilla activity. Significant differences were inferred using Kruskal–Wallis one-way ANOVA Test K for independent samples ($P < 0.05$). Columns carrying different capital letters refer to *P. dumerilii* transactivation data statistically different between concentrations of 9-*cis*-RA; whereas the columns carrying different small letters refers to comparison of human transactivation data.

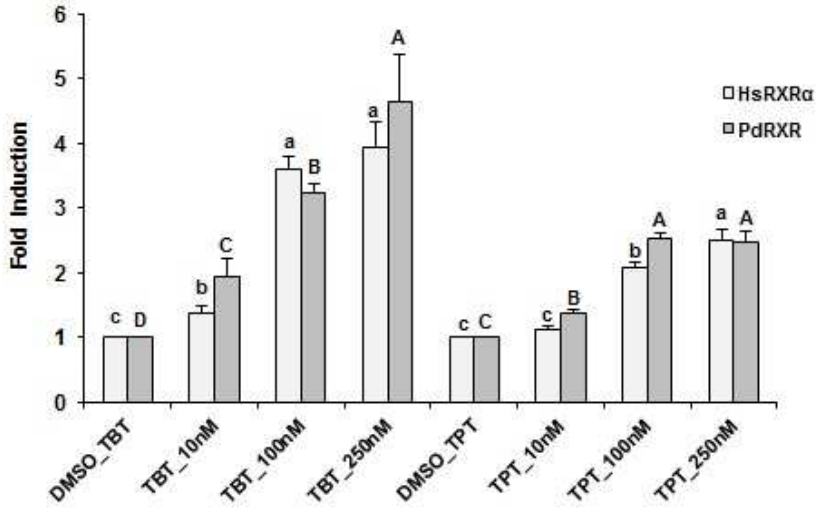

Figure 7.7: Transactivation activity of luciferase reporter gene mediated by GAL4 DBD-RXR LBD fusion constructs in COS-1 cells in the presence of TBT and TPT. The results are expressed as average fold activation after normalization to Renilla (means ± S.E.; at least n = 3). Significant differences were obtained using Kruskal–Wallis one-way ANOVA Test K for independent samples ($P < 0.05$). Columns carrying different capital letters refer to *P. dumerilii* transactivation data statistically different between concentrations of TBT or TPT; whereas the columns carrying different small letters refers to comparison of human transactivation data.

7.5 Discussion

RXR is a highly conserved member of the NRs superfamily and plays key roles in the physiology of metazoans, including reproduction and development (Szanto et al., 2004). In spite of the increasing number of studies reporting the isolation and functional characterization of RXR/USPs (e.g. Iwema et al., 2007; Tocchini-Valentini, et al., 2009; Bridgham et al., 2010; Urushitani et al., 2011), the range of examined *taxa* is still limited. In mollusks, RXR orthologues have been isolated and functional characterized in several classes; and 9-*cis*-RA, the putative natural ligand, has been reported to activate the molluscan RXR (Nishikawa et al., 2004; Bouton et al., 2005; Castro et al., 2007; Urushitani et al., 2011). On the other hand, although the presence of a putative RXR orthologue has been genome predicted in *Capitela* sp (Annelid), no functional characterization has been carried out (Campo-Paysaa et al., 2008; Albalat and Cañestro, 2009). Here, we aim to fill this knowledge gap by evaluating the ability of the annelid RXR to be activated in the presence of its putative natural ligand, 9-*cis*-RA. Hence, we report for the first time the isolation and functional characterization of a RXR orthologue from an annelid species, the polychaeta *P. dumerilii*. We also evaluated the transcriptional activity *of P. dumerilii* RXR in the presence of two organotin compounds, TBT and TPT. The aim was to provide further evidences supporting RXR-dependent signaling cascades as prime targets of organotins across metazoans.

We successfully cloned the entire ORF of a RXR orthologue from *P. dumerilii* (Fig. 7.2). The complete RXR cDNA coding sequence encodes a protein with 466 amino acid residues (Fig. 7.2). In *Capitela* sp genome only one RXR orthologue isoform has been reported (Campo-Paysaa et al., 2008; Albalat and Cañestro, 2009). Similarly, in the present work we are also able to isolate a single RXR isoform for the *P. dumerilii*. During the RACE-PCRs and ORF PCR, no variant transcripts were identified. Still, we cannot exclude the existence of additional undetected isoforms. In other metazoan species, a variable number of RXR isoforms were reported (Kostrouch et al., 1998; Bouton et al., 2005; Castro et al., 2007; Lv et al., 2013). For instance, in the mollusk *B. glabrata* only one RXR transcript was isolated, and no variants were identified (Bouton et al., 2005), whereas in *N. lapillus* and *T. clavigera* two RXR isoforms were cloned (Castro et al., 2007; Urushitani et al., 2011). Also, in the bivalve *Chlamys farreri*, four RXR isoforms were cloned (Lv et al., 2013). The reported isoforms differ only in the amino acid insertion/deletion in the T-box of the DBD, possibly generated via alternative mRNA splicing (Castro et al., 2007; Urushitani et al., 2011; Lv et al., 2013).

Phylogenetic analysis shows that the *P. dumerilii* RXR groups together with mollusks, the annelid sister group (Keay and Thornton, 2009). Additionally, sequence alignment analysis (Fig. 7.3 and Fig. 7.4A) shows high levels of identity with RXRs from *H.*

sapiens and the mollusks *N. lapillus* and *B. glabrata*. For human it has been shown that 9-*cis*-RA is a high affinity ligand for RXRα (Heyman et al., 1992) and capable to induce a transcriptional response *in vitro* (Hiromori et al., 2015). Until recently, the role of 9-*cis*-RA as a natural ligand for vertebrates RXR has been questionable since it is very difficult to detect on tissues (Ulven et al., 2001; Dawson and Xia, 2012). However, 9-*cis*-RA presence has been detected in mouse pancreas (Kane et al., 2010), in the liver and ovary of juvenile rainbow trout (Gesto et al., 2012a), and in the gonads of several gastropod species (Gesto et al., 2012b, 2013 and 2016).

Critical amino acid residues in the LBD that confers the ability for binding and transactivation response to 9-*cis*-RA has been identified and described in human RXRα (Egea et al., 2000, 2002; Hiromori et al., 2015). Residues located on helices H3, H5, H7 and H11, and the β-turn contacts to amino acids including I268, C269, A271, A272, Q275, W305, N306, L309, F313, R316, L326, A327, V342, I345, V349, R371, C432 and H435 formed the hydrophobic pocket where the RA isomer molecule is buried (Egea et al., 2000, 2002). The 9-*cis*-RA contains a carboxylate group and a long aliphatic chain. *In silico* crystallographic studies show that the carboxylate group of 9-*cis*-RA specifically interacts with Q275 in helix 3, R316 in helix 5 and L326 in the β-strand, while the long aliphatic chain interacts with a subset of the binding-pocket residues in helix H11 of human RXRα (Wurtz et al., 1996; Egea et al., 2000, 2002). Loss of function studies suggested that only two of the reported residues are crucial for ligand recognition and RXR transactivation, the R316 and L326. The single mutation of R316A or L326A significantly decreased the ability of human RXRα to be transactivated in the presence of 9-*cis*-RA, whereas transcriptional responsiveness is completely lost in the double residue of R316A/L326A mutations (Harada et al., 2015; Hiromori et al., 2015). The key residues in the LBD known to interact with 9-*cis*-RA are present in *P. dumerilii* RXR, suggesting that this molecule is also a putative ligand for *P. dumerilii* RXR (Fig. 7.3). This most likely reflects functional conservation of RXRs between these lineages regarding ligand-binding properties. The LBD also harbors the ligand-dependent activation function AF-2, a major dimerization interface (Germain et al., 2003) that has the key residues fully conserved in *P. dumerilii* compared to *H. sapiens* RXRα, differing to the molluscan RXR in only one amino acid residue (Fig. 7.3). Besides the LBD, the DBD, including the P-, T- and D-boxes, is extremely well conserved (Fig. 7.3) in *P. dumerilii* RXR. The P-, D- and T-boxes are important short regions of the DBD that contribute to response element specificity, dimerization interface within the DBD, and contacts with the DNA backbone and residues flanking the DNA core recognition sequence (Zhao et al., 2000; Germain et al., 2003, 2006b). In the *P. dumerilii* RXR, P-box and T-box are 100% identical to other lophotrochozoan species and human RXRα, while the D-box has one amino acid

substitution when compared to the human RXRα orthologue (Fig. 7.3). Overall, the obtained amino acid sequence suggested the conservation of *P. dumerilii* RXR ability towards 9-*cis*-RA binding. Our subsequent luciferase reporter gene assay confirmed that *P. dumerilii* RXR is, in fact, responsive to 9-*cis*-RA; although less efficiently when compared to the positive control, the human RXRα (Fig. 7.6). A weaker activation by 9-*cis*-RA was also observed for the mollusk *B. glabrata* (Bouton et al., 2005) and cephalochordata (Tocchini-Valentini et al., 2009).

Altogether, our data suggests that RXR ability to bind to 9-*cis*-RA and subsequently regulate target gene transcription is evolutionary conserved among Lophotrochozoans. Our work gives further support to the hypothesis that RXRs ability to bind and respond to 9-*cis*-RA occurred early during metazoan evolution, being present in the common ancestor of Cnidaria and Bilateria. A response that seems to be conserved throughout evolution with exception of Ecdysozoan lineages (Escriva et al., 2000; Billas et al., 2001; Clayton et al., 2001; Iwema et al., 2007; Tocchini-Valentini et al., 2009; Wang and LeBlanc, 2009).

The basal tissue expression indicates that *P. dumerilii* RXR is ubiquitously expressed along the body axis in both genders, indicating a possible role in multiple physiological functions (Fig. 7.5). However, we were not able to dissect individual tissues because of their small size. Hence, to refine the expression pattern presented here, *in situ* hybridization should be performed in future studies.

In vertebrates, RXR is upstream of several signaling pathways involved in a range of physiological functions (Mark and Chambon, 2003; Szanto et al., 2004; Mark et al., 2006). Therefore, it is not surprising that disruption of the RXR-dependent signaling pathway can affect the endocrine system. One interesting example is the impact of organotins. Endocrine disruption by organotin compounds has been reported in metazoans belonging to different *taxa*; evolutionary conserved modulation across *taxa* (Santos et al., 2012; Castro and Santos, 2014; Wu et al., 2014). In gastropods, exposure to TBT and/or TPT at rather low concentrations (~1 ng/L) impacts sexual development and reproduction, with the imposex phenomenon in females being the most drastic adverse effect reported (Horiguchi et al., 1997; Matthiessen et al., 1998; Santos et al., 2000; Schulte-Oehlmann et al., 2000; Oehlmann and Schulte-Oehlmann, 2003; Sternberg et al., 2010). Imposex has been observed in more than 200 gastropod species (Gibbs et al., 1997; Matthiessen et al., 1998; Abidli et al., 2013). Strong evidences points to the involvement of the RXR-dependent signaling in imposex induction, with TBT and/or TPT mimicking the action of RXR natural ligands (Nishikawa et al., 2004; Castro et al., 2007; Sternberg et al., 2010; Lima et al., 2011; Urushitani et al., 2011; Stange et al., 2012). *In vitro* binding assays showed that TBT and TPT bind to human RXRs and rock shell *T.*

clavigera with similar affinity as 9-*cis*-RA (Nishikawa et al., 2004). It has also been shown that *in vivo* injections of 9-*cis*-RA, at similar concentration of organotin compounds (1 µg/g body weight), leads to female rock shell imposex development (Nishikawa et al., 2004). This hypothesis was further supported in the dogwhelk *N. lapillus* where 9-*cis*-RA, TBT and HX630 (synthetic agonist) induced imposex development (Castro et al., 2007; Stange et al., 2012).

For many other metazoan *taxa* with reported effects after organotins exposure, the signaling cascades involved remain unknown. A few studies in the international literature show that annelids seem to be sensitive to low TBT levels. For instance, when juveniles of the polychaete *Armandia brevis* were exposed to sediment-associated TBT (93 ng/g) for 42 days, a concentration found in many moderately contaminated TBT sediments (Gómez-Ariza et al., 2006; Antizar-Ladislao, 2008), the growth was inhibited by approximately 25% (Meador and Rice, 2001). Similarly, early life stages of *P. dumerilii* are also sensitivity to TBT. Hagger et al. (2002) exposed embryo-larvaes of this worm to different TBT concentrations (0.31, 0.54, 1.75 and 3.11 µg/L) for 24, 48 and 72 h. Even a short-term exposure led to genotoxic, cytotoxic, survival, and developmental effects at concentrations 0.31, 0.54, 1.75 and 3.11 µg/L. A strong correlation was found between the development abnormalities and mortality in TBT exposed embryo-larvae in a concentration and age related manner. For the endpoint mortality, LC_{50} values for 24, 48 and 72 h were found to be 5.89, 1.34 and 0.61 µg/L, respectively (Hagger et al., 2002). Hence, it is plausible to hypothesize that chronic exposures, as those performed with gastropods, could even yield impacts in ecologically relevant endpoints at even lower concentrations. TBT is particularly persistent in water column and sediments, with greater concentrations found in the later environmental compartment at 0.6 to 16816 ng Sn/g (Santos et al., 2002; Gómez-Ariza et al., 2006; Antizar-Ladislao, 2008). Our data suggests that the reported effects resultant from TBT exposure, at the level of *P. dumerilii* embryos-larvae development might be mediated through RXR signaling pathway. *P. dumerilii* RXR was transactivated in the presence TBT at the three tested concentrations 10, 100 and 250 nM (Fig. 7.7).

In contrast to TBT, only a few studies evaluated the effects of TPT in aquatic organisms, addressing mostly the impact in gastropods (Schulte-Oehlmann et al., 2000; Barroso et al., 2002; Santos et al., 2006; Yi et al., 2012; Laranjeiro et al., *in press*). Recent studies in fish, however, suggest that TPT might also target phylogenetically conserved pathways at rather low concentrations (Zhang et al., 2008; Hu et al., 2009). These findings were confirmed by field and laboratory experiments. Zhang et al. (2008) exposed *Oryzias latipes* (Medaka) to different TPT concentrations (1.6, 8, 40, 200, and 1000 ng/L) for 5 weeks. From the exposure reproductive and transgenerational negative effects were

observed. TPT exposure significantly suppressed the spawning frequency, spawned egg number, egg quality and gonad development; and induced teratogenesis, such as hemorrhaging, morphological malformation, conjoined twins and reduces hatchability (Zhang et al., 2008). It has been shown that field TPT-exposed sturgeon *Acipenser sinensis* accumulate the compound in different tissues, with higher concentration being found in liver (31-128 ng/g wet weigh). It was also reported that accumulated TPT in maternal tissue can be transferred to the offspring (Hu et al., 2009). In eggs, TPT was found at concentrations of 25.5-13.0 ng/g wet weigh, that later resulted in larvae ocular abnormal development and severe skeletal/morphological deformations (Hu et al., 2009). The reported TPT effects were suggested to be mediated through RXR signaling pathway since the compound efficiently activates target gene transcription at low concentrations (Hu et al., 2009).

To date only a single study demonstrated that annelids are also sensitive to TPT exposure. The survival of a benthonic freshwater annelid *Tubifex tubifex* is affected after 96 h of TPT water exposure, with a LC_{50} value at 1 µg/L (Fargasová, 1998). Our work shows that in addition to TBT, *P. dumerilii* RXR can also be transactivated in the presence of TPT at the lowest concentration tested (10 nM), indicating that RXR signaling pathways may be disrupted by TPT, a compound that also shows a tendency to bio-accumulate in sediments (Yi et al., 2012). In the aquatic environment, annelids play fundamental roles in the maintenance of food chains, recycling nutrients, and affect sediment geochemical processes via bioturbation (Gillett et al., 2007; Beckman, 2012). Some annelids are benthonic with part of the life cycle taking place on the water column. *P. dumerilii*, for instance, has a benthic/pelagic life cycle. The fertilization is external and takes place in the water column as well as the subsequent formed embryo and early larvae development stages (Fischer and Dorresteijn, 2004). Then, at young immature worms (atoke) stage, they leave the water column and settle on macro-algae foot or thalli inside self-made mucus tubes (Gambi et al., 2000; Fischer and Dorresteijn, 2004). Therefore, during its life cycle *P. dumerilii* and other annelids are exposed to organotin compounds via more than one route: water and sediment, and probably food consumption. Thus, it is not unreasonable to propose that annelids might be also affected by TPT, an agonist of *P. dumerilii* RXR, in accordance with the presented data.

Human RXRα LBD C432 residue on helix H11 is crucial for the binding mechanisms and organotin-induced transactivation (Hiromori et al., 2015). The affinity of RXR towards TBT and TPT is associated with the interaction between the tin moiety and the sulfur atom of the cysteine residue; mutation of this residue leads to loss of transactivation by both TBT and TPT (Grün et al., 2006; le Maire et al., 2009; Harada et al., 2015; Hiromori et al., 2015). An additional residue, L326, has also been suggested to

participate in organotin binding in human RXRα, although less effectively than C432. Mutation of this residue to alanine retained organotin-mediated transactivation activity, yet concentrations required to elicit a response were about 1 order of magnitude higher than the concentrations required for the wild type (Hiromori et al., 2015). Both residues are present in the sequences of RXR orthologues of mollusks and *P. dumerilii* (Fig. 7.3), suggesting a conserved ability to respond to organotin compounds. A hypothesis corroborated by our *in vitro* transactivation assays (Fig. 7.7).

Urushitani et al. (2011) reported that the transcriptional activation responses of *T. clavigera* RXR in the presence of TBT and TPT were very similar. This observation is in accordance to the reported binding affinities of the compounds towards RXR, and identical severity of both organotin on promoting imposex development on the rock shell females (Nishikawa et al., 2004). In the present study, the transactivation response mediated by *P. dumerilii* RXR was higher in the presence of TBT when compared to TPT (Fig. 7.7). This data suggests that both TBT and TPT seem bioactive in *P. dumerilii*, but probably the former organotin has a higher affinity for the receptor and is, possibly, more harmful. Taking in consideration the results presented here, it will be relevant to address the *P. dumerilii* RXR ligand affinity for the organotin compounds in future studies and evaluate possible biological effects from their exposure. Considering that RXR serves as a heterodimeric partner to several NRs, such as the retinoic acid receptor (RAR), the peroxisome proliferator-activated receptor (PPAR), the ecdysone receptor (EcR), the thyroid hormone receptor (ThR), etc., multiple endocrine cascades can potentially be targeted by organotin compounds. To date, with respect to *P. dumerilii*, a RAR orthologue was the sole reported partner NR (Gutierrez-Mazariegos et al., 2014). A true PPAR orthologue seems to be missing in annelids. So far, presence of a closely related gene the ecdysone-induced proteins 75B was reported in the *Capitella* sp. (Albalat and Cañestro, 2009). Yet, additional heterodimeric partners must exist; for instance, EcR orthologues were genome predicted in two other annelids, *Capitela* sp. and *Helobdella robusta* (Laguerre and Veenstra, 2010). In fact, TBT was shown to modulate the EcR/RXR heterodimer in two crustacean species, *Daphnia magna* and the brown shrimp *Crangon crangon* (Wang et al., 2011; Verhaegen et al., 2011). In *D. magna*, combined exposure with TBT and 20-hydroxyecdysone, an EcR natural ligand, promoted incomplete molting and increased mortality (Wang et al., 2011). Thus, future studies should focus on the isolation of the heterodimeric partners of RXR in annelids and access the signaling cascades modulated by organotins.

7.6 Conclusion

In the present study, we isolated a cDNA encoding a RXR orthologue for the *P. dumerilii* and functional characterized it using an *in vitro* transactivation assay. The deduced amino acid sequence showed that *P. dumerilii* RXR had high levels of identity with known vertebrate and mollusk RXRs. Key residues for interaction and binding to 9-*cis*-RA and organotin compounds are present and conserved in LBD. The *P. dumerilii* RXR orthologue is capable of inducing a transcriptional response *in vitro* upon 9-*cis*-RA exposure, in accordance with the conservation of key residue of the LBD related to interaction and binding. These findings advocate for a role in RXR-dependent gene regulation in the annelid endocrinology mediated by 9-*cis*-RA. Next, we show that *P. dumerilii* RXR is also able to promote transcription of a reporter gene in the presence of increasing concentrations of TBT and TPT. These findings emphasize the existence of a common, and evolutionary conserved, mechanism for endocrine disruption by TBT and TPT through modulation of RXR-dependent signaling in metazoans. Still, further studies are needed to evaluate *P. dumerilii* RXR ligand affinity towards natural ligands and organotin compounds. Also, the predicted sensitivity of *P. dumerilii* towards organotins should be validated through *in vivo* approaches in combination with gene expression analysis, for the assessment of RXR-dependent signaling pathways. Finally, our findings emphasize the need to evaluate TBT and TPT binding abilities and RXR-mediated target gene activation in other species with non-reported effects.

7.7 Acknowledgments

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CHAPTER 8

8 *Nucella lapillus* retinoid X receptors isoforms activate transcription of reporter genes in response to imposex-induction compounds

8.1 Abstract

Tributyltin (TBT) and triphenyltin (TPT) are ubiquitous aquatic environmental contaminants. Gastropods are very sensitive species to the toxic effects of such compounds, particularly the development of male sexual characteristics in females, a phenomenon termed to imposex. The molecular cascade involved in such phenomenon remains to be fully characterized. Yet, compiling evidences indicate that TBT and/ or TPT bind and are high affinity ligands of the retinoid X receptor (RXR), transactivating target gene transcription of RXR isoforms in a similar manner to the putative natural ligand 9-*cis*-retinoic acid. These findings strongly support the involvement of RXR-dependent signaling pathways in gastropod masculinization. Here we provide more evidences that reinforce the postulated hypotheses. Through a target gene transactivation *in vitro* approach using COS-1 cell lines we shown that in RXR-signaling cascade from the TBT-sensitive gastropod, *Nucella lapillus*, is responsive to several RXR agonists, including the organotins, TBT and TPT.

In contrast to *Thais clavigera*, female *N. lapillus* are less sensitive to TPT developing female penis induction only at extremely high levels. Here we proposed that differences regarding RXR binding affinity to TPT might be implicated with species-specific sensitivity response to TPT. In fact we could observe that compared to *T. clavigera* RXR, *N. lapillus* RXR has a lower binding affinity to TPT. Overall, data presented here not only supports our postulated hypothesis, but also suggest that although TPT does not induce female penis development in *N. lapillus* at environmental relevant levels, due to its lower RXR affinity, is capable of modulating RXR-dependent signaling pathway, which agrees with imposex data available in the literature.

8.2 Introduction

Since the mid-1960s the organotin compounds tributyltin (TBT) and triphenyltin (TPT) have been extensively used in many applications as agricultural fungicides, wood preservatives, antifouling for ships and fishing nets (Fent, 1996). Due to its extensive use increasing amounts have been released and rapidly spread into the environment mainly to the aquatic ecosystems (Matthiessen and Gibbs, 1998; Yi et al., 2012; Wu et al., 2014). These compounds are known to cause severe health effects on humans and wildlife (Santos et al., 2012; Wu et al., 2014). Imposex, i.e., the imposition of male's secondary sexual characteristics (such as the penis and vas deferens) in female gastropods, is the most well-known example of endocrine disruption induced by TBT and TPT at very low

concentrations (~1 ng/L) (Smith, 1971; Horiguchi et al., 1997; Matthiessen and Gibbs, 1998; Castro et al., 2007; Lima et al., 2011; Abidli et al., 2012, 2013). In more severe stages, female gastropods are unable to reproduce due to oviduct blockage by vas deferens formation, resulting in population decline or even in local species extinction (Smith, 1971; Bryan et al., 1986; ten Hallers-Tjabbes et al., 1994).

Although TBT and TPT had a simultaneous introduction and occurrence in the aquatic environment, the imposex phenomenon was initially (early 1980s) broadly associated to TBT (Fent, 1996; Santos et al., 2002; Yi et al., 2012). Since then, it has been reported to affect more than 200 species of gastropods, such as *Nucella lapillus*, *Ilyanassa obsolete*, *Nassarius reticulates* and *Thais clavigera* (Gibbs et al., 1997; Matthiessen and Gibbs, 1998; Santos et al., 2000, 2002, 2006; Abidli et al., 2013). Only later, studies started also to associate TPT to imposex development affecting species such as *T. clavigera*, *Bolinus brandaris* and *Hinia reticulata* (Horiguchi et al., 1994, 1997; Schulte-Oehlmann et al., 2000; Barroso et al., 2002; Santos et al., 2006). Interestingly, not all gastropod species show the same response to TBT and TPT exposure (Bryan et al., 1988; Gibbs et al., 1997; Schulte-Oehlmann et al., 2000). No imposex promotion has been observed when *N. lapillus* was injected (1 µg TPTCl g^{-1} body weight) or via water exposure to TPT (5-100 ng TPT-Sn/L) (Bryan et al., 1988; Schulte-Oehlmann et al., 2000), whereas severe imposex development was observed when *N. lapillus* were exposed to the same TBT levels (Castro et al., 2007; Lima et al., 2011). Nevertheless, TPT produced an adverse effect on the extension of the male genitalia, reducing the mean length of the prostate gland and penis, and also induces tissue excrescences mainly epithelial hyperplasiae on the gill, osphradium or pallial sex organs (Schulte-Oehlmann et al., 2000). A recent experiment with *N. lapillus* shows that TPT injection at environmental relevant levels leds to vas deference development, but no penis (Laranjeiro et al., *in press*). In contrast to *N. lapillus*, *T. clavigera*, a close species from a phylogenetic stand point, develops imposex at a similar level when injected with both organotins (Horiguchi et al., 1997; Nishikawa et al., 2004). TBT and/or TPT also alters the shell thickening, and affects egg-laying fecundity and snail growth of the hermaphrodite *Lymnaea stagnalis* (Giusti et al., 2013). Under the influence of TPT and TBT exposure *M. cornuarietis* also develops imposex (Schulte-Oehlmann et al., 1995, 2000). In addition, lipid accumulation and fatty acid homeostasis is also affected in *Marisa cornuarietis* by both organotin compounds (Janer et al., 2007; Lyssimachou et al., 2009). While TBT exposure results in an increasing of lipid and fatty acids total levels (Jenner et al., 2007), the TPT exposure leads to a decrease (Lyssimachou et al., 2009).

Despite several postulated hypotheses on the cause of imposex induction, the full cascade of molecular events through which organotin compounds induce imposex is still

no fully clarified (Castro et al., 2007; Lima et al., 2011). In the last years, a growing number of studies has reported that endocrine disruption by organotin compounds might be mediated by the modulation of retinoid X receptor (RXR) (Nishikawa et al., 2004; Castro et al., 2007; Lima et al., 2011; Urushitani et al., 2011; Stange et al., 2012). Retinoid X receptor is a transcription factor that belongs to the nuclear receptor (NR) superfamily and is implicated in many biological functions due to its unique features: (1) is a ligand-dependent *bone fine* NR that is believed to bind with high affinity to its putative natural ligand the retinoid biological active isomer 9-*cis*-retinoid acid (9-*cis*-RA) and thus may be involved in retinoid transduction signals; (2) is capable to form heterodimers with other NRs, and in such association can be activated by ligand agonists of its own and its partner receptors (permissive heterodimers) or only activated by agonist of its partners (non-permissive heterodimers) (Germain et al., 2006). Like other member of NRs superfamily comprises a modular structure of five main domains: The A/B region is a variable N-terminal domain; a highly conserved DNA-binding domain (DBD or C region); the D region which serves as a flexible hinge permitting RXR to bind to other NRs; and a moderately conserved E region, also known as the ligand binding domain (LBD) (Germain et al., 2006).

Nishikawa et al. (2004) isolated a RXR gene orthologue from *T. clavigera* that share a high sequence similarity to human RXRα, and like this it binds to 9-*cis*-RA and organotin compounds. A single *in vivo* injection of either 9-*cis*-RA or TPT at similar concentrations (1 µg/g body weight) into morphologically normal female results in the development of imposex within 1 month (Nishikawa et al., 2004). Obtained evidences suggested that TBT and TPT may mimic the role of RXR natural ligand (Nishikawa et al., 2004). Later, these findings were supported by the study of Castro et al. (2007). A RXR orthologue was isolated from *N. lapillus* and shown that it binds to 9-*cis*-RA. Moreover, through a combination of exposure experiments, they showed that the 9-*cis*-RA induces imposex in females of *N. lapillus* to the same degree as TBT, when administered at similar concentrations (1 µg/g body weight) within two months. In addition demonstrated that methoprene acid (16 µg/g body weight), a selective ligand for RXR, also induces imposex although to a lower degree (Castro et al., 2007). More recently, a study revealed that *T. clavigera* RXR isoform has the ability to activate transcription of a reporter gene in the presence of 9-*cis*-RA, TBT and TPT (Urushitani et al., 2011). The compiling results indicate that RXR is a clearly endocrine prime target for organotin compounds.

Hence, the present study aims to expand the previous findings which pointed to a central role of RXR signaling cascade into female imposex induction development by organotin compounds. We investigated if *N. lapillus* RXR isoforms are responsive to the presence of organotin compounds and other putative ligands that are known to induce

imposex development in this species using luciferase transactivation reporter gene assays. We also performed an *in vitro* ligand binding assay to evaluate the affinity ability of TPT for *N. lapillus* and *T. clavigera* RXR. The aim is to understand if the affinity for RXR *in vitro* might relates with the different species-sensitivity to TPT previous reported for *N. lapillus* and *T. clavigera*.

8.3 Material and methods

8.3.1 Chemical compounds

9-*cis*-RA (≥ 98%), Methoprene acid (≥ 98%), Tributyltin chloride (TBT-Cl; 96%), and Triphenyltin chloride (TPT-Cl; 95%) and sterile-filtered dimethyl sulfoxide (DMSO) were purchase from Sigma-Aldrich. The 4-(7,8,9,10-Tetrahydro-7,7,10,10-tetramethylbenzo[b] naphtho[2,3-f][1,4] thiazepin-12-yl-benzoic acid (HX630; > 98%) a RXR selective agonist were acquired from Tocris Bioscience.

8.3.2 Sequence analysis

Using Clustal Omega sequence alignment tool, with default parameters to infer about conserved key residues (http://www.ebi.ac.uk/Tools/msa/; Sievers et al., 2011), the two full protein sequence of *N. lapillus* RXR isoforms was aligned with the RXR sequences of other species obtained from NCBI's GenBank database (http://blast.ncbi.nlm.nih.gov; Geer et al., 2010; Benson et al., 2013). Full RXR gene protein sequences were retrieved from GenBanK: *Homo sapiens* RXRα (HsRXRa; BAH02296.1), *T. clavigera* isoform 1 (TcRXR1; BAJ76722.1), *T. clavigera* isoform 2 (TcRXR2; BAJ76723.1), *N. lapillus* RXRa isoform (NlRXRa; ABS70715.1), *N. lapillus* RXRb isoform (NlRXRb; ABS70716.1), *Biomphalaria glabrata* RXR (BgRXR; NP_001298239.1).

8.3.3 Fusion protein construction

For the *N. lapillus* RXRa isomer, the sequence range from the T-box, starting in "KREA" sequence (Fig. 8.1), of DNA-binding domain (DBD) to ligand binding domain (LBD) including the stop codon. This was amplified by polymerase chain reaction (PCR) with Phusion Flash high-fidelity polymerase PCR Master Mix (Fisher Scientific) using cDNA from male gonads as template. The PCR profile had the following conditions: 98℃ of initial denaturation for 10 s; thereafter, 35 cycles of amplification were carried out with a denaturation at 98℃ for 10 s, annealing at 50℃ fo r 5 s and extension at 72℃ for 15 s, and a final extension step at 72ºC for 1 min. The PCR product was gel-purified and restricted digested with XbaI and KpnI restriction enzymes (Promega; Table 8.1) for sub

cloned into pBIND expression vector fusing it to the C-terminal end of the GAL4 DNA-binding domain. The enzyme T4 ligase (Promega) was used for overnight ligation of the insert into pBIND following the manufactures instructions. Constructs were transformed into Nzy5α *Escherichia coli* cells (Nzytech). In the case of NlRXRb isoform some difficulties were faced to amplify the T-box + LBD (Fig. 8.1). Thus, in order to get its sequence we synthesized (IDTDNA) and performed a restriction digestion XbaI and KpnI, sub cloned into pBIND with T4 ligase and transform into Nzy5α *E. coli* cells (Nzytech). Constructs were extracted from bacteria positive colonies using the Nzytech miniprep kit (Nzytech). All cloned inserted DNA sequences were verified by sequencing (GATC) to check if there were in reading frame without any amino acid substitutions or deletions, before performed Midiprep (Nzytech) according to manufacture protocol to use for the transactivation assays. Retinoid X receptors nucleotide sequence accession numbers used are as follows: HsRXRα NM_002957.5, NlRXRa EU024473.1 and NlRXRb EU024474.1.

Table 8.1: List of primers used to amplify the sequence to be cloned into pBIND expression vector.

Specie and isoform	Sequence	Restriction enzymes used
*Nlapillus*RXRa	F: ACTG**TCTAGA**AAGAGAGAGGCCGTGCAGGAG	XbaI
	R:TATA**GGTACC**AACCCAAGACACAACTGACGATGG	KpnI
*Hsapiens*RXRα	F: AATT**TCTAGA**GCCGTGCAGGAGGAGCGGCA	XbaI
	R: AATT**GGTACC**AGTCATTTGGTGCGGCGCCT	KpnI

8.3.4 Cell cultures

COS-1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) with phenol red and supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Cell culture was kept at 37℃ in a humidified atmosphere containing 5% of CO_2.

8.3.5 Transativation assays

The day before transfection, cells were seeded on 24-well culture plates at a density of 2 x 10^5 cells per well and cultured in DMEM (PAN-Biotech) supplemented with 10% fetal bovine serum (Invitrogen, Thermo Fisher), and 1% penicillin/streptomycin (Invitrogen, Thermo Fisher). COS-1 cells transfection was performed with Lipofectamine® 2000 Transfection Reagent (Invitrogen, Thermo Fisher) and Opti-MEM reduced serum medium (Gibco, Thermo Fisher) following the manufacturers' instruction. Briefly, 0.5 µg pBIND RXR-(T-box + LBD) constructs and 1 µg of the pGL4.31 luciferase reporter vector

were added for a final Opti-MEM volume of 25 µL. In another tube, 2 µL of Lipofectamine were added to Opti-MEM medium to a final volume of 25 µL and left for incubation 5 min at room temperatures. Then, diluted plasmids pBIND RXR-(T-box + LBD) and pGL4.31 luciferase were mixed to Lipofectamine Transfection reagent and left incubated for 20 min at room temperature. Once complexes were formed 300 µL more of Opti-MEM medium was added. Cells were washed once with Phosphate buffered saline (PBS) to eliminate DEMEM serum traces, and the mixture (total of 350 µL) was added to the cells and incubated at 37ºC in a humidified atmosphere contai ning 5% of CO_2. After 5 h of incubation, cells were washed once with PBS and exposed to the test ligands in 1 mL phenol red-free DMEM supplemented with 10% dextran-coated charcoal-treated serum (Invitrogen, Thermo Fisher) and 1% penicillin/streptomycin (Invitrogen, Thermo Fisher). All working compound stock solutions were prepared in DMSO, and the concentration in the culture medium did not exceed 0.1%. The DMSO was also used as a vehicle control. Selected test concentration of 9-*cis*-RA, TBT, TPT, MA and HX630 were based on previous published works (Harmon et al., 1995; Kanayama et al., 2005; Gumy et al., 2008; le Maire et al., 2009; Urushitani et al., 2011; Stange et al., 2012). The cells were lysed and harvested 24 h later, with 100 µL of Passive Lysis Buffer (Promega) after being washed once with PBS (PAA Biotech). The cell lysates were incubated at 37ºC for 15 min with gentle agitation (90 rpm) and store at -80ºC (no longer than a week) until data reading.

Luciferase activity and Renilla activities were determined using the dual-luciferase reporter assay system (Promega) in accordance with the manufacturer's instructions. The light luminescence intensity was determined with a plate reader Synergy HT Multi-Mode Microplate reader (BioTek) after injecting 50 µL of LARII reagent (fire fly luciferase substrate) followed by addition of 50 µL of Stop and Glo reagent (Renilla luciferase substrate). Firefly luciferase activity was normalized with the Renilla activity (efficiency of transfection).

8.3.6 Ligand binding assay

The LBD of dogwhelk NlRXRa (codons 186-441) and of rock shell TcRXR-1 (codons 177-431; NR accession number AAU12572.1 from Nishikawa et al. (2004)), were subcloned into pGEX-4T expression vector (Pharmacia, Uppsala, Sweden). GST-RXRs fusions were expressed in *E. coli* BL21 and purified according to the standard procedure (Pharmacia, Uppsala, Sweden). The purified proteins (30 µg/mL) were incubated with increasing concentrations of [^{14}C]-TPT (nM) with or without a 100-fold nM excess of unlabeled TPT. After incubation at 4ºC for 1 h, specific binding was determined by hydroxyapatite binding assay (Clagett-Dame and Repa, 1997).

8.3.7 Statistical analysis

All data from transactivation assays were obtained from the same numbers of replicated experiments carried out independently at least 3 times. The results from fold induction did not meet ANOVA assumptions (homogeneity of variances and/or normal distributed data). Then, Kruskal–Wallis one-way ANOVA and Test K for independent samples was applied to the raw data using IBM Spss version 22.1 software (license and software provided by the University of Porto). In all cases, means were taken as statistically at different $P < 0.05$ was. All data are reported as means ± Standard Error (StE).

8.4 Results

8.4.1 Sequences alignment analysis

For the present study we used the previously isolated and published cDNA encoding amino acid sequence for both *N. lapillus* RXR isoforms by Castro et al. (2007). The two NlRXR isoforms are identical except for an amino acid residue substitution in the LBD, NlRXRa has a Glu228, whereas NlRXRb has a Gly233 (Fig. 8.1); and amino acid short sequence insertions/deletions located in the T-box of the DNA binding domain (Fig. 8.1). This T-box insertion/deletion is a feature also reported to exist in *T. clavigera* RXR isoforms (Urushitani et al., 2011). Multiple sequence alignment of RXR amino acid sequence analysis showed that NlRXR isoforms had all the main features typical of the RXR family including key residues in the ligand binding domain proposed to interact with 9-*cis*-RA (Egea et al., 2000, 2002) and the key cysteine (C432 in human RXRα protein sequence) for organotin compounds binding and interaction in humans RXRα (le Maire et al., 2009; Hiromori et al., 2015) (Fig. 8.1). It has been reported that, due to the LBD key residues conservation, NlRXRs has retained the ability to bind to 9-*cis*-RA, something that has been proved through an *in vitro* binding assay (Castro et al., 2007).

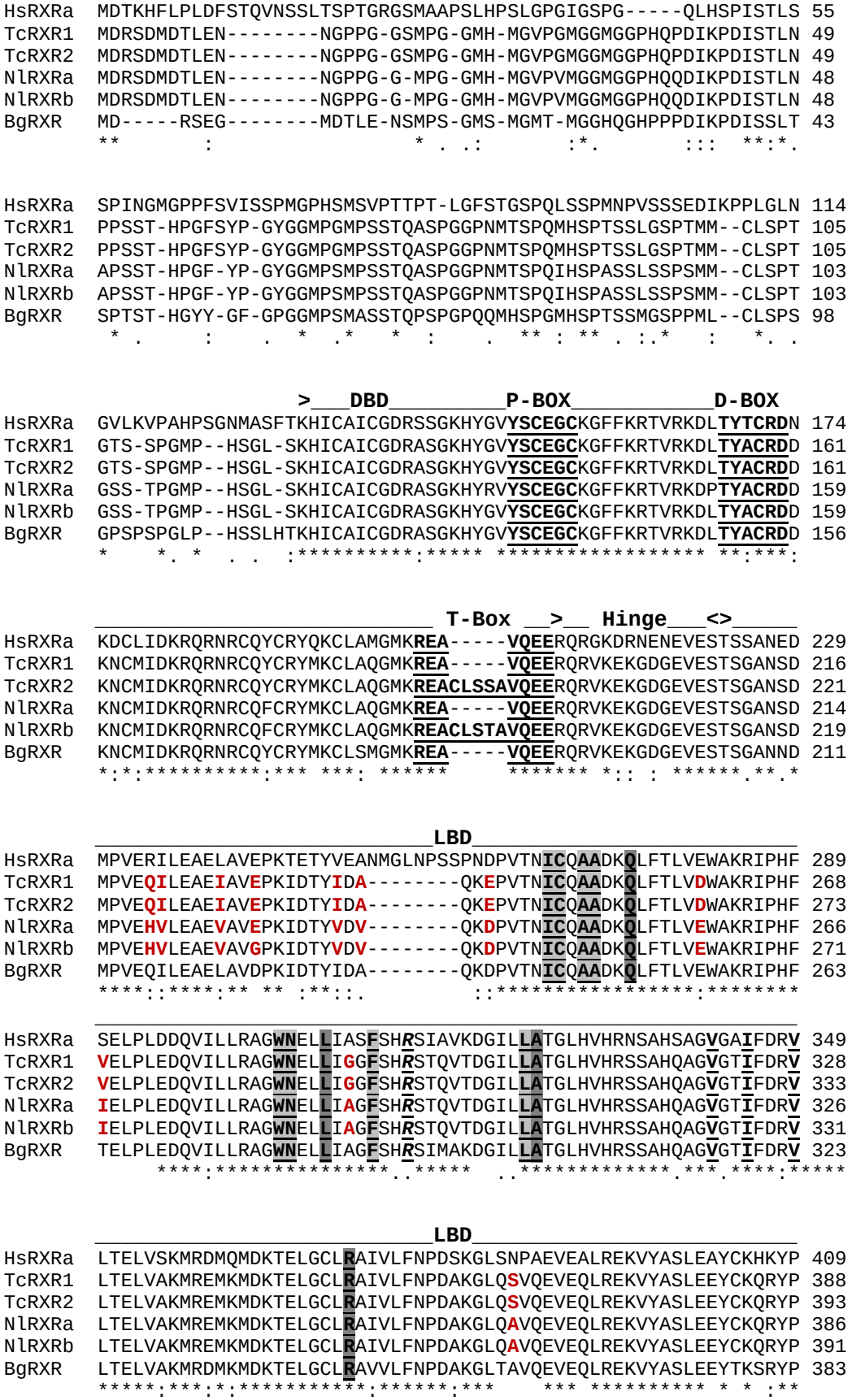
HsRXRa MDTKHFLPLDFSTQVNSSLTSPTGRGSMAAPSLHPSLGPGIGSPG-----QLHSPISTLS 55
TcRXR1 MDRSDMDTLEN--------NGPPG-GSMPG-GMH-MGVPGMGGMGGPHQPDIKPDISTLN 49
TcRXR2 MDRSDMDTLEN--------NGPPG-GSMPG-GMH-MGVPGMGGMGGPHQPDIKPDISTLN 49
NlRXRa MDRSDMDTLEN--------NGPPG-G-MPG-GMH-MGVPVMGGMGGPHQQDIKPDISTLN 48
NlRXRb MDRSDMDTLEN--------NGPPG-G-MPG-GMH-MGVPVMGGMGGPHQQDIKPDISTLN 48
BgRXR MD-----RSEG--------MDTLE-NSMPS-GMS-MGMT-MGGHQGHPPPDIKPDISSLT 43
SPINGMGPPFSVISSPMGPHSMSVPTTPT-LGFSTGSPQLSSPMNPVSSSEDIKPPLGLN 114
PPSST-HPGFSYP-GYGGMPGMPSSTQASPGGPNMTSPQMHSPTSSLGSPTMM--CLSPT 105
PPSST-HPGFSYP-GYGGMPGMPSSTQASPGGPNMTSPQMHSPTSSLGSPTMM--CLSPT 105
APSST-HPGF-YP-GYGGMPSMPSSTQASPGGPNMTSPQIHSPASSLSSPSMM--CLSPT 103
APSST-HPGF-YP-GYGGMPSMPSSTQASPGGPNMTSPQIHSPASSLSSPSMM--CLSPT 103
SPTST-HGYY-GF-GPGGMPSMASSTQPSPGPQQMHSPGMHSPTSSMGSPPML--CLSPS 98
>___DBD_____P-BOX_____D-BOX
GVLKVPAHPSGNMASFTKHICAICGDRSSGKHYGVYSCEGCKGFFKRTVRKDLTYTCRDN 174
GTS-SPGMP--HSGL-SKHICAICGDRASGKHYGVYSCEGCKGFFKRTVRKDLTYACRDD 161
GTS-SPGMP--HSGL-SKHICAICGDRASGKHYGVYSCEGCKGFFKRTVRKDLTYACRDD 161
GSS-TPGMP--HSGL-SKHICAICGDRASGKHYRVYSCEGCKGFFKRTVRKDPTYACRDD 159
GSS-TPGMP--HSGL-SKHICAICGDRASGKHYGVYSCEGCKGFFKRTVRKDLTYACRDD 159
GPSPSPGLP--HSSLHTKHICAICGDRASGKHYGVYSCEGCKGFFKRTVRKDLTYACRDD 156
T-Box __>__ Hinge___<>_____
KDCLIDKREQRNRCQYCRYQKCLAMGMKREA-----VQEERQRGKDRNENEVESTSSANED 229
KNCMIDKREQRNRCQYCRYMKCLAQGMKREA-----VQEERQRVKEKGDGEVESTSGANSD 216
KNCMIDKREQRNRCQYCRYMKCLAQGMKREACLSSAVQEERQRVKEKGDGEVESTSGANSD 221
KNCMIDKREQRNRCQFCRYMKCLAQGMKREA-----VQEERQRVKEKGDGEVESTSGANSD 214
KNCMIDKREQRNRCQFCRYMKCLAQGMKREACLSTAVQEERQRVKEKGDGEVESTSGANSD 219
KNCMIDKREQRNRCQYCRYMKCLSMGMKREA-----VQEERQRVKEKGDGEVESTSGANND 211
LBD
MPVERILEAELAVEPKTETYVEANMGLNPSSPNDPVTNICQAADKQLFTLVEWAKRIPHF 289
MPVEQILEAEIAVEPKIDTYIDA--------QKEPVTNICQAADKQLFTLVDWAKRIPHF 268
MPVEQILEAEIAVEPKIDTYIDA--------QKEPVTNICQAADKQLFTLVDWAKRIPHF 273
MPVEHVLEAEVAVEPKIDTYVDV--------QKDPVTNICQAADKQLFTLVEWAKRIPHF 266
MPVEHVLEAEVAVGPKIDTYVDV--------QKDPVTNICQAADKQLFTLVEWAKRIPHF 271
MPVEQILEAELAVDPKIDTYIDA--------QKDPVTNICQAADKQLFTLVEWAKRIPHF 263
SELPLDDQVILLRAGWNELLIASFSHRSIAVKDGILLATGLHVHRNSAHSAGVGAIFDRV 349
VELPLEDQVILLRAGWNELLIGGFSHRSTQVTDGILLATGLHVHRSSAHQAGVGTIFDRV 328
VELPLEDQVILLRAGWNELLIGGFSHRSTQVTDGILLATGLHVHRSSAHQAGVGTIFDRV 333
IELPLEDQVILLRAGWNELLIAGFSHRSTQVTDGILLATGLHVHRSSAHQAGVGTIFDRV 326
IELPLEDQVILLRAGWNELLIAGFSHRSTQVTDGILLATGLHVHRSSAHQAGVGTIFDRV 331
TELPLEDQVILLRAGWNELLIAGFSHRSIMAKDGILLATGLHVHRSSAHQAGVGTIFDRV 323
LBD
LTELVSKMRDMQMDKTELGCLRAIVLFNPDSKGLSNPAEVEALREKVYASLEAYCKHKYP 409
LTELVAKMREMKMDKTELGCLRAIVLFNPDAKGLQSVQEVEQLREKVYASLEEYCKQRYP 388
LTELVAKMREMKMDKTELGCLRAIVLFNPDAKGLQSVQEVEQLREKVYASLEEYCKQRYP 393
LTELVAKMREMKMDKTELGCLRAIVLFNPDAKGLQAVQEVEQLREKVYASLEEYCKQRYP 386
LTELVAKMREMKMDKTELGCLRAIVLFNPDAKGLQAVQEVEQLREKVYASLEEYCKQRYP 391
LTELVAKMRDMKMDKTELGCLRAVVLFNPDAKGLTAVQEVEQLREKVYASLEEYTKSRYP 383

Figure 8.1: Amino acid sequence alignment of the RXR from human and mollusk species. Key residues interacting with 9-*cis*-RA, are highlighted at bold. Hydrogen bonds, which direct or mediated by water molecules, between the substrate and pocket residues are highlighted at bold and shading in grey. In bold and black grey hydrophobic and Van der Waals interactions. The arginine bold italic underlined forms an ionic interaction with the ligand. Cysteine known to interact with TBT is highlighted in shading black. Underline the P-, D- and T-boxes; underlined transactivation function AF-2, (*) - shows identical residues; (:) shows one conservative amino acid substitution; and (.) shows two conservative amino acid substitutions. Different amino acids residues within the LBD between *N. lapillus* isoforms and *T. clavigera* RXR isoform are highlighted in red. Domains and key residues were inferred from de Groot et al. (2005), Castro et al. (2007) and Urushitani et al. (2011). *H. sapiens* RXRα (HsRXRa), *T. clavigera* isoform 1 (TcRXR1), *T. clavigera* isoform 2 (TcRXR2), *N. lapillus* RXRa isoform (NlRXRa), *N. lapillus* RXRb isoform (NlRXR), *B. glabrata* RXR (BgRXR).

8.4.2 Transcriptional activity of NlRXRa and b isoforms expressed in mammalian COS-1 cells lines

To evaluate whether the putative imposex induction ligands are in fact able to activate target gene transcription mediated by a NlRXR isoforms dependent signaling cascade we use a transactivation reporter luciferase gene assay approach using mammalian COS-1 cell lines. Both NlRXR isoforms induced luciferase reporter gene activity when 9-*cis*-RA was added to the medium. However, the transactivation activity of NlRXRb is lower than NlRXRa, with no activity reporter for it to the lowest 9-*cis*-RA concentration (0.01 µM) (Fig. 8.2).

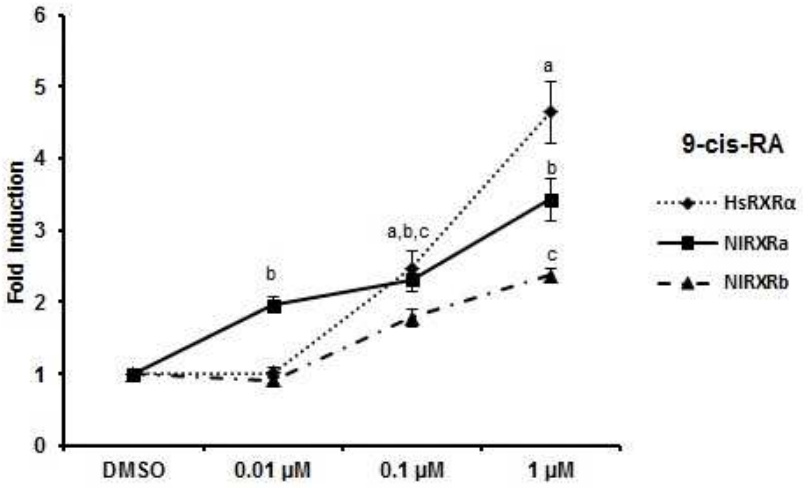

Figure 8.2: Transactivation activity of luciferase reporter gene mediated by GAL4 DBD-RXR LBD fusion constructs in COS-1 cells in the presence of 9-*cis*-RA for HsRXRα, NlRXRa and NlRXRb. The results are expressed as average fold activation after normalization to Renilla (means ± S.E.; at least n = 3). Significant differences were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples ($P < 0.05$): (a) Human RXRα, (b) NlRXRa and (c) NlRXRb response is significant different from control vehicle.

Figures 8.3 and 8.4 display the luciferase reporter gene transactivation results after COS-1 cells exposure to TBT and TPT, respectively. The NlRXRa isoform were able to induce the luciferase activity when exposed to TBT and TPT (Fig. 8.3). In contrast for NlRXRb a target gene transcription repression was obtained (Fig. 8.4). The activation response of NlRXRa to TBT was two-fold higher compared to TPT for the same tested concentrations (Fig. 8.3 and 8.4). Also, the 0.01 µM TBT treatment led to a similar transactivation as a 0.1 µM of TPT (Fig. 8.3 and 8.4). Moreover, for NlRXRa a significant gene transcription activation is already observed for TBT at 0.01 µM, whereas a significant gene transcription repression is observed at 0.01 µM for TPT (Fig. 8.3 and 8.4). In contrast, Urushitani et al. (2011) reported for *T. clavigera* RXR-1 isoform that following exposure to TPT and TBT transactional activity responses were very similar at the concentration of 0.1 µM.

The TBT or TPT chosen concentrations of 0.01 and 0.1 µM that induced transcriptional activation of NlRXRa isoform are considered to be nearly pharmacologically relevant, reported to induce imposex in gastropods (Nishikawa, 2006).

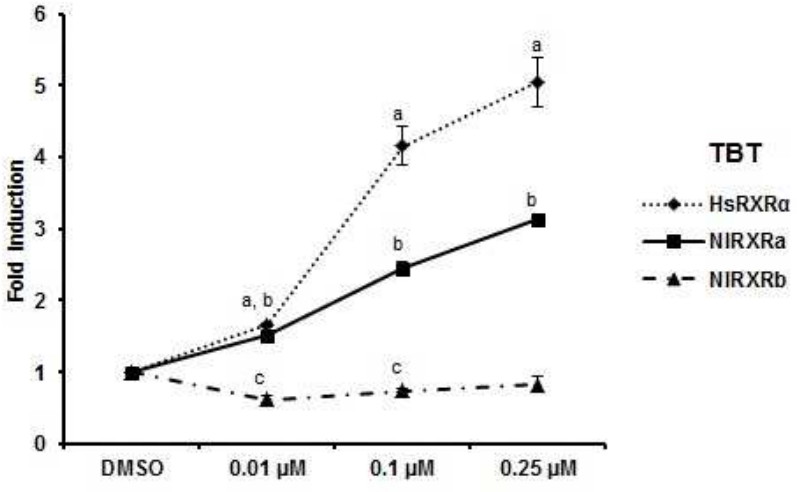

Figure 8.3: Transactivation activity of luciferase reporter gene mediated by GAL4 DBD-RXR LBD fusion constructs in COS-1 cells in the presence of TBT for HsRXRα, NlRXRa and NlRXRb. The results are expressed as average fold activation after normalization to Renilla (means ± S.E.; at least n = 3). Significant differences were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples (P < 0.05): (a) Human RXRα, (b) NlRXRa and (c) NlRXRb response is significant different from control vehicle.

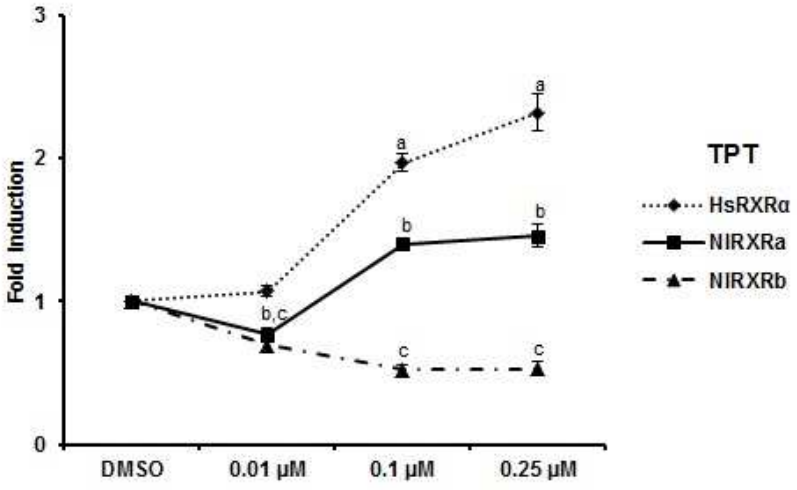

Figure 8.4: Transactivation activity of luciferase reporter gene mediated by GAL4 DBD-RXR LBD fusion constructs in COS-1 cells in the presence of TPT for HsRXRα, NlRXRa and NlRXRb. The results are expressed as average fold activation after normalization to Renilla (means ± S.E.; at least n = 3). Significant differences were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples (P < 0.05): (a) Human RXRα, (b) NlRXRa and (c) NlRXRb response is significant different from control vehicle.

In figure 8.5 are presented the results of human RXRα, NlRXRa and b following exposure to the RXR synthetic agonist HX630. Human RXRα, and both *N. lapillus* RXR isoforms were capable of transactivation reporter gene. Though, for NlRXRa fold transactivation induction were higher compared to the isoform b and the human RXRα.

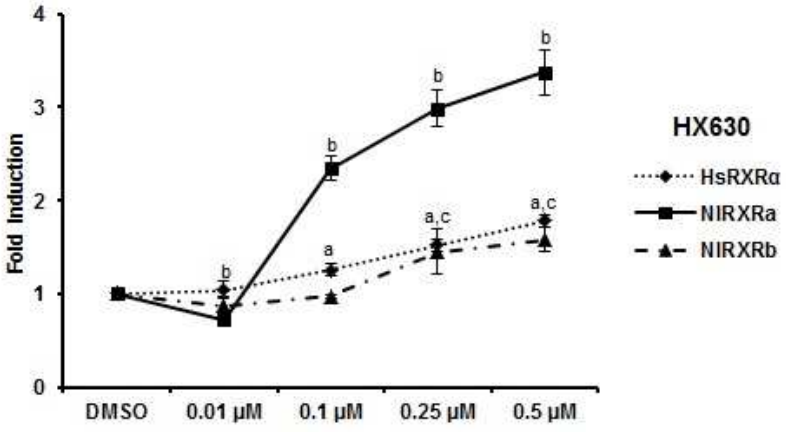

Figure 8.5: Transactivation activity of luciferase reporter gene mediated by GAL4 DBD-RXR LBD fusion constructs in COS-1 cells in the presence of a RXR selective agonist the HX630 for HsRXRα, NlRXRa and NlRXRb. The results are expressed as average fold activation after normalization to Renilla (means ± S.E.; at least n = 3). Significant differences were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples ($P < 0.05$): (a) Human RXRα, (b) NlRXRa and (c) NlRXRb response is significant different from control vehicle.

A previous study showed that methoprene acid is also able to induce the initial stages of imposex in *N. lapillus*, although at a 16-fold higher concentration than TBT (Castro et al., 2007). Hence, for a comparison purpose we selected for this putative RXR agonist five concentrations: three are identical to those used for organotin compounds (0.01, 0.1 and 0.25 µM) and two additional concentrations, 10 and 50 µM. No transactivation response by MA occurred for both NlRXR isoforms and human RXRα at 0.01-10 µM (Fig. 8.6). A significant repression has been observed at the 10 µM used concentration for the NlRXRb (Fig. 8.6). A significant transcriptional activity was only observed for all the three RXR at 50 µM. At this concentration the NlRXRa transactivation activity were 4.5 fold in comparison with the control.

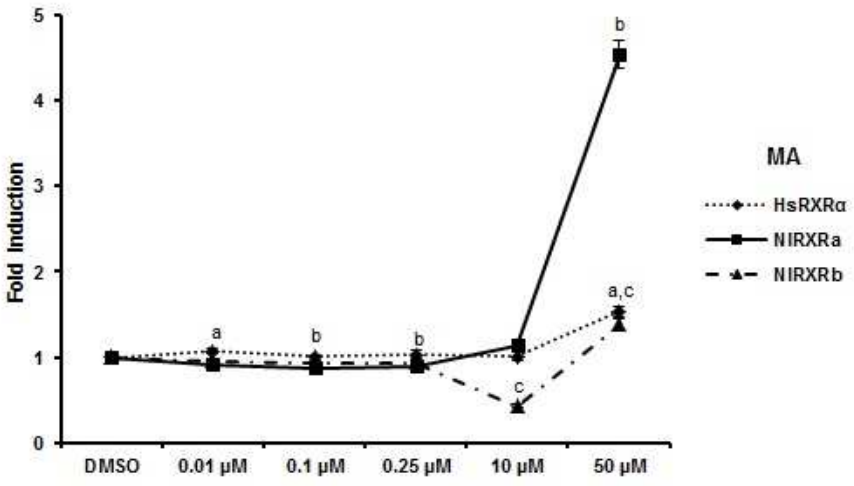

Figure 8.6: Transactivation activity of luciferase reporter gene mediated by GAL4 DBD-RXR LBD fusion constructs in COS-1 cells in the presence of a methoprene acid for HsRXRα, NlRXRa and NlRXRb. The results are expressed as average fold activation after normalization to Renilla (means ± S.E.; at least n = 3). Significant differences were obtain using Kruskal–Wallis one-way ANOVA Test K for independent samples ($P < 0.05$): (a) Human RXRα, (b) NlRXRa and (c) NlRXRb response is significant different from control vehicle.

8.4.3 TPT Ligand binding assay

The LBD of TcRXR-1 and NlRXRa protein was expressed in *E. coli* as a fusion with GST and tested the binding ability to TPT. As results, we found that NlRXRa binds to TPT although less efficiently than TcRXR-1 (Fig. 8.7). Scatchard analysis of the binding of [^{14}C]-TPT to NlRXRa yielded a Kd values of 369 nM (Fig. 8.7D), whereas TcRXR yielded a Kd values of 125 nM (Fig. 8.7C).

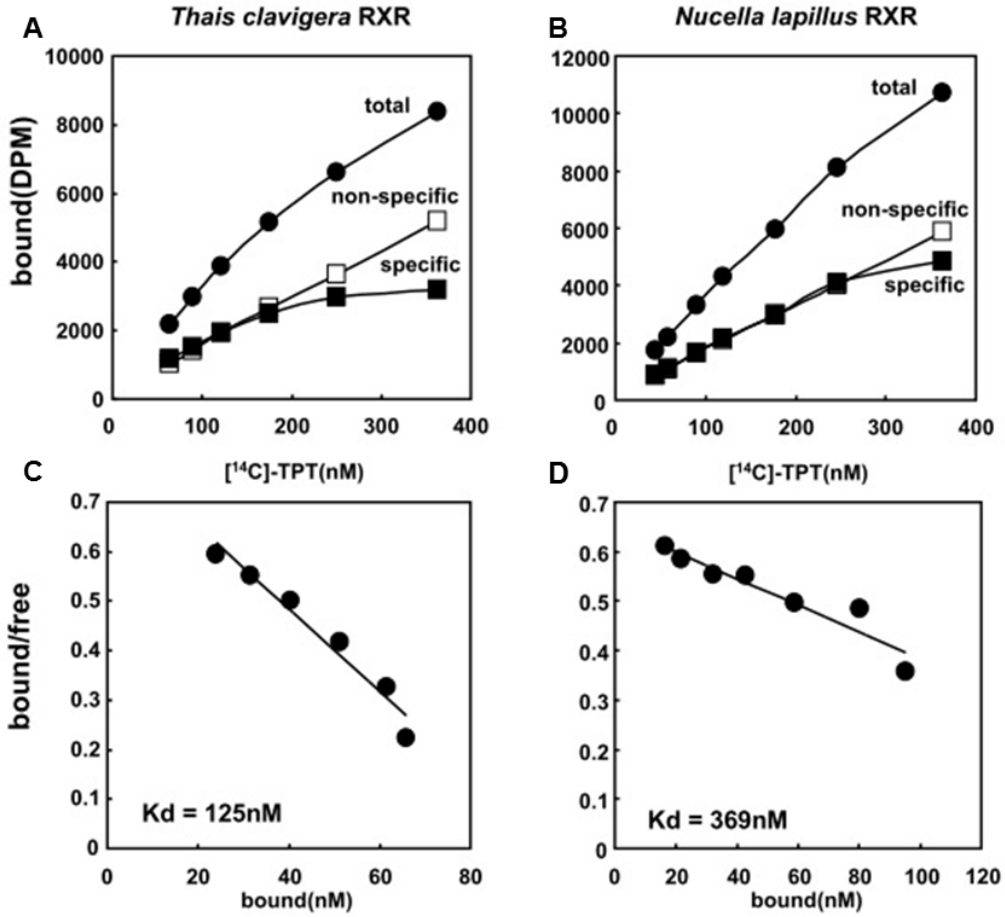

Figure 8.7: The LBD of *N. lapillus* RXRa and *T. clavigera* RXR-1 expressed in *E. coli* was incubated with increasing concentrations of [^{14}C]- TPT in the absence (total binding) or presence of 400-fold non-labeled TPT (nonspecific binding): (A) *T. clavigera* and (B) *N. lapillus* RXR nonspecific binding was subtracted from total binding and plotted as specific binding. Scatchard analysis, specific TPT binding to gastropods RXR was transformed by Scatchard analysis and plotted (C) *T. clavigera* linear regression yielded Kd = 125 nM and (D) *N. lapillus* linear regression yielded Kd = 369 nM. *T. clavigera* and *N. lapillus* RXR sequence used for binding assay was retrieved from Nishikawa et al. (2004) and Castro et al. (2007), respectively.

8.5 Discussion

The molecular mechanism behind imposex development is still not fully understood (Lima et al., 2011; Abidli et al., 2013). Though, in the last decade important evidences have emerged, establishing a crucial role of the RXR-dependent signaling cascade on the imposex development on female gastropods. It is now largely accepted that TBT and TPT have the ability to mimic the action of the putative natural ligand of RXR, the 9-*cis*-RA, and inappropriately modulate their signaling cascade (Nishikawa et al., 2004; Castro et al., 2007; Lima et al., 2011; Abidli et al., 2013). It has been shown that like 9-*cis*-RA, TBT and TPT are high-affinity ligands for human RXRα and *T. clavigera*

(Nishikawa et al., 2004; Kanayama et al., 2005). Also, *in vivo* injection of 9-*cis*-RA induces imposex development in females of *N. lapillus* and *T. clavigera* species as well as the TBT and/or TPT, when administered at similar concentrations (Nishikawa et al., 2004; Castro et al., 2007; Horiguchi et al., 2008). Additionally, selective RXR agonist, MA and HX630 also induced female *N. lapillus* masculinization (Castro et al., 2007; Stange et al., 2012). More recently, a study revealed that *T. clavigera* RXR isoforms has the ability to activate transcription of a reporter gene in the presence of 9-*cis*-RA, TBT and TPT (Urushitani et al., 2011).

Even though the link between imposex induction under organotins exposure and RXR is well established, it is still not clear why phylogenetically close gastropod species show different sensitivity towards TPT (Horiguchi et al., 1997; Santos et al., 2000; Schulte-Oehlmann et al., 2000; Wang et al., 2010). *T. clavigera* develops imposex after injection or exposure to TPT at the same level as TBT (Horiguchi et al., 1997). In contrast, TPT induces very mild levels of imposex in female *N. lapillus*, particularly a penis growth, after *in vivo* injection to high TPT levels (Laranjeiro et al., *in press*); whereas in studies involving exposure at environmentally relevant TPT levels, no imposex could be observed (Bryan et al., 1988; Schulte-Oehlmann et al., 2000). Given that RXR has a central role in mediating organotin endocrine effects, we hypothesize that differences in binding affinity, between the receptor in the two gastropod species, should be the key feature that determines species-sensitivity to TPT.

In the present study we provided more evidences that reinforce the major role of RXR-dependent signaling pathways in imposex induction by evaluating dogwelk *N. lapillus* RXR isoforms responsiveness to putative natural ligand 9-*cis*-RA, TBT, TPT, MA and HX630 by using luciferase reporter gene assays. We also evaluated through an *in vitro* ligand binding assay the affinity of TPT for *N. lapillus* and *T. clavigera* RXR, in order to get insights towards different species-sensitivity to the compound.

We first evaluated the ability of NlRXR isoforms to activated target gene transcription in the presence of 9-*cis*-RA. Both NlRXR isoforms showed transcriptional activation response in the presence of 9-*cis*-RA. Similar transactivation response to 9-*cis*-RA has also been reported for mollusks *B. glabrata* and *T. clavigera* RXR isomers in mammalian cell lines (Bouton et al., 2005; Urushitani et al., 2011). We could observe significant induction of transcriptional activity by NlRXR isoforms in the presence of 1 µM of 9-*cis*-RA, although lower in the case of NlRXRb (Fig. 8.2). Urushitani et al. (2011) also reported a different transcriptional activity response by 9-*cis*-RA between TcRXR isoforms. The authors reported a rock shell RXR-2 transcriptional activity lower than that of RXR-1. NlRXRa isoform obtain data is in agreement with previously published work that reports the ability of receptor to bind and activate the transcription of the luciferase

reporter in the presence of 9-*cis*-RA (Castro et al., 2007; Gutierrez-Mazariegos et al., 2014). It became recently clear that the 9-*cis*-RA, might be in fact a natural ligand for imposex-susceptible gastropod RXRs (Gesto et al., 2013). For *N. lapillus*, the retinoic acid isomer has been detected in several tissues and its biosynthesis has been demonstrated *in vivo* (Gesto et al., 2013).

Regarding exposure to TBT, different luciferase reporter gene responses were obtained for NlRXR isoforms. The NlRXRa isoform were able to induce the luciferase activity, whereas NlRXRb a target gene transcription repression was obtained when exposed to TBT (Fig. 8.3). RXR signaling cascade also seems to respond to TBT differently in *N. lapillus* and *T. clavigera*. TBT, at similar environmentally relevant levels, significantly induced imposex in both *N. lapillus* and *T. clavigera* after via water and/or *in vivo* injections (Horiguchi et al., 1997; Nishikawa et al., 2004; Castro et al., 2007; Horiguchi et al., 2008; Lima et al., 2011). Yet, at a same TBT concentration of 0.1 µM, transactivation response on *T. clavigera* were ~2 fold higher that the response we observed for NlRXR isoform a (Fig. 8.3; Urushitani et al., 2011). This data suggests that differences in target gene transcription response between the two gastropods might be due to different binding affinity for TBT.

For TPT we first analyzed the RXR isoforms ability to mediate target gene transcription. Similar to TBT, the two dogwhelk RXR isoforms responded differently to TPT. NlRXRa had significant transactivation response activation, whereas NlRXRb had a significant response repression (Fig. 8.4). Importantly, TPT requires a concentration 10-fold higher than TBT to induce a similar transactivation response (Fig. 8.3 and 8.4). In contrast for *T. clavigera* RXR-1 isoform it has been reported a similar transcriptional activity induction when exposed to TPT and TBT at an identical concentration (Urushitani et al., 2011). The NlRXR isoforms transactivation responses for TPT at 0.1 and 0.25 µM are similar. The results show that TPT at 0.25 µM reaches a “plateau” in transactivation (Fig. 8.4). Next, to determine if NlRXRa can bind to TPT we performed an *in vitro* analysis. The analysis was also performed for *T. claivera* RXR-1 isoform for comparison (Fig. 8.7). The aim was to evaluate if differences of affinity between the two gastropod’s RXR is related to species-specific sensitivity to TPT. The Kd values of TPT for NlRXRa are ~29 fold higher than those observed for 9-*cis*-RA (Castro et al., 2007), whereas for TcRXR-1 were only ~8 fold (Nishikawa et al., 2004). Our results indicate that NlRXRa LBD indeed binds to TPT although at a lower level compared to TcRXR-1, and with low affinity compared to the putative natural ligand, 9-*cis*-RA (Castro et al., 2007). This different affinity might explain species-specific sensitivity to TPT and the ability to promote female masculinization. Altogether, these findings suggested a common disrupting molecular

signaling mediated pathway through RXR that can lead to different intensities of imposex induction according to the compound binding affinity and transcriptional activity.

Our data suggest that TBT has a higher ligand affinity than TPT for NlRXRa. In future studies it will be also important to explore TBT binding affinity to get further evidences to understand the differences observed between the transactivation results in the presence of the two organotin compounds. In the case of *N. lapillus* we expect that RXRs should have less affinity for TPT compared to TBT.

The incidence of imposex in female gastropods has been strongly associated with TBT and/or TPT accumulation on tissues (Shim et al., 2000; Oliveira et al., 2009; Horiguchi et al., 2010; Wang et al., 2010). Moreover, changes on RXR gene expression in females gastropod tissues has also been linked and positive correlated to organotins exposure and/or tissue accumulation (Horiguchi et al., 2010; Lima et al., 2011). For instance, TPT is able to accumulate in tissues of exposed *T. clavigera* females (~1200 ng/g wet weight) leading to a significantly increased expression of the RXR gene in the penis/penis-forming area (PFA) and head ganglia, which resulted in induction of imposex (Horiguchi et al., 2010). TBT-exposed *N. lapillus* females also experience changes in the NR gene expression in a tissue specific manner (Lima et al., 2011). A significant RXR gene down-regulation was observed in females both before and after imposex initiation, in the head ganglia. Before imposex development, in the penis/PFA no differences in transcription of RXR gene between control and TBT exposed female snails were observed. Only after imposex induction a significant increased of gene transcription was observed (Lima et al., 2011). *N. lapillus* also accumulates TPT in their tissues (Oliveira et al., 2009). So, it should be interesting in future studies evaluate if the accumulation of TPT in tissues also alters RXR gene expression levels.

HX630 is a specific and selective RXR agonist (Umemiya et al., 1997). Recently, it has been showed that HX630 when injected at a TBT similar concentration (1 µg/g wet weight soft tissue) induced imposex development in *N. lapillus*. Furthermore, the RXR antagonist HX531 was able to suppress HX630 induced imposex, supporting the involvement of RXR mediated pathway in female masculinization development (Stange et al., 2012). Our data confirmed the *N. lapillus* RXR isoforms ability to efficiently induced reporter gene transactivation by to RXR synthetic agonist HX630 (Fig. 8.5).

Transcriptional activity activation of NlRXR isoforms was also obtained with MA albeit at high concentration (Fig. 8.6). MA promotes *N. lapillus* imposex, although to lower degree than that observed for TBT, and at higher concentrations, supporting the results of the transactivation assays (Castro et al., 2007). The compound is a selective ligand for RXRs that binds and is capable of transcriptional activation in both insect and mammalian cells (Harmon et al., 1995). Although, in mammalian cells only activates RXR at much

higher concentrations (10^{-4} M). Here, we used a concentration of MA that was lower than that required for the induction of higher transcriptional activity of RXR in mammals (Harmon et al., 1995). In addition, for other gastropod species *B. glabrata* it was demonstrated *in vitro* that MA binds to RXR, but the binding is not with high affinity since higher concentrations are required (Bouton et al., 2005). This lower affinity might explain our transactivation results for NlRXR isoforms.

NlRXRb transactivation activity was repressed in the presence of TBT and TPT The biological relevance of this finding in the context of imposex development is unknown but deserves further investigation. Also, responses to the presence of 9-*cis*-RA, MA and HX630 were lower compared to the NlRXRa isoform. Crystallographic and mutagenic studies has identified 18 key amino acid residues (see Fig. 8.1) within the ligand binding pocket of the human RXRα LBD critical for binding and transactivation response to 9-*cis*-RA (Egea et al., 2000, 2002; Harada et al., 2015; Hiromori et al., 2015). These amino acids are fully conserved in NlRXR isoform as well as in other mollusks such as *B. glabrata* RXR and *T. clavigera* RXR isoforms (de Groot et al., 2005). The key amino residue cysteine known to interact with organotin compounds is also conserved in the two isoform sequence (le Maire et al., 2009; Hiromori et al., 2015). The difference between NlRXR isoforms only lies in one amino acid substitution in the LBD: NlRXRb Gly233 whereas NlRXRa has a Glu228; also differing within five amino acid residues insertion in the DBD, similar to the reported in *T. clavigera* RXR isoform 1 and 2 (Urushitani et al., 2011). *T. clavigera*, RXR-2 has a lower transcriptional activity in the presence of 9-*cis*-RA. Urushitani et al. (2011), suggests that the lower activity might be caused by the insertion of the five amino acids in the T-box since is the only observed difference between RXR isoforms. The T-box, is a small motif from the DBD, that plays an important role mediating hormone response element binding interactions with RXR homodimers complexes in Direct repeats (DR1) 1; and for the formation of heterodimers when RXR occupies the site downstream of DR-1 (Zechel et al., 1994; Zhao et al., 2000). Recently it has been shown that NlRXRa is able to bind as a homodimer to DR-1 elements, whereas in heterodimer with partner retinoic acid receptor (RAR) is able to bind specific sequences of DNA to DR-1, DR-2, or DR-5 elements (Gutierrez-Mazariegos et al., 2014). In contrast, as a homodimer or in heterodimer with NlRAR, the NlRXRb isoform shows impaired DNA binding (Gutierrez-Mazariegos et al., 2014). RXR isoform with a five-amino acid insertion/deletion located in the T-box has also been reported in the fiddler crab *Uca pugilator*, which has also a different ability to bind to specific DNA responsive elements in both homo- and heterodimer (Wu et al., 2004). These findings might explain the lower and repressed luciferase transcriptional activation determined. Castro et al. (2007) reported that NlRXRb has lower tissue transcriptional expression compared to NlRXRa isoform. All

together the finding suggests that different isoforms of RXR in mollusks have different transcriptional activities and probably regulate different biological functions.

8.6 Conclusion

Overall, the results of the present study with the imposex-susceptible mollusks *N. lapillus* contributes to our understanding of the importance of RXR-dependent signaling cascade in gastropods imposex induction, clearly supporting the assumption that is a major molecular target of organotins. Our results also highlight that TPT can act as an endocrine disrupter in *N. lapillus* through RXR signaling pathway. The findings reported here support the hypothesis that the low imposex-induction capability of TPT in *N. lapillus* female is associated to the lower binding affinity to RXR. We also found that transcription regulation activities of *N. lapillus* RXRa and b isoforms differed from each other, suggesting that difference regarding its physiological functions in the regulation of the gastropod endocrine system might exist.

8.7 Acknowledgements

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CHAPTER 9

9 General discussion

9.1 Background

With the increasing number of studies focusing on the characterization of retinoid cascade across metazoans, i.e., detection of endogenous retinoids, isolation and functional characterization of the metabolic and signaling modules, further evidences points to an early emergence of these pathway in metazoan evolution (e.g. Albalat, 2009; Theodosiou et al., 2010; André et al., 2014). Available results suggest that these pathways were already present in the last common ancestor of all bilateria (André et al., 2014). In fact, some of the components of retinoid pathway were described from porifera to chordates (André et al., 2014). However, experimental determination of the precise role of the majority of retinoic metabolic and signaling molecular components is missing for most invertebrates lineages. Yet, the presence of a molecular component orthologue by itself does not indicate a similar physiological role as described for vertebrates, and such lack of information limits our understanding of the evolutionary origin of retinoid pathway. In the present thesis we approach this issue. We aimed to get new insights regarding retinoid system evolutionary origin outside vertebrates, focusing in particular in lophotrochozoans. Hence, we focus our attention in the isolation and functional characterization of key molecular components of the retinoid pathway. In this thesis we provide one of the most detail efforts in the cloning and functional characterization of putative key molecular components involved in retinoid cascade in marine lophotrochozoans (Fig. 9.1). In addition, we have also approach the possible involvement of retinoid pathways in the regulation of a physiological process in mollusks: the gametogenesis.

The lack of information regarding the presence of an active retinoid pathway on lophotrochozoans also hampers our knowledge regarding the putative impact of endocrine disrupting chemicals known to cause adverse effects on some vertebrate *taxa* by modulating the retinoid cascade. Here we also investigate the modulation of the retinoid system by EDCs, focusing mostly in the signaling cascade in lophotrochozoan.

9.2 Retinoid metabolic cascade evolution in lophotrochozoans

Animals can obtain retinoic acid precursors by intake of pro-vitamin A carotenoids rich food. The β-carotene (βc) is the carotenoid most common in the diet (Theodosiou et al., 2010). Two major βc metabolic oxidative cleavage routes are known to exist in metazoans: 1) βc central cleavage by BCO-I, the main and “classical” path since it gives rise to retinal a precursor for other active and non-active retinoids; and 2) BCO-II eccentric βc cleavage, considered to be a part of an alternative cascade leading to retinoic acid

(RA) isomers synthesis (Simões-Costa et al., 2008). It was suggested that this last route might represent the most ancient pathway in RA synthesis, that should be present in those animals for which the “classical” cascade is absent; but like vertebrates other metazoans can also have both routes (Simões-Costa et al., 2008; Theodosiou et al., 2010). It has also been proposed that in protostomes BCO enzyme might have the ability to performed more than one role at the level of retinoid metabolism (Simões-Costa et al., 2008; Albalat, 2009). At least this seems to be a true scenario for insects (Oberhauser et al., 2008). A BCO orthologue from *Drosophila melanogaster* and *Galleria mellonella* symmetric cleaved βc into all-t*rans*-retinal and subsequent can converted it to 11-*cis*-retinal (Oberhauser et al., 2008). For lophotrochozoans no functional characterization or isolation of a BCO had been performed before. In Chapter 2, we presented the first attempt to characterize a BCO like enzyme in lophotrochozoans. For the annelid *Platynereis dumerilii* we were able to successfully isolate the entire open reading frame (ORF) of a BCO-like gene (Chapter 2). Taking in consideration our preliminary functional test we believe that the enzyme resembles the BCO-II like function. We demonstrate that the *P. dumerilii* BCO enzyme is able to use βc as a substrate. No retinal production could be detected suggesting that the enzyme might have metabolized the βc into β-apocarotenal instead.

In vertebrates, the major enzyme involved in retinoid storage is lecithin retinol acyltransferase (LRAT). Yet, other enzymes with acyl CoA: retinol acyltransferase (ARAT) activity such as diacylglycerol O-acyltransferase 1 (DGAT1) must have a role in this process (Albalat et al., 2011). LRAT orthologues are absent from chordates (cephalochordates and tunicates) to cnidarian genomes (Fig. 9.1). However, DGAT1 is present (Fig. 9.1) and represents the most likely candidate for this role, being proposed to be the ancestor route at least in chordates (Albalat et al., 2011; André et al., 2014). In Chapter 3 we reported the presence of this enzyme orthologue in *P. dumerilii* and *Patella depressa*, with both proteins displaying conserved amino acid required for functionality. To assess its possible role during retinol esterification we evaluated its gene expression in selected tissues on non- and injected retinol *P. depressa* specimens in both genders. *Dgat1* gene expression levels are kept unchanged on treated groups compared to controls, for both sexes and analyzed tissues (gonads and digestive gland), which does not give further support to the involvement of this enzyme in retinol esterification. Previously, in the gastropod *Osilinus lineatus* it has been demonstrated that the esterification cascade must be present and active. After 48 h of retinol injection high levels of retinyl ester could be detected (Gesto et al., 2012). Later, in *P. depressa*, Gesto et al., (2013) detected the presence of endogenous levels of retinol (as free and total) and also retinyl esters (though only in males), proposing that a metabolic pathway for retinoid

metabolism must be presence. The results suggested that retinyl esters synthesis by retinol esterification could be mediated by a still unidentified enzyme. Yet, future functional characterization of the isolated DGAT1 will clarify its role in protostomes.

The aldehyde dehydrogenases 1 (ALDH1s) is the enzyme in vertebrates that plays a major role in metabolizing the irreversible conversion of retinal into RA (Theodosiou et al., 2010). In Chapter 4, we were able to retrieve two isoforms for both *P. dumerilii* (ALDH1b and c) and *P. depressa* (ALDH1b and c). Sequence analysis revealed conservation of key residues characteristic of ALDHs superfamily members relevant for enzymatic biochemical activity, for the catalysis, co-factor and substrate binding (Sobreira et al., 2011). The key amino acid in the subtract entry channel (SEC) for all the isolated ALDH1s resemble residues typical of an ALDH2 instead of an ALDH1 (Sobreira et al., 2011). The functional characterization confirms the enzyme inability to use retinal isomers as a substrate for RA synthesis. It cannot be excluded the existence of a third ALDH1 that can display this role, or alternatively, another still unidentified enzyme might catalyze this conversion.

Overall, our data suggests that the annelid *P. dumerilii* seems to have retained a route for direct biosynthesis of retinoid active form not requiring retinal as an intermediate precursor: 1) it holds a functional BCO orthologue, apparently from type 2 since it is able to catalyzed βc with no retinal formation indicative that β-apocarotenal might have been produced. 2) *In vitro* functional studies with the isolated ALDH1s indicates that this protein is unable to use retinal isomers for RA synthesis; and ALDH1s presence in *Capitella* sp genome also seems to have other role than RA synthesis according to the SEC amino acid residues signature (Chapter 4). In the case of mollusks the existence of a functional pathway for retinoid biosynthesis vertebrate-like still needs further evidences (Chapter 3 and 4; Coelho et al., 2012). Recent *in vivo* data strongly supports the existence of active metabolic route for retinoid biosynthesis and storage in mollusks (Gesto et al., 2012, 2013; Chapter 5). Our data suggests that the enzymes involved in such cascade for mollusks might not be the same as described for vertebrate *taxa*: 1) after 48 h of retinol *in vivo* injection in *P. depressa* individuals, *Dgat1* gene expression were found to be constant between treatment groups (Chapter 3); 2) The alcohol dehydrogenases (ADHs) are a group of enzymes that catalyzes the first sept of RA biosynthesis: the reversible oxidation of retinol into retinal. While vertebrates have at least three ADHs (e.g. ADH1, 3 and 4) protostomes have a single orthologue, the ADH3 (Theodosiou et al., 2010). for *N. lapillus*, the expression pattern of *Adh3* after retinol injections do not reflect a true role on the retinoid routes as well is high expression on the digestive tract (Coelho et al., 2012); and 3) in addition *P. depressa* ALDH1s orthologues *in vitro* do not catalyzed the conversion of RAL isomers to RA (Chapter 4).

Taking into consideration the findings presented here, it is clear that for lophotrochozoans it remains uncertain what enzymes might play a role on retinoid metabolism (Fig. 9.1). It has been proposed that initially the ALDHs and ADHs enzymes had other metabolic roles (i.e. participate in metabolism of aldehydes, nitric oxide and xenobiotic compounds) and only during evolution might have acquired in vertebrates lineages a function on retinoid biosynthesis (Cañestro et al., 2003; Simões-Costa et al., 2008; Albalat, 2009; Sobreira et al., 2011). Our results seem to support this assumption, but further work needs to be performed to evaluate the actual role of these enzymes. For vertebrates, in addition to ALDH1s and ADHs enzymes, it has been shown that some cytochrome P450 (CPY) family members are able to catalyze the oxidations of retinol to retinal and of retinal to RA (Duester, 1996; Campo-Paysaa et al., 2008; Albalat, 2009), although it's actual role on retinoid biosynthesis remains to be determined (Campo-Paysaa et al., 2008; Albalat, 2009). For invertebrates alternative routes might exist. Future studies should address this issue. In vertebrate's short-chain dehydrogenase/reductase (SDR) such as retinol dehydrogenase (RDH) are involved in RA biosynthesis representing a rate-limiting step on the molecule production (Theodosiou et al., 2010). SDRs orthologues have been reported in different invertebrate *taxa* including lophotrochozoans and might be involved in RA synthesis but the function of such enzymes remains to be determined (Albalat, 2009; Theodosiou et al., 2010). In the case of DGAT1 further functional studies should be carried out *in vitro* and/or *in vivo* through protein purification and cell-based biochemical assays for both *P. dumerilii* and *P. depressa*, in order to clarify its involvement in retinoids storage. Also, like vertebrates, other unknown ARAT enzymes might exist performing the retinol esterification and storage. We cannot exclude the possibility that DGAT1 could have a role in the triacylglycerol metabolic cascade instated (André et al., 2014).

9.3 Retinoid homeostasis and reproduction in metazoans: vertebrates and invertebrates insights

In vertebrates, retinoids have essential roles in many physiological processes such as vision, immune system regulation, reproduction and embryonic development (Campo-Paysaa et al., 2008; Theodosiou et al., 2010). Because of their crucial role, their levels require a tightly balance and signaling (Theodosiou et al., 2010). Outside vertebrates some of the key molecular components implicated in retinoid homeostasis have not been described to date (Theodosiou et al., 2010; André et al., 2014). Though, endogenous retinoid content has been described in invertebrates indicating that metabolic routes are present (e.g. Gesto et al., 2012, Gesto et al., 2013), but the ability to maintain a retinoid strictly balance remains unknown for most metazoans (André et al., 2014). Hence,

contrasting with vertebrates, invertebrate evidences for retinoid functional roles are scarcer. Still, it seems that some physiological processes appear to be conserved (Campo-Paysaa et al., 2008; Theodosiou et al., 2010). For instance, in vertebrates, RA has a prominent role in determining sex-specific timing of meiosis entry on developing gonads and/or gametogenesis; and represents a clearly example of how tightly level of retinoids in cells relies on the balance between its metabolism and signaling routes (Anderson et al., 2008; Bowles et al., 2006; Bowles and Koopman, 2007). Studies showed that during mouse embryogenesis, regulation of RA signaling levels by CYP26 oxidative degradation route controls the sex-specific initiation of germ cell meiosis (Rodríguez-Marí et al., 2013). For instance, the up-regulation of *Cyp26b1* expression during male embryo testis development leads to RA degradation, thus preventing germ cells from initiating meiosis; whereas in female embryos, RA stimulates germ cells to enter into meiosis in ovaries due to a down-regulation of *Cyp26b1* expression (Rodríguez-Marí et al., 2013). In anurans and mammals it has been shown that inhibition of either RA synthesis by ALDH1a2 or RA-signaling mediated by retinoic acid receptor (RAR) prevented meiotic entry in larval or embryo gonads in both genders (Griswold et al., 2012; Piprek et al., 2013).

A similar role can be conserved in mollusks. Sternberg et al. (2008) showed that retinoid X receptor (RXR) gene expression levels increased during gonads recrudescence phase in both genders of *Ilyanassa obsoleta*, but the receptor expression over time differed between male and female. Additionally, in the gastropod *O. lineatus* endogenous retinoid levels were determined on specimens at different gonadal developing stages. In male snails nonpolar retinoids (retinol and retinyl ester) increased with the gonad maturation and decreased during spawning (Gesto et al., 2012). In contrast, in females, nonpolar retinoids were not detected in any gonad maturation phase (Gesto et al., 2012) although an active metabolic storage and synthesis path seems to be present in both genders. After *in vivo* injection with retinol and retinal both active and nonpolar retinoids could be detected in both sexes, but with higher levels detected in males (Gesto et al., 2012). Taken together, these evidences support a role of RXR and retinoid metabolism in the gonads development of mollusks with a marked difference between genders.

In the Chapter 5 we provide additional evidences for a function of retinoids in gametogenesis regulation of mollusks. We have addressed the involvement of the retinoid metabolic and signaling cascade during gonad maturation cycle in the gastropods *Patella vulgata*. We found that RA isomers endogenous levels in gonads differ between mature and resting stages, with significantly higher concentration detected in the latter. Interesting, higher concentration of total RA isomers were found in mature males compared to mature female gonads. Regarding nonpolar retinoids, total retinol and retinyl

palmitate levels were found to be higher in males and ripe animals that in females. The differences observed on retinoid content between genders and gonad developmental stages could reflect different progression timing during gametogenesis cycle suggesting a specific role of retinoids in mollusk reproduction. To further support the involvement of retinoid system on *P. vulgata* gametogenesis, we evaluated the transcription levels of RXR and RAR, during the gonad cycle development. We could not find significant differences in the expression of *P. vulgata* retinoid receptors between mature male and female, and ripe limpets. In addition, in Chapter 6 we demonstrate that like in other gastropods species, *P. vulgata* RAR is unresponsive to retinoids as a monomer. This might explain the stable RAR expression in mature and immature animals. Taken together, these evidences, suggest that retinoid homeostatic balance might exist and is regulated by a metabolic pathway along mollusks gametogenesis.

9.4 Retinoid X receptors and retinoic acid receptors isolation and functional characterization in lophotrochozoans

Retinoid X receptor have a more widespread phylogenetic distribution compared to RAR (Fig. 9.1), and is considered one of the most basal components of retinoid signaling systems (Kostrouch et al., 1998; Thornton et al., 2003; Simões-Costa et al., 2008). RXR orthologues have been genome predicted or cloned, and functional characterized in metazoans from cnidarians to protostomes lineages; have arisen during evolution before the establishment of bilateral symmetry (Thornton, 2003; Theodosiou et al., 2010). Available data points to a mostly conserved binding profile to putative natural ligands 9-*cis*-RA across metazoans (Castro and Santos et al., 2014; Gutierrez-Mazariegos et al., 2014b). Though, it seems that during evolution in some metazoan groups such as insects and crustaceans (ecdysozoan) RXR lost the ability to bind 9-*cis*-RA and have acquired affinity for other signaling molecules (Gutierrez-Mazariegos et al., 2014b), although, functional characterization is still missing for several *taxa*. For instance, before the present work no isolation and functional characterization of a RXR orthologue in an annelid species had been performed. Only a single RXR variant orthologue was genome predicted in *Capitella sp.* (Albalat and Cañestro, 2009). In Chapter 7 we described the isolation and functional characterization of a RXR orthologue from *P. dumerilii*. The obtained sequence suggests RXR binding ability to the putative natural ligand 9-*cis*-RA, because key residues in the ligand binding domain are conserved (Egea et al., 2000). Additionally, we showed that *P. dumerilii* RXR is able to induce target gene transcription activation in response to 9-*cis*-RA. Though, in the future it will be important to evaluate the binding ability and affinity to 9-*cis*-RA. Further studies are also needed to characterize the physiological role of retinoid pathways in this group. In Chapter 6 we also provide further

evidences that support the idea that in mollusks the receptor kept a vertebrate-like response for retinoids. *Crassostrea gigas* and *P. vulgata* RXR activated target gene transcription in the presence of all-*trans*-RA. 9-*cis*-RA is the putative *bone fine* ligand for RXR receptors. Yet, RXR has lower affinity for all-*trans*-RA (Levin et al., 1992). We hypothesize that during our experiment all-*trans*-RA isomerization to 9-*cis*-RA must have occurred (Levin et al., 1992). These studies support that the ability of RXR to be responsive to retinoids is conserved along lophotrochozoan lineages.

Retinoid acid receptor is one of the NR members that initially were believed to be a chordate/vertebrate innovation (Bertrand et al., 2004; Campo-Paysaa et al., 2008; Albalat and Cañestro, 2009). Though, with the genome sequencing of many protostomes it become clear that has a more basal origin, being already present in Urbilateria, the ancestor of all bilaterian (Bertrand et al., 2004). The presence of RAR orthologues has been genome predicted and/or isolated in from deuterostomes to protostomes (lophotrochozoan) invertebrate *taxa* (Campo-Paysaa et al., 2008; Gutierrez-Mazariegos et al., 2014b; Fig. 9.1). Yet, specific gene loss seems to have occurred in cnidarian and ecdysozoan lineages (Campo-Paysaa et al., 2008; Albalat and Cañestro, 2009). Still, information regarding its function remains limited. Most advances have been made for chordate invertebrates, such as the tunicates and cephalochordates that show the ability to bind and activate target gene transcription in the presence of RA isomers (Gutierrez-Mazariegos et al., 2014b). RAR orthologues from *Lottia gigantea* and *C. teleta* display the conservation of some residues in the ligand binding doming identical to human RARs and other chordates (Campo-Paysaa et al., 2008). Yet, no experimental evidences were provided to confirm the formulated assumption. Only recently the first's evidences have emerged. For two mollusks gastropod, *Thais clavigera* and *N. lapillus*, it has been demonstrated that isolated RAR orthologue does not activate target gene transcription and/or binds to retinoids (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014a). On the contrary the *P. dumerilii* RAR orthologue does (Gutierrez-Mazariegos et al., 2014a). These evidences suggest that during lophotrochozoan evolution mollusks RAR may have lost the ability to transactivate in the presence of retinoids whereas annelids maintained the ability to transctivate in the presence of RA isomers. Interestingly, although mollusks RAR do not activate gene transcription in the presence of retinoids, the ability to form heterodimer with RXR is conserved (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014a).

In Chapter 6 we address if the loss of ability to activate target gene transcription was lost in the entire mollusks phyla or if it is clade or species-specific. The ability of mollusks RAR/RXR heterodimer to activate target gene transcription was also evaluated. We aimed to get new insights to clarify the RAR evolutionary history in lophotrochozoans

particularly mollusks. To address this aim we focus our study in RAR orthologues from *N. lapillus*, *C. gigas*, *P. vulgata* and *Acanthochitona crinita*. Our transactivation assays revealed that none of mollusks RAR were transactivated by all-*trans*-, 9-*cis*- and all-*trans*-4-oxo-RA (4-oxo-RA), consistent with the poorly conservation of key residues on LBD that interact with retinoids. It was observed a target gene transcription repression that is consistent with the previously formed assumption that RAR in mollusks might act as a gene constitutive repressor (Gutierrez-Mazariegos et al., 2014a). For *N. lapillus* RAR it has previously been demonstrated that it is not able to bind to retinoids and to recruit coactivators, but efficiently interacts with corepressor (Gutierrez-Mazariegos et al., 2014a).

Next, we demonstrated (Chapter 6) that mollusk RAR is able to efficiently form heterodimer with RXR, and this feature seems to be not only conserved along the mollusk phylum, but also between chordates and lophotrochozoan. Due to time restriction we were not able to isolate the ORF or at least the hinge and LBD required for the assays, for *A. crinita* RXR orthologue. This is a work that is still in progress. Apart from forming heterodimer with RAR, RXR also form dimeric protein complex with other NRs family members (Germain et al., 2006). The heterodimers are classified as permissive or non-permissive. Non-permissive heterodimers are not activated by RXR ligands, whereas a permissive heterodimer can be activated by ligands from RXR and/or its partner independently or together to induce a synergistic activation (Germain et al., 2006). In vertebrates, the heterodimer RAR/RXR function as non-permissive, and is only activated when a ligand binds to RAR leading to a heterodimer conformation exchange, release of corepressors and recruitment of activators to initiated target gene transcription (Germain et al., 2006). Taking in consideration the heterodimer RAR/RXR mode of action on vertebrates, we aimed to understand how this protein complex behaves in mollusks since RAR was demonstrated to be unresponsive to the putative *bone fine* ligands tested so far (Urushitani et al., 2013; Gutierrez-Mazariegos et al., 2014a; Chapter 6). Remarkably, we observed for *N. lapillus*, *P. vulgata* and *C. gigas* heterodimer significant transcription repression activation in the presence of all-*trans*-RA, whereas in the presence of 4-oxo-RA this was significant for *N. lapillus* only. Regarding *P. vulgata*, repression was not significant and for *C. gigas* no transactivation or repression occurred. Interesting, for *N. lapillus* RAR/RXR heterodimer the repression response were higher than the observed for RAR alone. The data obtained raise fundamental questions: what is the biological significance of mollusks RAR/RXR heterodimer? Which physiological process(s) are mediated through this pathway?

				Storage		Synthesis				Signaling	
				LRAT	DGAT1	BCO	ADHs	SDHs	ALDHs	RAR	RXR
			Porifera	unk	+	+	unk	unk	+	-	-
			Cnidarians	unk	+	+	+	+	+	-	a
Protostomes	Lophotrochozoan	Mollusks	*L. gigantea*	unk	+	+	+	+	+	+	+
			P. depressa	unk	b	unk	unk	unk	b	unk	unk
			P. vulgata	unk	unk	unk	unk	unk	unk	+	+
			C. gigas	unk	unk	unk	unk	unk	unk	+	+
			L. stagnalis	unk	unk	unk	unk	unk	+	+	+
			T. clavigera	unk	unk	unk	unk	unk	unk	+	+
			N. lapillus	unk	+	unk	b	unk	unk	+	+
			A. crinita	unk	unk	unk	unk	unk	unk	+	unk
		Annelids	*P. dumerilii*	unk	b	+	unk	unk	b	+	+
			C. teleta	unk	+	+	+	+	+	+	+
		Platyhelminthes	*H. robusta*	unk	+	unk	unk	unk	+	-	+
			S. mansoni	unk	+	unk	unk	unk	+	-	+
	Ecdyzoan	Crustaceans	*Decapoda*	unk	+	unk	unk	unk	unk	unk	+
			D. pulex	-	+	+	+	+	+	unk	+
		Insects	*T. castaneum*	unk	+	unk	unk	unk	+	-	+
			A. mellifera	unk	+	unk	unk	unk	+	-	+
			A. gambiae	unk	+	unk	unk	unk	+	-	+
			D. melanogaster	-	+	+	unk	+	+	-	+
		Nematoda	*B. malayi*	unk	+	unk	unk	unk	-	-	+
			C. elegans	unk	+	+	+	+	+	-	-
Deuterostomes		Ambulacraria	*S. purpuratus*	-	+	+	+	+	+	+	+
			S. kowalevskii	-	+	-	+	+	+	+	+
	Chordata	Cephalochordate	*B. floridae*	-	+	+	+	+	+	+	+
		Tunicates	*O. dioica*	unk	+	unk	unk	unk	-	-	+
			C. intestinals	-	+	+	+	+	+	+	+
			Vertebrates	+	+	+	+	+	+	+	+

Figure 9.1: Cladogram summary of the current knowledge concerning the presence and/ or absence of key molecular retinoid modules in different metazoan groups. (+) indicates that molecular components are present in the phylum or species; (−) means that molecular components are absent from genome for analyzed species; (unk) absence of evidences; (a) indicates that the molecular component is present in some species but absent in others from the same phylum; (b) indicates that the molecular component is present in the species but its functional role in retinoid metabolism remains unclear; highlighted in dark grey is indicated isolated and/or functional molecular component characterized in the present work for the selected species (adapted from André et al., 2014).

9.5 Mechanisms of endocrine disruption of retinoid cascade in lophotrochozoans

Another major goal of the present thesis was to get new insights about the modulation of lophotrochozoan retinoid system by EDCs, focusing mostly in the signaling cascade. Available data indicates that retinoid metabolic and singling cascade are major targets of EDCs, with clear reported adverse effects in vertebrate (including humans) and invertebrate *taxa* (Novák et al., 2008; Santos et al., 2012). In general, to date most studies

have focused on vertebrate retinoid cascade disruption (Novák et al., 2008; Santos et al., 2012). The lack of knowledge about retinoid system has hampered the use of a similar approach for invertebrates (Novák et al., 2008; Santos et al., 2012; Castro and Santos, 2014). RXR orthologues from lophotrochozoans *taxa* seems to have conserved a similar function (ability to bind and activate target gene transcription in response to 9-*cis*-RA) to vertebrate *taxa*. A shared feature that suggests that lophotrochozoans RXR signaling cascade is also a potential target for modulation by the same EDCs as vertebrate *taxa*, such as the organotin compounds (OTs) (Chapter 1, 6, 7 and 8; Santos et al., 2012; Castro and Santos, 2014). The OTs tributyltin (TBT) and triphenyltin (TPT) are ubiquitous aquatic environmental organic pollutants characterized primarily by its reproductive toxicity in human and wildlife (Antizar-Ladislao, 2008; Yi et al., 2012). Occurrence of both OTs has been reported in different compartments of the aquatic environment such as water, sediments and biotas even after their use has been banned (Antizar-Ladislao, 2008; Yi et al., 2012). The best known and most studied effect is the masculinization of gastropod females, a phenomenon named as imposex. A growing number of evidences strongly supports that the induction of imposex involves the negative modulation of the RXR-dependent signaling pathway. Although the whole downstream molecular cascade involved remains unknown (Lima et al., 2011). RXR from imposex-sensitivity gastropods, like humans RXRs, binds to 9-*cis*-RA with high affinity (Nishikawa et al., 2004; Castro et al., 2007). Additionally, *in vitro* ligand binding assays revealed that TBT and TPT are also affinity ligands for human RXRs and *T. clavigera* (Nishikawa et al., 2004; Kanayama et al., 2005). Furthermore injections of 9-*cis*-RA, TPT and/or TBT resulted in induction of imposex in *T. clavigera* and/or *N. lapillus* (Nishikawa et al., 2004; Castro et al., 2007). After OTs exposure, RXR gene expression in gastropod female tissues undergoes alteration (Horiguchi et al., 2010; Lima et al., 2011). For instance, RXR gene transcription expression significant increase in penis/penis forming area in imposex induced female gastropods compared to the control ones (Horiguchi et al., 2010; Lima et al., 2011). Additionally, it has also been demonstrated that TBT and TPT have the ability to activate target gene transcription mediated by RXR in *T. clavigera* to a similar level that 9-*cis*-RA (Urushitani et al., 2011). Altogether data advocate that OTs induces imposex by mimicking the action of retinoids modulating the RXR-dependent signaling pathway.

In Chapter 8 we provided evidences that give support to the postulated assumption. We demonstrate that *N. lapillus* RXR isoforms (a and b) are responsive to those compounds that were previously pointed to promote imposex development in this female gastropods (Castro et al., 2007; Stange et al., 2012). *N. lapillus* RXRa in the presence of the putative natural ligand 9-*cis*-RA, TBT, HX630 and methoprene acid (MA) activated target gene transcription. In the case of RXR isoform b transcription, is only

activated in the presence of 9-*cis*-RA, HX630 and MA although at lower levels compared to RXR isoform a; while in the presence of TBT a gene transcription repression was observed. The biological importance of such observation remains to be study, but this evidence suggests that the two isoforms might step in different physiological process. Our data integrate well with the findings of Urushitani et al. (2011), suggesting that the different response to the tested compounds is related with the 5 amino acid insertion/depilation at the T-box. In Chapter 8, we also demonstrate that *N. lapillus* RXRa is able to activate the transcription of reporter genes in response to stimulation by TPT. In addition we evaluated the binding affinity for *N. lapillus* RXRa and *T. clavigera* RXR-1 to TPT. The affinity to binding TPT was higher for *T. clavigera* then for *N. lapillus* RXRa (Chapter 8). Contrary to what is reported to *T. clavigera*, TPT- exposed *N. lapillus* females develop mild levels of imposex (Laranjeiro et al., *in press*). The different affinities for TPT between the two species might explain the different sensitivity to TPT, particularly in relation to imposex development. Yet, since RXR binds and transactivated reported gene in the presence of TPT, it is a direct indication that this pollutant can also modulate RXR signaling cascade in *N. lapillus*. However, given that transactivation occurs at higher concentrations than those of TBT, the ecological relevance in gastropods is likely to differ (Chapter 8).

Given the degree of RXR conservation between lineages, many more metazoans might be affected by the action of OTs. Data clearly suggested that the taxonomic scope of OTs-mediated RXR-dependent signaling pathway may be larger than initially anticipated. Indeed, OTs also affects other mollusks in addition to gastropods and other metazoan lineages. For example, lipid and fatty acid level profile in mollusks undergoes change after short or long term exposure to OTs, but the signaling cascade involved remains unknown (Janer et al., 2007; Lyssimachou et al., 2009). In addition, in Chapter 6 we demonstrate the *C. gigas* RXR and *P. vulgata* also responsive to TBT. For the oyster *C. gigas* it has been reported that TBT exposure is responsible for the abnormal shell growth and abnormal larval development (Heral et al., 1989; Higuera-Ruiz and Elorza, 2011). Our data suggests that the oyster reported effects might be mediated to a RXR-dependent signaling pathway. TBT exposure through sediment inhibited juvenile growth of the deposit feeder polychaete *Armandia brevis* (Meador and Rice, 2001). Results from a TBT- exposed experiment of embryos and larvae of the *P. dumerilii* showed that the OTs had dose-dependent genotoxic, cytotoxic, and developmental effects (Hagger et al., 2002). In Chapter 7 we provided evidences that support the hypothesis that the effects from TBT exposure on *P. dumerilii* that might also be mediated by a RXR dependent pathway. *P. dumerilii* RXR when exposed to the OTs leads to target gene transcription activation. TPT also induced target gene transactivation activity via *P. dumerilii* RXR

although at higher concentrations when compared with TBT. Although few studies described the effects of TPT exposure in annelids, our data suggests that *P. dumerilii* is sensitive to both OTs. Further studies are required to determined possible biological implications of TPT exposure and link it to RXR-mediated signaling pathways. Interesting, the target gene transcription activation in the presence of TBT and TPT were higher for *P. dumerilii* compared to the *N. lapillus* RXRa suggesting that the former species might be highly sensitive to OTs (see Chapter 7 and 8). TPT and TBT also causes effects on embryo-larval development leading to body deformities and delayed growth in sea urchin *Lytechinus variegatus* (Perina et al., 2011). In tunicates TBT and/or TPT exposure leads to larva development arrest at distinct stages and/or to morphological abnormalities development (Cima et al., 1996; Dolcemascolo et al., 2005). At environmentally relevant levels, TBT also affects teleost fish reproduction and embryonic development. An embryonic reduced hatchability rate and morphological abnormalities development could be observed for *Sebastiscus marmoratus* after TBT exposure (Zhang et al., 2011). Also TBT seems to induce RXRα expression in *S. marmoratus* embryos, which would be responsible for the increasing apoptotic cells (Zhang et al., 2011).

In the case of imposex induction and other reported effects on mollusks by OTs a key question remains: are the effects mediated through RXR as a homodimer or through signaling pathways involving other heterodimeric partners such as RAR?. In Chapter 6 we approach this issue. We aimed to understand if mollusks RAR/RXR heterodimer signaling pathway might be also a target of TBT. To do so, we evaluated the protein complex transcription response in the presence of TBT. In Chapter 6 we obtained an interesting result with our *in vitro* transactivation assay. In general, a target gene transcription repression response, for both human and mollusks RAR as a monomer and in heterodimer with RXR was obtained, being more pronounced as heterodimers (Chapter 6). It seems that in RAR/RXR heterodimer, TBT is able to interact with RXR. In order to clarify the results observed with TBT, we evaluated the response of the RAR and RAR/RXR heterodimer in the presence of HX630, a compound known to be a weaker human RAR-antagonistic and a selective RXR-agonist (Umemiya et al., 1997a, b). As expected HX630 induces target gene transcription activation via human and mollusks RXR. With the exception of *P. vulgata*, in general, for human and other mollusks RAR no transcriptional activation or repression were obtain in the presence of HX630 consistent with the available data showing that this compound is a RAR weaker antagonist (Umemiya et al., 1997a, b), whereas in the heterodimer RAR/RXR a significant transcriptional repression has been observed. Additionally, to understand the obtained results of RAR/RXR heterodimer in the presence TBT, a mutation for loss of function regarding activation of RXR by TBT has been performed in both human and *N. lapillus*

RXRa. The reported RXR key cysteine at the LBD that interacts and binds to TBT was substituted by an alanine (le Maire et al., 2009). A transcriptional activation repression of both human and *N. lapillus* mutant RAR/RXR in presence of TBT has been observed, but at a lower extent when compared with wild-type RAR/RXR heterodimer, and only significant for *N. lapillus*. Also, for the mutated human and *N. lapillus* RXR a transcription repression has been detected. This information suggests that RXR has a role in the RAR/RXR and might not be silent as previously suggested.

Studies with mammalian models have revealed that, in the heterodimeric complex both RAR and RXR can efficiently bind their ligands. Yet, only after ligand bind to RAR gene transcription mediated by the heterodimer complex is activated. The RXR ligands contribute synergistically to the transcriptional activity of the heterodimer only when the RAR is in the presence of a ligand (Minucci et al., 1997; Germain et al., 2002). In the heterodimer complex, RXR when bounded to its ligand is not able to release the corepressors and recruit coactivators, this only occurred when RAR is bounded to a ligand (Germain et al., 2002). In addition, RAR with no ligand enhance the RXR bounding interaction with corepressors (Lammi et al., 2008). The RAR/RXR heterodimer has also been reported to act has a transcription repressor. This happens when RAR is in the presence of its own agonist ligands and the heterodimer complex is bonded to the downstream half-site of responsive elements of direct repeats spaced by 1 base par. In this situation repressors are not released (Kurokawa et al., 1995; DiRenzo et al., 1997). *In vitro* studies demonstrated that TBT has no ligand affinity and is unable to activate target gene transcription via human RARs (Kanayama et al., 2005; Kamata et al., 2008). Yet, another independent study suggests that TBT has the ability to interact with RAR/RXR heterodimer dependent signaling pathway by bind to RXR in the presence of an RAR agonist ligand (Yonezawa et al., 2007). Taking in account the reported evidences for mammalian models, we hypothesize that within mollusks RAR/RXR heterodimer RXR ligands (TBT, HX630 and probably the all-*trans*-RA) bind efficiently to the receptor. Since RAR/RXR heterodimer might only be activated by putative RAR ligands, no corepressors were released leading to the observed result. Overall, altogether the findings propose not only that RXR might have a major role on the mollusks RAR/RXR heterodimer signaling pathways when its own ligands are present, but also that the heterodimer transactivation repression seems to be an ancient behavior that might be conserved with mammals. To help clarifying this finding, future studies focusing in studying TBT and HX630 binding affinity should be performed with the mollusks RARs and understand how the compounds interacts in the presence of co-factors.

In Chapter 6, we also evaluated the ability of other five common environmental pollutants to activate target gene transcription by *N. lapillus* RAR. These pollutants have

been reported to activate target gene transcription mediated by human RARs (Lemaire et al., 2005; Kamata et al., 2008). Based on previously studies (Lemaire et al., 2005; Kamata et al., 2008), we have selected two organochlorine pesticides, Endrin and Dieldrin, an alkylphenols 4-n-nonylphenol, a monoalkylphenols 4-*n*-heptylphenol and a polycyclic aromatic hydrocarbon the Naphthalene. None of the tested pollutants were capable of induce transactivation response of a reporter gene in *N. lapillus* RAR, only a significant repression has been observed by Dieldrin exposure. Since we notice a significant transactivation repression for *N. lapillus* RAR in the presence of Dieldrin, we decided next to test RAR/RXR heterodimer response. Interesting for *N. lapillus* RAR/RXR significant transcription activation was observed, whereas for RXR a transactivation repression was obtained. Further studies needs to be conducted to evaluated *N. lapillus* RXR and RAR ligand affinity and the biological response by exposure to this pollutant. The biological relevance of this response is as yet unknown, but should be a subject for future studies.

9.6 Conclusion and future perspectives

Overall, the results presented in this thesis contribute to bridge knowledge gaps regarding the retinoid metabolic and signaling module repertoire in lophotrochozoans. Taken together, our data adds not only important information to understand the evolutionary history of retinoid system in metazoans, but also contributes with new insights regarding retinoid signaling pathway as a potential target for negative modulation by priority environmental pollutants, mainly OTs.

In conclusion, invertebrates have a repertoire of metabolic and signaling retinoid modules but for most of them functional characterization is still missing, hampering a better understanding of their role. Our data suggests that, in fact, at least for lophotrochozoans the presence of an orthologue does not imply a conserved function of retinoid metabolism with vertebrates. A retinoid metabolic pathway vertebrate like might not be present and conserved in lophotrochozoans. It seems that during evolution some metabolic components, such as ADH3 and ALDH1s in vertebrates have acquired a function on retinoid metabolism, whereas in lophotrochozoans it probably kept the ancestral function. Yet, for these two enzymes and others molecular metabolic components it cannot be rolled out the involvement in the metabolic cascade of retinoids, additional functional studies are needed. Nevertheless, for other *taxa* the presence of key metabolic players should be investigated to account for possible species-specific losses or gains and allow a more accurate understanding of the evolution of retinoids system in metazoans. For instance, in the case of ALDH1s, it should be investigated if in chordate invertebrate species (cephalochordate and tunicates), for which a conserved SEC amino acid residue signature has been reported, are in fact able to use retinal to produce RA. In

addition, it would be pertinent in future studies to focus in the isolation and functional characterization of other known vertebrate-like retinoid metabolic players' not only in lophotrochozoans but also in other invertebrate lineages, to infer about its conservation across metazoans. For example, isolation of CYP26 orthologues known to be implicated in RA catabolism; isolation of SDR orthologues involved in retinol and retinal metabolism. Additionally, also addressing the putative involvement of CPY 450 family members in invertebrates that might have a contribution on retinoid metabolism as in vertebrates.

A retinoid metabolic route must exist in lophotrochozoans since evidences revealed the presence of retinoid content and described RA biological effects. Data presented in this work also supports the existence of an active retinoid system in the mollusks *P. vulgata* that might have a role along the gonad maturation and reproductive process as in vertebrates; a physiological function that seems to require a fine balance in retinoid levels.

Our data also indicates that contrary to the metabolic routes, the retinoid signaling pathways, particularly RXR, appear to be conserved throughout evolution among vertebrates and lophotrochozoans *taxa*.

Although it is unquestionable that a RXR-dependent signaling pathway has a major role on imposex development being a prime target of OTs, the full elucidation of the involved cascade remains to be demonstrated. Our work with *N. lapillus* provides further evidences that imposex development is mediated through a RXR-dependent signaling pathway. In addition, we provided new evidence that might contribute to understand how OTs interacts with retinoid signaling pathways. TBT appears to modulate the target gene transcription mediated by RAR/RXR heterodimer in both human and mollusk, although in the mollusks RAR has a different mode of action as monomers. We believe that within the heterodimer RAR/RXR, TBT can bind to RXR and interact with RAR leading to a gene transcription repression. Yet, further studies needs to be performed in order to understand exactly how TBT interacts with the heterodimer complex by evaluating their receptor binding affinity and interaction with co-factors (coactivators and corepressors). Also, it will be interesting to evaluate the transcription of reporter genes in response to stimulation by TBT-exposed RAR/RXR heterodimer of other invertebrate *taxa* such as annelids and chordates to understand if the response might be conserved across metazoans.

Our findings also highlight the existence of a common, and evolutionary conserved, mechanism for endocrine disruption by OTs through modulation of RXR-dependent signaling in metazoans. We provided the firsts evidences that primary action of OTs in annelids might be mediated by RXR. As a follow up of this study it will be relevant to evaluate the ligand binding affinity to retinoids and OTs. *In vivo* assays focus on *P. dumerilii* life-cycle exposure to OTs and retinoids should be carry out to evaluate

biological effects and gene expression alteration profile to support the obtained data. Future studies should elucidate whether annelids have endogenous retinoids levels and have the ability to synthesize active retinoids.

In the mollusks RAR orthologue, LBD alteration of several residues interacting with the ligand has apparently led to an overall decrease in the strength of the interaction with the retinoids and it seems to be conserved along gastropods, bivalves and polyplacophora classes. It seems that the same amino acid residues alteration on mollusks RAR LBD that make the receptor unresponsive to retinoids might also make them unresponsive to certain EDCs. Our findings emphasize the need to evaluate EDCs binding abilities to mollusks RAR-mediated target gene activation. In addition, it would be pertinent in future studies to focus in RAR-mediated signaling pathway modulation by EDCs in other invertebrate species such as chordates and annelids where the receptor has the ability to respond to retinoids in a similar manner as vertebrates.

9.7 References

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