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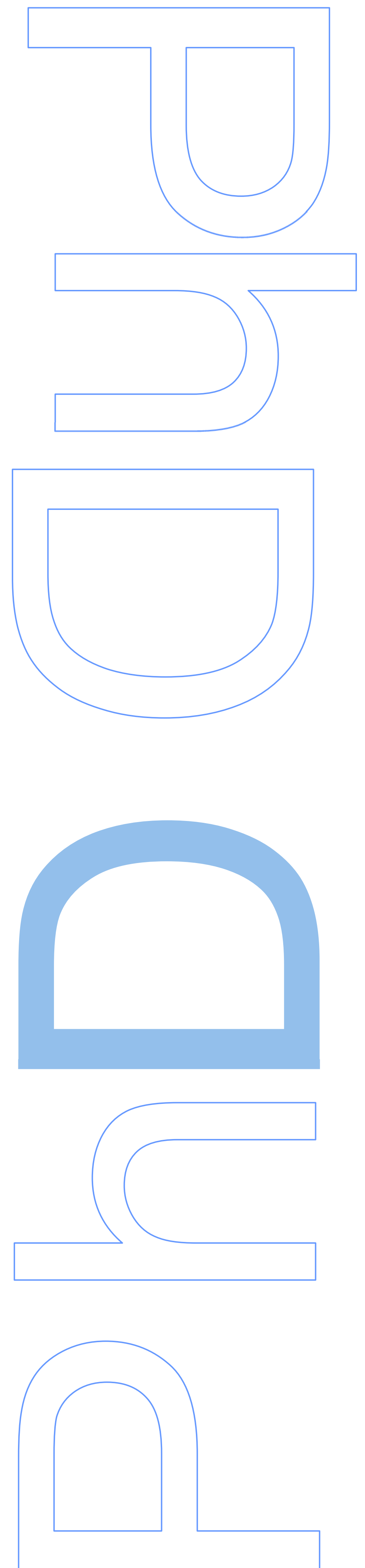

Screening obesogens in aquatic model organisms

Ana Maria Ferreira Capitão,
Tese de Doutoramento apresentada à
Faculdade de Ciências da Universidade do Porto
Biologia
2017

Screening obesogens in aquatic model organisms

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Doutoramento em Biologia

Faculdade de Ciências

2017

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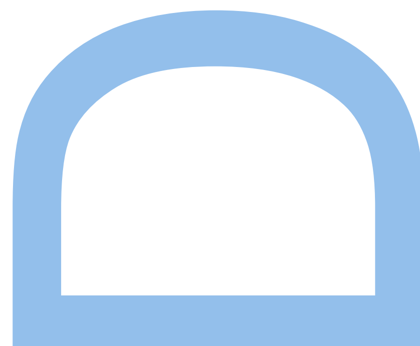

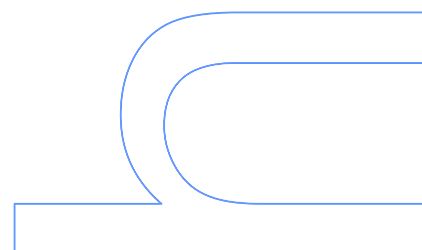

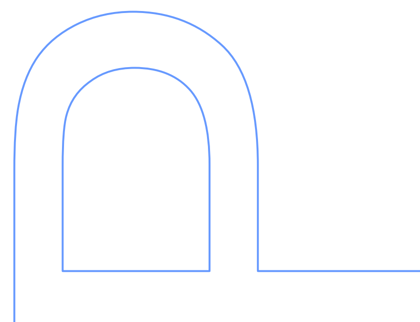

This thesis was suported by FCT (ref: SFRH/BD/90664/2012), Norte2020 and FEDER (Coral—Sustainable Ocean Exploitation—Norte-01-0145-FEDER-000036).

The work included in this thesis (including 1 article published, 2 submitted and 1 in preparation) was performed in collaboration with co-authors. I declare that I have contributed to the development of the ideas in this thesis, to produce and analyse the data presented and to the writing of all chapters.

Capitão, A., Lyssimachou, A., C. Castro, L.F., Santos, M.M., 2017. Obesogens in the aquatic environment : an evolutionary and toxicological perspective. Environ. Int. 106, 153–169. doi:10.1016/j.envint.2017.06.003 (published)

Capitão, A., Marques, M., Ruivo, R., Mendiratta, N., Fonseca, E., C. Castro, L.F., Santos, M.M. The sea urchin PPAR/RXR heterodimer is a target of the model obesogen TBT. (submitted to: Environmental Science & Technology);

Capitão, A., Marques, M., Ruivo, R., Fonseca, E., Nakanishi, T., Santos, M.M., C. Castro, L.F. The transphyletic exploitation of PPAR orthologues by organotins in gnathostome lineages.
(submitted to: Environmental Health Perspectives).

Capitão et al.. The molluscan PPAR is implicated in lipid metabolism and is exploited by organotins.
(in preparation).

Para as minhas afilhadas Carolina e Gabriela

e para o meu sobrinho Lucas.

Que a curiosidade nunca vos abandone.

Agradecimentos

Gostaria de agradecer ao meu orientador principal, Doutor Miguel Santos e ao meu coorientador Doutor Filipe Castro por me terem recebido e integrado nas suas equipas. Ambos me proporcionaram a oportunidade de realizar um trabalho estimulante e de crescer enquanto investigadora durante estes quatro anos. O seu conhecimento e orientação foram essenciais na realização deste trabalho.

Agradeço ao CIIMAR, à Faculdade de ciências e à FCT por me terem proporcionado as infraestruturas e o financiamento necessário à realização deste trabalho. Agradeço à equipa do BOGA e em especial ao Ricardo Branco pelo apoio dado na montagem dos ensaios.

Não posso deixar de agradecer a todos os meus colegas e amigos que fazem com que os grupos, EDEC, AGE e METOX sejam tão especiais. Durante estes quatro anos cruzei-me com várias pessoas que me marcaram e que fizeram com que esta viagem fosse ainda mais interessante. Ana André um obrigada especial por me teres mostrado os “cantos à casa”. Ana André, Cristina Rocha, Inês Pascoa, Mónica Marques, Raquel Ruivo, Teresa Neuparth, Marta Correia, Angeliki Lyssimachou, Elza Fonseca, Ricardo Capela e Tiago Torres obrigada por todos os momentos em que me fizeram sentir em casa e por darem sentido à palavra equipa. Quis o destino que trabalhasse em colaboração com duas pessoas fantásticas que se revelaram verdadeiras amigas, Raquel Ruivo e Mónica Marques obrigada. Inês Páscoa e Maria João obrigada por terem cruzado o meu caminho e por me terem ajudado a ser uma pessoa melhor e mais positiva.

Por fim agradeço ás pessoas que amo e que fizeram de mim a pessoa que sou hoje, uma pessoa feliz. Não poderia ser de outra forma tendo à minha volta uma família fantástica que sempre me apoiou, os meus pais que sempre fizeram o que consideraram ser o melhor para a minha felicidade. Os meus irmãos que sempre estiveram ao meu lado, sempre me protegeram e fizeram rir. As minhas afilhadas e o meu sobrinho que me mostram o que realmente importa na vida. Ao meu Nico que me apoia, que demonstrou muita paciência durante este percurso e que está sempre pronto a dar-me a sua opinião sincera. E a todos os meus amigos e amigas que tornam a minha vida muito melhor.

Resumo

Os lípidos são um dos principais componentes nos seres vivos. Estes possuem, entre outras, funções de armazenamento de energia, participam na constituição das membranas biológicas, são utilizados como mensageiros intracelulares, cofatores enzimáticos e percursores na síntese de produtos celulares. Nos últimos anos, o aumento da incidência da obesidade nos países ocidentais, esteve na base de uma nova hipótese que sugere a existência de fatores de risco ambientais, tais como poluentes, que poderão promover este fenómeno. Vários químicos, designados de disruptores endócrinos (EDCs), possuem a capacidade de interferir com a fisiologia dos organismos, alterando diversos processos biológicos. Recentemente, alguns EDCs foram descritos como tendo a capacidade de modelar a homeostasia lipídica em várias espécies de vertebrados e invertebrados. Estes químicos foram designados obesogénios. O principal mecanismo associado à desregulação da homeostasia lipídica parece envolver a modelação inapropriada de recetores nucleares (NRs). Os poluentes com propriedades obesogénicas mais estudados em mamíferos são os organoestanhos tributilestanho (TBT) e trifenilestanho (TPT), estes são reconhecidos como ligandos dos NRs, recetor ativado *pelo* proliferador de peroxissomas gamma (PPARγ) e recetor X do ácido retinoico (RXR). O PPARγ, que forma um heterodímero com o RXR, possui um papel crucial na adipogénese. Enquanto o ortólogo do PPAR foi reportado apenas em deuterostómios e moluscos, o RXR encontra-se presente na grande maioria dos metazoários. Contudo, a informação relativa ao PPAR em invertebrados é escassa, o que dificulta a compreensão da(s) função(s) deste fator de transcrição e a sua possível dirupção por EDCs.

O trabalho desenvolvido durante esta tese pretende preencher as lacunas identificadas anteriormente e expandir o conhecimento relativo à perturbação da homeostasia lipídica associado com EDCs, focando-se em particular na modelação de NRs pelos organoestanhos, obesogénios modelo em mamíferos. Primeiramente focou-se na caracterização funcional dos PPARs num amplo conjunto de *taxa* vertebrados e invertebrados, explorando os resultados num contexto evolutivo e toxicológico. Inicialmente isolou-se e efetuou-se a caracterização funcional de um ortólogo do PPAR em moluscos recorrendo a análises de genómica comparativa, filogenia e ensaios de transativação. A espécie selecionada para o estudo foi o gastrópode *Patella depressa,* cujo PPAR transativou na presença de presumíveis ligandos naturais, como o ácido araquidônico (ARA) e eicosapentaenoico (EPA). Posteriormente avaliou-se a resposta deste recetor na presença de TBT e TPT. Ambos os organoestanhos reprimiram o PPAR de *P. depressa* tanto em monómero como em heterodímero (PPAR/RXR).

Seguidamente testaram-se os efeitos *in vivo* no perfil de ácidos gordos de *P. depressa* quando exposto a níveis ambientalmente relevantes de TBT. A exposição a diferentes concentrações de TBT (100 e 250 ng Sn/L) revelou alterações estatisticamente significativas no perfil de ácidos gordos, reforçando as observações *in vitro.*

Antes do presente trabalho não existia informação disponível relativa aos efeitos dos organoestanhos no metabolismo lipídico de equinodermes ou qualquer caracterização do PPAR nestes organismos. Tendo em conta que o *PPAR* está previsto no genoma deste *taxa*, e considerando a sua posição filogenética, foi isolado e funcionalmente caracterizado um PPAR de *Paracentrotus lividus*. À semelhança dos moluscos, observou-se que a mistura de ácidos gordos, presumíveis ligandos naturais, tem a capacidade de transativar o heterodímero PPAR/RXR. Curiosamente, o TBT e TPT também se comportam como repressores do PPAR/RXR em *P. lividus.* A exposição *in vivo* de *P. lividus* a TBT (100 e 250 ng Sn/L) causou alterações no perfil de ácidos gordos e nos níveis de transcrição de *rxr* e *acsl* (acil-coa sintetase de cadeia longa); nos mamíferos ambos os genes são regulados pelos PPARs.

De forma a expandir o conhecimento relativo ao funcionamento dos PPARs numa perspetiva evolutiva recorreu-se a análises de genómica comparativa, filogenia e ensaios de transativação para caracterizar os PPARs das principais linhagens de cordados (cefalocordados, *condropterígeos*, actinopterígeos-lepisosteiformes e perciformes, anfíbios e mamíferos). Os nossos resultados demonstram que o PPARγ é transativado pelo TBT em todos os grupos com exceção dos actinopterígeos. Adicionalmente, os PPARα e β são reprimidos na maioria dos *taxa* de gnastostomados (mamíferos, anfíbios e actinopterígeos) mas são ativados em *condropterígeos*. A transativação do PPAR de um cordado basal, o cefalocordado *Branchiostoma lanceolatum* não foi afetada significativamente pela exposição a TBT; contudo foi modelada na presença de potenciais ligandos naturais.

Em conclusão, este trabalho caracterizou funcionalmente, pela primeira vez, o PPAR em linhagens basais de metazoários sugerindo que a capacidade de ativar a transcrição na presença de lípidos encontra-se conservada em termos evolutivos entre vertebrados e invertebrados. Ao contrário do comportamento dos PPARs na presença de lípidos, que demonstraram uma resposta semelhante em todos os organismos testados, as respostas obtidas na presença de TBT e TPT variaram entre grupos. As alterações no perfil de ácidos gordos observadas tanto em *P. depressa* como em *P. lividus,* contribuem para o aumento do conhecimento relativo à disrupção do metabolismo lipídico pelo obesogénio modelo, TBT. Numa perspetiva de avaliação de

risco, os resultados do presente trabalho apresentam uma elevada relevância dado que realçam a importância de se utilizar espécies de diferentes grupos, com diferente constituição genómica, nos ensaios ecotoxicológicos.

Abstract

Lipids display key functions in all living organisms. They are used as energy storage, building blocks of biological membranes, precursors in the synthesis of important cellular products, intracellular messengers, enzymes cofactors, among others. In recent years the rise in the obesity rates in western countries led to the hypothesis that environmental risk factors, such as pollutants, are potentially involved in the prevalence of the phenomenon. Today, it is well established that several chemicals, known as endocrine disrupting chemicals (EDCs), can interfere with organism's physiology, impacting key biological processes. Recently, some EDCs have been described to modulate lipid homeostasis in both vertebrates and non-vertebrates species. These chemicals have been named as obesogens. The main mechanism of action associated with lipid homeostasis disruption seems to involve the inappropriate modulation of nuclear receptors (NRs). One of the best-known examples of obesogens in mammals are organotins. Tributyltin (TBT) and triphenyltin (TPT) are known ligands for mammalian NRs peroxisome proliferator-activated receptor gamma (PPARγ) and retinoid X receptor (RXR), which displays a key role in adipogenesis. Whereas PPAR orthologues have been reported in deuterostomes and molluscs, RXR is present in most of metazoans. Yet, there is a paucity of data on PPAR functional characterization outside vertebrates, which hampers a better understanding of the role of this transcription factor and its putative disruption by EDCs.

The work developed in the frame of this thesis aims to fill the gaps identified above and expand our knowledge on the taxonomic scope of lipid homeostasis perturbation by environmental chemicals, focusing in particular in the modulation of NRs by the model mammalian obesogens organotins. The present work is the first to functionally characterize PPARs in a large set of vertebrate and non-vertebrate *taxa* and discuss the findings in an evolutionary and toxicological context. Our first approach involved the isolation and the first functional characterization of PPAR orthologues in molluscs using comparative genomics, phylogeny and transactivation assays. The gastropod, *Patella depressa,* was the target species. PPAR alone transactivated in the presence of putative natural ligands, arachidonic acid (ARA) and eicosapentaenoic acid (EPA). We then addressed the response in the presence of TBT and TPT. Both organotins repress *P. depressa* PPAR alone and when tested in heterodimer PPAR/RXR. We then tested the *in vivo* effects of environmentally relevant levels of TBT in *P. depressa* fatty acid profile. Exposure to TBT at 100 and 250 ng Sn/L supported the *in vitro* findings, since statistically significant alterations were observed in the fatty acid profile.

Before the present work no information was available regarding the effect of organotins in lipid metabolism or PPAR characterization in echinoderms. Given that PPAR was also genome predicted in this *taxa*, and considering its phylogenetic position, we isolated and functionally characterized one PPAR from *Paracentrotus lividus*. Similar to molluscs, transactivation assays showed that a mixture of fatty acids, the putative natural ligands, transactivate expression via the heterodimer PPAR/RXR. Interestingly, TBT and TPT also behaved as repressors of *Paracentrotus lividus* PPAR/RXR. *In vivo* exposure of *P. lividus* to TBT (100 and 250 ng Sn/L) led to alterations in the fatty acid profile and in the transcription of *rxr* and *acsl* (long-chain acyl-CoA synthetase); in mammals both genes are known to be regulated by PPARs.

To expand the knowledge on the function of PPAR in an evolutionary perspective we then used comparative genomics, phylogeny and transactivation assays to characterize the PPARs of the main chordate lineages (cephalochordates, condrichthyes, actinopterygii – lepisosteiformes and perciformes, amphibians and mammals). Our results revealed that PPARγ is activated by TBT in all groups with the exception of the actinopterygii. Moreover, PPARα and β are repressed in most gnathostome taxa (mammals, amphibians and actinopterygii) but activated in the condrichthyes. The transactivation of PPAR of the basal chordate, *Branchiostoma lanceolatum* was not significantly affected by TBT; however it was modulated by the putative natural ligands tested.

Overall, the present work functionally characterized, for the first time, PPAR in basal metazoan lineages suggesting that the ability to activate transcription in the presence of lipids is evolutionary conserved across vertebrates and in the tested non-vertebrates. In contrast to lipids, that show a similar response between the tested vertebrates and non-vertebrates, the response of PPARs towards the model obesogens, TBT and TPT, were lineage-specific. Changes in lipid profile were observed in both *P. depressa* and *P. lividus* , which expands our knowledge on lipid disruption by the model obesogen, TBT. From a risk assessment stand-point these findings are highly relevant as they highlight that genomic diversity at ecosystem scales is fundamental to infer the impact of endocrine disruptors, such as obesogens, in physiological and functional modules, notably in the context of the Anthropocene. An evolutionary perspective is therefore critical to better estimate the risk of obesogens at an ecosystem scale.

Keywords: Obesogens, Endocrine disruption, Evolution, Nuclear receptor, Peroxisome proliferator-activated receptor, Lipid metabolism, Aquatic animals

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List of abreviations

ACA - Acetyl-CoA Acyltransferase

ACACa - acetyl-CoA carboxylase 1

ACACb - acetyl-CoA carboxylase 2

ACC - Acetyl CoA Carboxylase

ACOX - Acyl-CoA Oxidase

ACS - Acetyl-CoA synthetase

APOA-1 - apolipoprotein A-I

ARA – Arachidonic acid

BBzP - Butyl benzyl phthalate

BPA - Bisphenol A

BZF - bezafibrate

CA - clofibric acid

C/EBPs - CCAAT/enhancer binding proteins

CPT - carnitine palmitoyltransferase

CYP27a - sterol 27-hydroxylase

CYP4 - cytochrome P450 4

DBD - DNA binding domain

DDT - dichlorodiphenyltrichloroethane

DEHP - di-2-ethylhexylphthalate

DHA – docosahexaenoic acid

DiDP - diisodecyl phthalate

ECR - Ecdysone Receptor

EDC - Endocrine disrupting chemicals

EPA - Eicosapentaenoic acid

EPs – emerging pollutants

ER - estrogen receptor

FA - fatty acid

FABP-fatty acid binding protein

FADS - fatty acid desaturase

FASN - Fatty Acid Synthase

FXR - farnesoid X receptor

GPAT1 - glycerol-3-phosphate acyltransferase 1

HL - hepatic lipase

HNF4A - Hepatocyte Nuclear Factor 4 A

HSL - hormone sensitive lipase

IPA - Ingenuity pathway analysis

KSI - kidney somatic index

LBD - ligand biding domain

LPL - Lipoprotein Lipase

LSI - liver somatic index

LXR - liver X receptor

MEHP - Mono-ethyl-hexyl phthalate

MOA – Mode of action

MUFA – monounsaturated fatty acids

NP - nonylphenol

NRs - nuclear receptors

OECD – Organization for Economic Co-operation and Development

PA - phthalic acid

PAHs - Polycyclic aromatic hydrocarbons

PBBs - polybrominated biphenyls

PCBs - polychlorinated biphenyls

PFOA - perfluorooctanoic acid

PPARs - peroxisome proliferator-activated receptors

PPCPs – Pharmaceuticals and personal care products

PUFA – polyunsaturated fatty acids

PXR - pregnane X receptor

Rosi - Rosiglitazone

RXR - retinoid X receptor

SCD1 - stearoyl-CoA desaturase 1

sod1 - superoxide dismutase

SREBPs - sterol regulatory element-binding proteins

STA - Steroidogenic Acute Regulatory Protein

t-OP - octylphenol

TAG - Triacylglycerol

TBBPA - Tetrabromobisphenol A

TBT - Tributyltin

TCBPA - tetrachlorobisphenol A

TPT – Triphenyltin

UFA – unsaturated fatty acids

WAT - White Adipose Tissue

ZOT - Zebrafish obesogenic test

Chapter 1 - General introduction

Environment International 106 (2017) 153–169

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Review article

Obesogens in the aquatic environment: an evolutionary and toxicological perspective

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General introduction

Endocrine disruption

The steep increase of chemical production and use since the 1940s coincides with the rise of several endocrine-related disorders in humans and wildlife populations, suggesting a relationship between both events (Bergman et al., 2013; Diamanti-Kandarakis et al., 2009; Grün and Blumberg, 2006; Kabir et al., 2015). The number of studies supporting this hypothesis is growing and it is now established that several of these chemicals have endocrine disrupting properties (Bergman et al., 2013). Endocrine disrupting chemicals (EDC) interfere with the normal function of the endocrine system by mimicking, blocking and/or altering hormone roles and metabolism (Diamanti-Kandarakis et al., 2009; Kabir et al., 2015; Schug et al., 2011). Although more than 1300 chemicals have been identified to potentially interfere with hormonal metabolism, very few have been screened for their capacity to cause endocrine effects *in vivo* (Bergman et al., 2013; “TEDX The Endocrine Disrupting Exchange,” 2017).

This vast number of compounds identified as EDCs have distinct chemical structures, proprieties and applications. Some of these compounds are used as synthetic hormones (e.g. ethynilestradiol), plastics (e.g. bisphenol A (BPA), phthalates), pesticides and fungicides (e.g.: organotins, methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT), vinclozolin), solvents (e.g.: polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins), pharmaceutical agents (e.g. thiazolidinediones, atypical anti-psychotics, antihistamines, antidepressants) and personal care products (e.g. triclosan). Besides synthetic chemicals, some natural compounds are also known EDCs (e.g. phytoestrogens, including genistein and coumestrol) (Castro and Santos, 2014; Kabir et al., 2015; Schug et al., 2016). Several of these compounds can undergo bioaccumulation and biomagnification through the food-chain, being persistent in the environment. In contrast, others are easily degraded but their continuous release into the environment still makes them a cause of concern (Fig1) (Bergman et al., 2013; Diamanti-Kandarakis et al., 2009; Kabir et al., 2015).

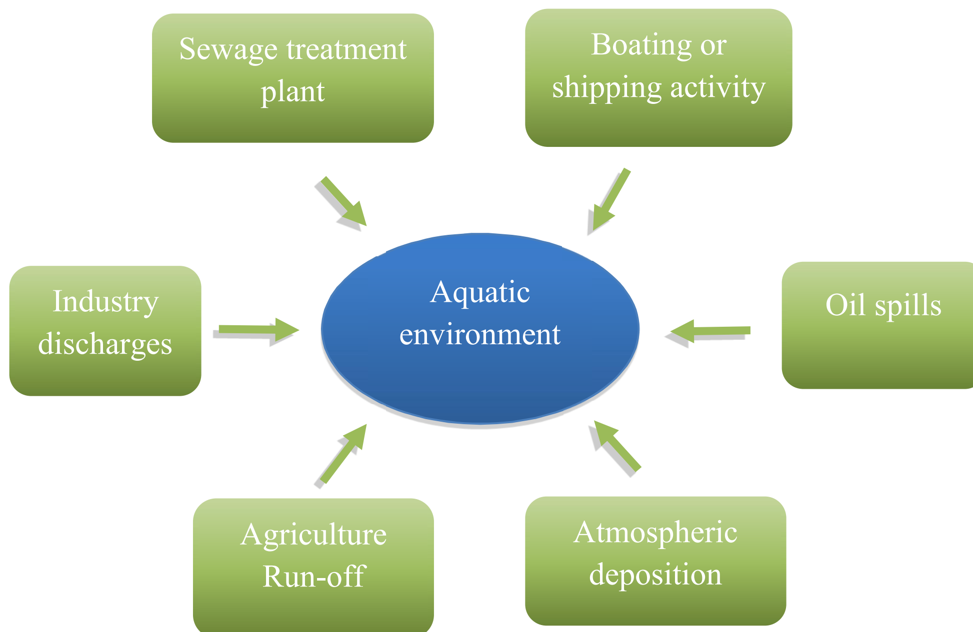

Fig. 1 - Schematic illustration of EDCs input in the aquatic environment (lakes, rivers and sea) (modified from Pait and Nelson, 2002; Sumpter, 2005).

The effects of different EDCs in non-target organisms have been well documented (Ferreira et al., 2009; Liu et al., 2014; Melvin, 2016; Rodrigues et al., 2006; Schug et al., 2016). Two well-known examples targeting the reproductive system are organotins that cause imposex in gastropods mollusks (a condition characterized by the development of male secondary sexual characteristics in females) (Abidli et al., 2009; Lima et al., 2011; Pascoal et al., 2013), and ethinylestradiol that alters the fecundity and sex ratio of fish (Runnalls et al., 2015; Soares et al., 2009). Alterations in gene and protein expression, as well as physiological and behavioral changes are also observed frequently as a consequence of EDC exposure (Brander, 2013; Sárria et al., 2013). More recently, evidences emerged regarding disruption in lipid homeostasis by EDCs. Since lipid metabolism dysregulation is related with several important diseases in the human population, the mode of action of these compounds - also known as obesogens- is now under strong scrutiny (Castro and Santos, 2014; De Cock and Van de Bor, 2014; Diamanti-Kandarakis et al., 2009; Grün and Blumberg, 2006; Ouadah-Boussouf and Babin, 2016; Santos et al., 2012). These compounds can increase the number of fat cells and/or the amount of fat stored in each cell by altering the pathways of energy metabolism and food intake (Holtcamp, 2012; Janesick and Blumberg, 2011). Several mechanisms of action have been suggested, including epigenetic changes that will be inherited by the future generations (Holtcamp, 2012). However, only a small portion of

chemicals has been tested so far for their potential to disrupt lipid homeostasis and a considerable amount of those fall in the obesogens category, such as organotins, BPA, perfluorooctanoic acid (PFOA), phthalates and some pharmaceuticals (Bašić et al., 2012; Bergman et al., 2013; Legler et al., 2015).

Lipid homeostasis

Lipid homeostasis is vital for the normal development, maintenance and reproduction of metazoans, given their transversal involvement in a great variety of metabolic processes, such as energy storage, membrane composition, as intracellular signaling molecules, enzyme cofactors and several others (Birsoy et al., 2013). In vertebrates, lipid metabolism is tightly regulated so that the organism can meet its physiological needs (Castro et al., 2016; Mello, 2010; Santos et al., 2012) (see fig.2). This metabolic regulation involves three major forms (Desvergne et al., 2006):

a. Allosteric control of enzyme activity along a metabolic pathway through the binding of an activator (for example, the enzyme-substrate);
b. Post-translational modifications, which activate/deactivate the enzyme;

One example is the phosphorylation/dephosphorylation of Acetyl CoA Carboxylase (ACC). Low glucose levels cause the dephosphorylation of ACC down-regulating the fatty acid synthesis, while high glucose levels cause the opposite response. This regulation is essential in the balance between β-oxidation and fatty acid (FA) synthesis. (Berg et al., 2002; Nelson et al., 2005).

c. Transcriptional regulation;

This regulation of lipid metabolism occurs through the action of several transcription factors, including nuclear receptors (NRs), sterol regulatory element-binding proteins (SREBPs) and CCAAT/enhancer binding proteins (C/EBPs) (Desvergne et al., 2006). The transcription factors can up or down-regulate the transcription of specific genes and protein synthesis (Lempradl et al., 2015; Lyssimachou et al., 2015). NRs include peroxisome proliferator-activated receptors (PPARs), pregnane X receptor (PXR), liver X receptor (LXR), farnesoid X receptor (FXR), all heterodimeric partners of retinoid X receptor (RXR), among others. The PPARs are crucial in the regulation of fat storage and FA β-oxidation. Mammals display three different PPAR genes: PPARα regulates enzymes involved in the up-take of fatty acids, fatty acid esterification and β-oxidation; PPARβ regulates FA oxidation in the muscles and PPARγ regulates and is

essential for adipogenesis. In vertebrates, in addition to function as an xenobiotic sensor, PXR is also involved in the regulation of lipid homeostasis in the liver and is capable of regulate PPARγ expression (Carazo et al., 2017; Mello, 2010). LXR, FXR and SREBP-2 play a key role in cholesterol homeostasis; LXR regulates the expression of SREBP-1c (Eberlé et al., 2004), while FXR activates the expression of PPARα. C/EBPs are involved in adipogenesis and can activate the expression of PPARγ (Nerlov, 2007) and SREBP-1c apart from cholesterol is also involved in the fatty acid synthesis (see Fig.2) (Reviewed by Alaynick, 2008; Berkenstam and Gustafsson, 2005; Desvergne et al., 2006; Mello, 2010).

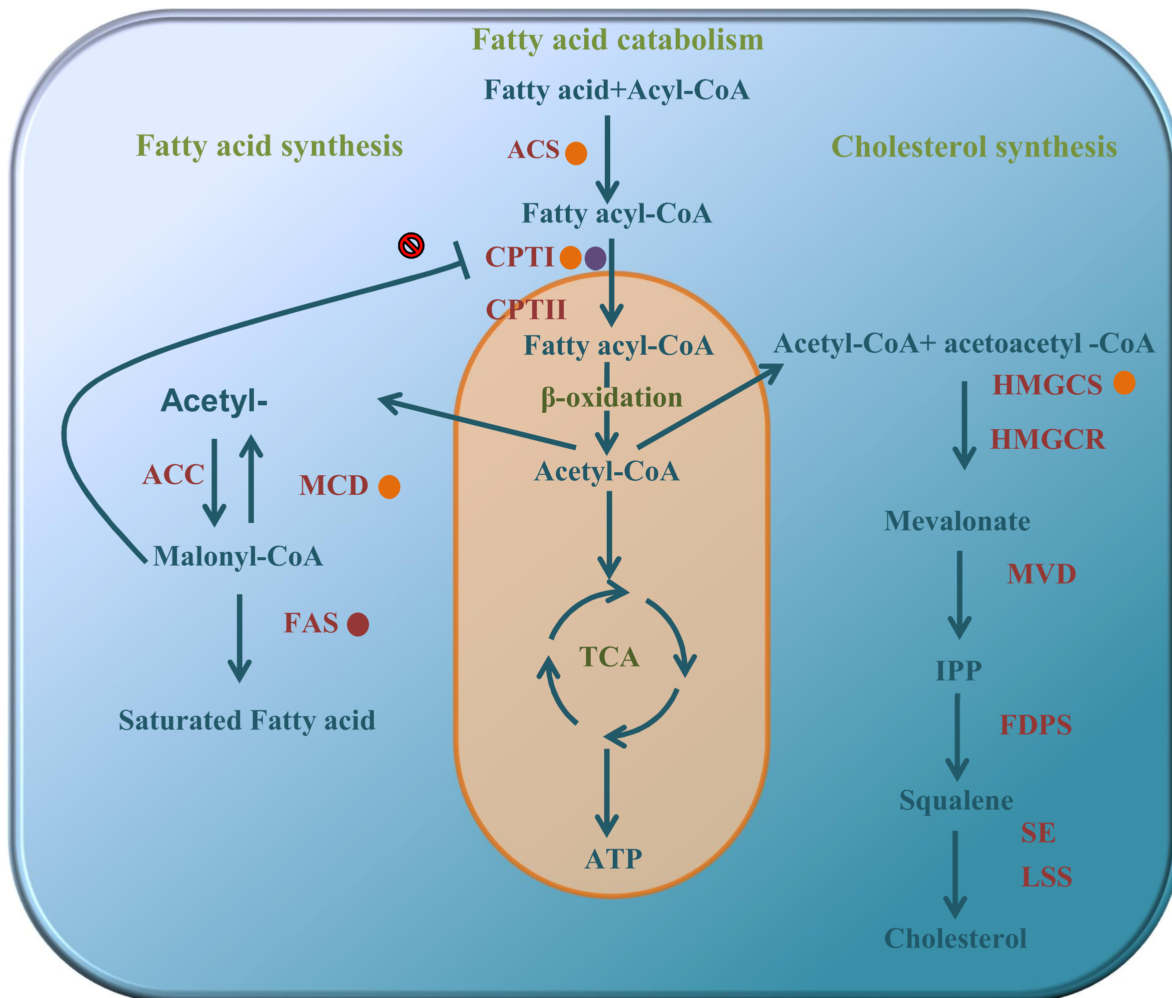

Fig. 2- Schematic representation of three key pathways involved in human lipid metabolism highlighting the involvement of Nuclear Receptors (PPARα ;PPARβ ; LXR). **Fatty acid synthesis** – acetyl CoA carboxylase (ACC) catalyses the synthesis of malonyl-CoA from acetyl CoA; fatty acid synthetase (FAS) uses malonyl-CoA, acetyl CoA and NADPH to synthetize saturated fatty acids. **Fatty acid catabolism** – acyl-CoA synthetase (ACS) catalyse the formation of fatty acyl-CoA from fatty acids and coenzyme A (CoA); carnitine palmitoyltransferase 1 and 2 (CPTI and CPT II) transport the fatty acyl-CoA across the mitochondrial membrane; the fatty acyl-CoA is degraded in the β-oxidation pathway producing acyl-CoA; acyl-CoA is the used in the citric acid cycle (TCA) to produce ATP. **Cholesterol synthesis** -Hydroxymethylglutaryl-Coenzyme A synthase (HMGCS) and Hydroxymethylglutaryl-Coenzyme A reductase (HMGCR) uses acetyl-CoA and acetoacetyl-CoA to produce Mevalonate; Mevalonate is transformed in isopentenyl pyrophosphate (IPP) through the enzyme diphosphomevalonate decarboxylase (MVD); Farnesyl diphosphate synthase (FDPS) transforms IPP in squalene; squalene is transformed in Cholestrol by squalene epoxidase (SE) and lanosterol synthase (LSS) (Based on (Alaynick, 2008; Berkenstam and Gustafsson, 2005; Birsoy et al., 2013; Desvergne et al., 2006; Mello, 2010; Shi and Burn, 2004).

Obesogens

Although this research area is relatively recent, several *in vitro* and *in vivo* studies have already shown the effect of obesogens in vertebrates (Chamorro-García et al., 2013; Grün and Blumberg, 2009a, 2009b). The differentiation of 3T3-L1 cells in adipocytes is stimulated in the presence of several putative obesogenic compounds such

as organotins (Tributyltin (TBT) and Triphenyltin (TPT)) (Pereira-Fernandes et al., 2013), phthalates (Mono-ethyl-hexyl phthalate (MEHP), mono-benzyl phthalate and mono-sec-butyl phthalate) (Hurst and Waxman, 2003) and BPA (Masuno et al., 2005). Several *in vivo* observations support these *in vitro* results. Chamorro-Garcia and co-workers studied the effect of TBT and Rosiglitazone (Rosi) (a therapeutic drug and PPARγ agonist) throughout 3 generations of C57/6J mice. The parental generation (F0) was exposed to TBT (5.42, 54.2 and 542 nM) or Rosi (500nM), the offsprings (F1) were exposed *in utero*, F2 was exposed as germ cells and F3 was not exposed at all. All 3 generations (F1, F2 and F3) from the TBT parental exposure presented, to some extent, an increase in the white adipocyte tissue (WAT), liver lipids and alteration in the expression profile of several genes involved in lipid metabolism (*Pparα*, *Pparγ*, Lipoprotein Lipase (*Lpl*), *Srebp1c*, Acyl-CoA Oxidase (*Acox*) and Fatty Acid Synthase (*Fasn*)). Similar results, although less pronounced, were observed with Rosi for epidydimal and perirenal WAT. The adipocyte number in F3 remained unchanged while in F1 and F2 it decreased particularly in epidydimal and perirenal WAT. It is important to highlight that the observations in F3 animals reveal transgenerational effects (Chamorro-García et al., 2013).

An increase in the offspring weight were also observed after exposure to phthalates (diethyl-hexyl-phthalate) (0.25 mg/kg b.w.) (Hao et al., 2013) and to BPA (70 μg BPA/kg/day) (Somm et al., 2009) in C57BL/6 mice and Sprague-Dawley rats, respectively. Importantly, in the last decades an increase in the medium weight has been observed in several other mammalian species living in the vicinity of human populations, e.g. macaques, chimpanzees, vervet monkeys, marmosets, mice, rats, dogs and cats (Klimentidis et al., 2011). This gives further support to the hypothesis that environmental factors of anthropogenic origin are likely to contribute to human obesity.

Interaction with NR

Several EDCs have been reported to interfere with the metabolism through interaction with NRs and the lipid metabolism is no exception (Santos et al., 2012). The NRs present two very conserved domains (Fig.3), the DNA binding domain (DBD) and the ligand biding domain (LBD). The DBD is responsible for the ligation of the NR to the DNA responsive element of target genes, while the LBD accommodate the ligand (Evans and Mangelsdorf, 2014; Mello, 2010; Thornton, 2003). The NRs ligands are very diverse and normally are small hydrophobic molecules. Although the NRs present specific natural ligands, their relation with the recent boom of chemicals seems to be

promiscuous (Thornton, 2003). Human and/or mouse PPARs, for example, were reported to interact with several groups of chemicals that include organotins (Harada et al., 2015; Hiromori et al., 2009; Kanayama et al., 2005), several phthalates (Bility et al., 2004; Cocci et al., 2015; Feige et al., 2007; Hurst and Waxman, 2003; Kanayama et al., 2005; Schlezinger et al., 2004) and some pesticides (Kanayama et al., 2005; Takeuchi et al., 2006). Other important NRs implicated in lipid homeostasis have also been reported to be modulated by EDCs, e.g., human FXR by alkylphenols, BPA and a phthalate (Butyl benzyl phthalate (BBzP)) (Kanayama et al., 2005), and LXRα was modulated by alkylphenols and phthalates (Kanayama et al., 2005; Mozzicafreddo et al., 2015).

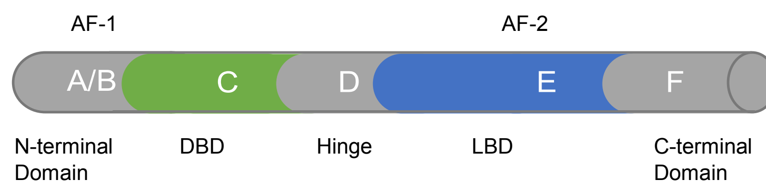

Fig. 3 - Modular structure of nuclear receptors. N-terminal is important for the interaction with co-regulaters and transcription factors. DBD – DNA binding domain is highly conserved, recognize and bind to DNA response element. LBD – Ligand binding domain is also conserved and responsible for the ligand-dependent activation of the transcripsion.

Metazoans, commonly known as animals, include a wide variety of organisms that can be organized into several groups on the basis of their evolutionary history (Fig.4) (Jenner, 2007). The key molecular components implicated in lipid metabolism have likely appeared in the ancestor of animals, although for the vast majority of animal lineages functional studies are still missing (Castro et al., 2016; Castro and Santos, 2014). This includes several transcription factors such SREBP and C/EBP, genes coding for metabolic enzymes, for example Acetyl-CoA Acyltransferase (*acaa1*) and carnitine palmitoyltransferse (*cpt*), and the storage of fat as Triacylglycerol (TAG) is also a common feature. When looking into fat storage we find an increasing complexity. Several early branching lineages do not possess a specific organ for fat accumulation, but for example arthropods present an organ with similar liver and adipose tissue functions, called fat body (Birsoy et al., 2013; Lavarías et al., 2009). In vertebrates, liver and adipose tissue are distinct and while in fishes, amphibians and reptiles the adipose tissue is concentrated in intra-abdominal regions, in mammals it is distributed widely in the body. In mammals the hepatic lipid stores are less relevant than in early-branching vertebrates (reviewed by Birsoy et al., 2013). The genomic and physiological evolution occurred in parallel (Brown, 2002). During the course of animal evolution, several duplications waves occurred (e.g. genome duplications in vertebrates), and these have likely contributed to shape the gene repertoire participating in lipid cascades (Brown, 2002; Crow and Wagner, 2006; Lundin, 1999). Within NRs, RXR is present in most

metazoans with exception of sponges and some cnidarian species (Sladek, 2011). In contrast, LXR and FXR seem to be deuterostome novelties (Bertrand et al., 2004; Fonseca et al., 2017). Mammals have three PPARs and up to five are observed in teleost genomes (Bertrand et al., 2004; Santos et al., 2012). Outside deuterostomes, PPAR has only been reported in mollusks (Vogeler et al., 2014), though without any functional characterization. The Ecdysone Receptor (ECR), a potential LXR/FXR orthologue (Ollikainen et al., 2006) was thought to be unique of arthropods but has recently been reported in nematodes, mollusks and annelids (Laguerre and Veenstra, 2010). The existence of this diversity of NRs seems to be linked with genome evolution. It appears that although several differences exist in lipid accumulation processes throughout metazoans, several important features are conserved. Therefore, it is not surprising that the obesogen effects are observed in other animal groups beyond mammals (Birsoy et al., 2013; Jordão et al., 2015; Lyssimachou et al., 2015).

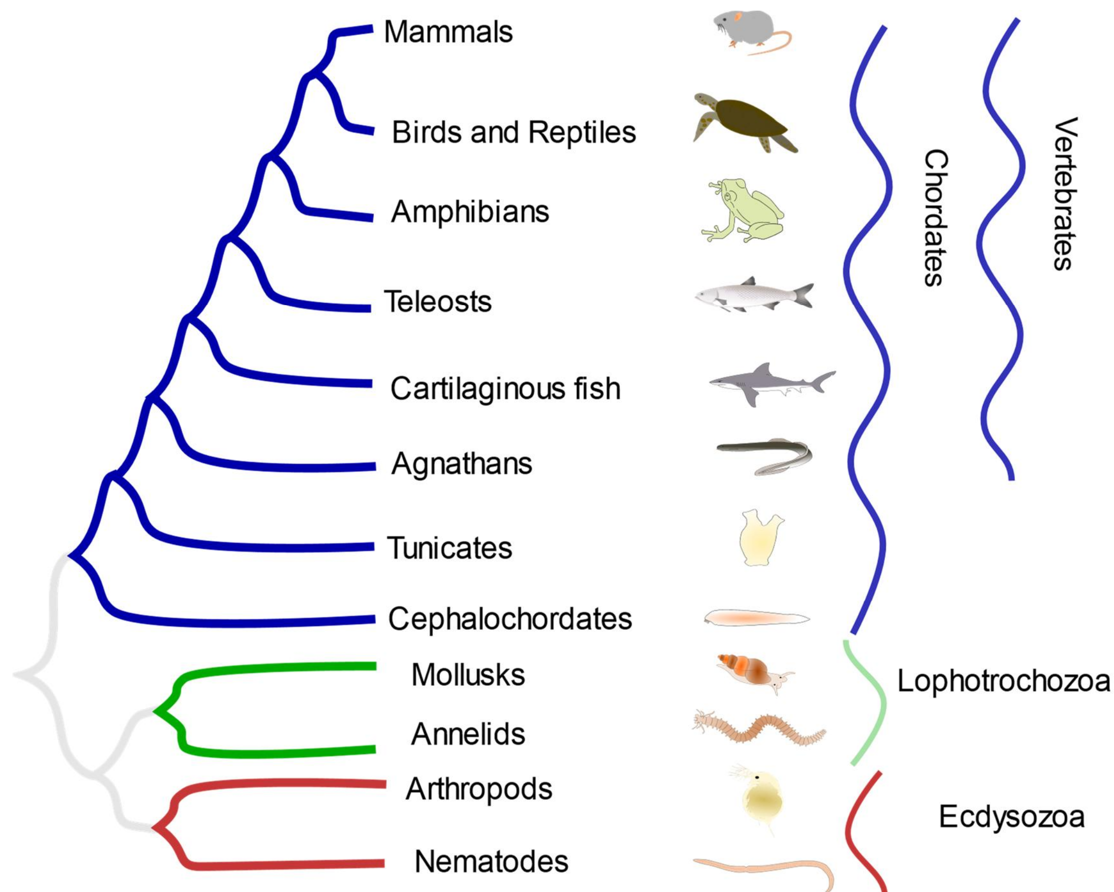

Fig. 4 – Phylogenetic tree highlighting the groups addressed in this review. The most basal metazoans don't show bilateral symmetry. Within those presenting a bilateral symmetry, in protostomes during the embryonic development the blastopore originate the mouth whereas in deuterostomes the blastopore originate the anus. The protostomes include the Ecdysozoa (such as arthropods and nematodes), animals with periodical molting process, and Lophotrochozoa (such as mollusks and annelids), most animals present a lophophore during some stage of their life cycle. The deuterostomes include Echinodermata, Hemichordata and Chordata (Tunicata), Cephalochordata, Vertebrata) (Jenner, 2007).

Obesogens outside mammals

Although aquatic animals are likely to be exposed to several obesogens in their habitat (Lyssimachou et al., 2015), the main focus of previous studies, including recent reviews, has been the modulation of lipid homeostasis by obesogens in mammalian models. However, a growing body of literature indicates that many more metazoan groups in addition to mammals are likely to be affected by this group of chemicals. An overview of the main effects of EDCs on lipid metabolism in aquatic animals is presented below.

Amphibians

Organotins

Organotins are persistent organic pollutants, introduced in the environment in 1960s (Antizar-Ladislao, 2008; Maynard, 2002), and were produced for diverse uses from antifouling agents in paints to the textile industry (Antizar-Ladislao, 2008; Bašić et al., 2012; García-Mayor et al., 2012; Maynard, 2002). Although TBT has been banned since 2008 by the European Union, this compound is still widely distributed in marine and freshwater environments (Antizar-Ladislao, 2008; Gipperth, 2009). A recent study in one of the largest arbor region in china revealed biological samples with concentrations of organotins ranging from non-detectable concentrations to 826.7µg/Kg. The TBT concentration reached 662.5µg/Kg and TPT in lower concentration but reaching the maximum of 92.4µg/Kg (Chen et al., 2017).

Organotins have been reported to alter amphibian lipid metabolism. *Xenopus laevis* when exposed (from stage 48 to metamorphosis) to different concentrations of TBT (0.33-3.3µg/L) revealed a dose-dependent increase in the formation of ectopic adipocytes in and around the gonads of both sexes. In males exposed to 3.3µg/L of TBT, the testicular tissue was replaced by adipocytes along the anterior-posterior axis (Grün et al., 2006). Exposure, under the same conditions, to the RXR-specific ligands (10 – 100 nM of LG100268 and AGN195203) and PPARγ agonist troglitazone (0.1 – 1 µM) resulted in similar alterations, pointing to RXR/PPARγ pathway as the action mechanism which is consistent with the observations in mammalian models (Grün et al., 2006). . Despite the lack of alterations in the whole-body weight, the exposure of the wood frog *Lithobates sylvaticus* from 0.1-5µg/L TPT from day 3 post-hatching up to 100-days, altered the expression of key lipid metabolism genes of the RXR/PPAR signalling pathway. A decrease in the expression of *rxrα* and *pparα* was observed in the animals exposed to both concentrations of TPT after 7 days and a decrease in *lpl* was observed in the animals exposed just to the lowest concentration. After 100 days, an increase in

the expression of genes such as *pparα* and *cyp4b1* was observed in the animals from both concentrations and an increase of *pparγ* and *lpl* was observed in the animals from the highest concentration (Higley et al., 2013). TPT and TBT were reported to activate the human RXRα and PPARγ, the results previous described suggest a similar effect in the amphibian RXRα and PPARγ (Grün et al., 2006; Kanayama et al., 2005).

Atrazine

The herbicide atrazine ((2-chloro)-4-(ethylamino)-6-(isopropylamino)-S-triazine), a photosynthesis inhibitor commonly detected in the water surface (McCarthy and Fuiman, 2008) also interferes with the lipid metabolism in amphibians. In Texas coast near agriculture areas were detected concentrations of 62.5 µg/L (Pennington et al., 2001), but immediately after application of the herbicide is common to detect concentrations of 400 µg/L in puddles (Zaya et al., 2011a).

Atrazine caused a decrease in the fat body size and in the weight of *Xenopus laevis* exposed to 200µg/L and 400µg/L from stage 47 until stage 66 (or 43 to 62) without observing any alteration in the feeding behaviour (Langerveld et al., 2009; Zaya et al., 2011a, 2011b). The animals exposed to 400µg/L presented an increase in the transcription of genes involved in lipid catabolism and carbohydrates metabolism and a decrease in the genes involved in lipid storage (Langerveld et al., 2009; Zaya et al., 2011a). The pathways were gene expression was altered are in line with the decrease in fat storage of the animals. One possible mechanism proposed for the response to atrazine was the down-regulation of the glucocorticoid hormone, other important alteration that can explain de results observed include an increase in the expression of *pparβ* involved in the utilization of fat for energy (Zaya et al., 2011a). The results observed are in accordance with previous studies in mammals, low-doses of atrazine can lead to weight gain through mild mitochondrial damage, while high doses can be toxic and prevent weight gain (Lim et al., 2009).

Other compounds

Several pharmaceuticals can be found in the aquatic ecosystems worldwide varying from ng/L or µg/L in urban environments to mg/L near production sites (Brodin et al., 2014). A mixture of pharmaceuticals (naproxen, diclofenac, atenolol and gemfibrozil) (100 and 1000 µg/L) caused a decrease in the hepatic triglycerides levels and an increase in the condition factor (body weight/ snout-vent length^3×100) of *Limnodynastes peronei* following 30-days exposure after reaching Gosner developmental stage 26. The triglycerides decrease is probably due to gemfibrozil since

this compound is used to regulate cholesterol levels in humans and is known to interact with lipid metabolism (Melvin, 2016).

BPA is a component of polycarbonated plastics widely used in different products like baby bottles, beverage containers and food cans (Bašić et al., 2012). The aquatic contamination with BPA is very variable, the concentrations can vary from non-detected to 17 mg/L in landfill leachate (Canesi and Fabbri, 2015; Flint et al., 2012). Although BPA has an obesogenic effect in several species (Guan et al., 2016; Somm et al., 2009), the exposure of *Xenopus tropicalis* embryos to BPA (3.4 mg/L), during 11 weeks, did not alter the *pparα* and *pparγ* gene expression neither the weight of the animals (Mathieu-Denoncourt et al., 2015). However, it would be important to evaluate a larger range of concentration.

Table 1- *Summary of effects of environmental chemicals in amphibian lipid homeostasis (green: up-regulated; red: down-regulated; n.d – not determined; bold-minimum effect concentration).*

Compound	Exposure	Species	Endpoints			Ref.
			Weight/Lipids alterations	Alteration in gene expression	Biochemical alterations	
TBT	0.33-3.3 µg/L (water); stage 48 to metamorphosis;	*Xenopus laevis*	Formation of ectopic adipocytes;	n.d	n.d	(Grün et al., 2006)
TPT	0.1-5.0 µg/L (water); 7 and 100 days;	*Lithobates sylvaticus*	7 days: weight; d snout-ventral-length;	7 days: *rxrα; pparα; scd1*(5 µg/L); *fas*(5 µg/L); *lpl*(0.1 and 5 µg/L); 100 days: *pparα; cyp4b; pparγ*(1 µg/L); *fas*(1 µg/L); *lpl*(1 µg/L);	n.d	(Higley et al., 2013)
Atrazine	200µg/L and 400µg/L(water); Stage 47 -66;	*Xenopus laevis*	Fat body size; Weight;	n.d	n.d	(Zaya et al., 2011b)
	400µg/L(water); Stage 47 -66;	*Xenopus laevis*	n.d	Microarray analysis: Genes involved in the lipid catabolism; Genes involved with lipid storage;	n.d	(Zaya et al., 2011a)
	400ppb (water); Stage 43 -62;	*Xenopus laevis*	Fat body size;	Microarray analysis: Genes involved with proteolysis, digestion and carbohydrates metabolism;	n.d	(Langerveld et al., 2009)
Mixture: naproxen, diclofenac, atenolol and gemfibrozil	0.1, 1.0, 10, 100 and 1000 µg/L (water); 30 days;	*Limnodynastes peronii*	Condition factor; LSI;	n.d	Hepatic triglycerides content (100 and 1000 µg/L);	(Melvin et al., 2016)
Compound	Exposure *in vitro*	System	Species	Endpoints		Ref.
TBT	2µg/L;	Cells-Cos 7; Luciferase assay;	*Xenopus laevis*	Transactivation RXRα and RXRγ;		(Grün et al., 2006)

Teleosts

Organotins

Alterations in lipid storage are among the effects reported in fishes exposed to TBT. Several species (*Oncorhynchus tshawytscha, Danio rerio, Carassius auratus* and *Sebastiscus marmoratus*) when exposed to TBT through water (1 ng/L-16,5 µg/L) or diet (0.4-150 ng/g/fish/day) exhibit an increase in lipid and/or TAG content, adiposity (adipocyte size) and/or altered body weight. The alterations reported were observed in acute (16,5 ng/L-16,5 µg/L for 1 day) and chronic experiments (1 ng/L-135 ng/L during 48 days -9 months) (Lyssimachou et al., 2015; Meador et al., 2011; Ouadah-Boussouf and Babin, 2016; Riu et al., 2014; Tingaud-Sequeira et al., 2011; Zhang et al., 2016). The alterations in the expression pattern of several genes involved in the lipid metabolism, in particular the alterations observed in the nuclear receptors *pparγ* and *rxr* and their downstream pathways is in line with the hypothesis that TBT alter the lipid metabolism through the interaction with the heterodimer PPARγ/RXR (Lyssimachou et al., 2015; Zhang et al., 2009). The impact of TBT in the metabolism of fish seems to be gender and tissue-specific. A different expression pattern of key genes in the lipid metabolism was observed in liver and brain and in females and males of *Danio rerio* when exposed to TBT (Lyssimachou et al., 2015). Is important to highlighted these differences to better understand the total impact of TBT in the lipid homeostasis.

The alterations in the expression of the NRs *rxr* and *ppar*, and their down-stream pathways are in line with the response of mammalian models (Harada et al., 2015; Kanayama et al., 2005; le Maire et al., 2009; Santos et al., 2012). More recently, new evidences suggests that TBT may impact lipid homeostasis not only through PPARγ/RXR but also through the LXR/RXR heterodimer (Ouadah-Boussouf and Babin, 2016). To enlighten these possibility the authors measured the TBT-induced adipocity by using a fluorescent dye (Nile Red), to quantify the lipid droplets in zebrafish; this test is known as the zebrafish obesogenic test (ZOT) (Tingaud-Sequeira et al., 2011). This study showed that only the agonists (or antagonists) of PPARβ/δ (GW501516/GSK3787), PPARγ (rosiglitazone/T0070907), RXR(DHA/UVI3003) and LXR (GW3965/GSK1440233A) could increase (or decrease) the adiposity, indicating that these NRs are the potential targets for TBT. The antagonists of those NRs where then used to rescue the obesogenic response of TBT; the RXR antagonist was fully successful, while the LXR antagonist reverted only partially the TBT effect. The other antagonists failed to rescue the TBT effects. The use of an antagonist of RXR homodimer, that is also an agonist of the heterodimer RXR/PPARγ, combined with LXR antagonist and TBT revealed that RXR/PPARγ is also involved in the action of TBT since

this mix could not rescue the effect of TBT, but the addition of PPARγ antagonist could decrease the adiposity. Taken together the effects of TBT seem to be mediated through RXR/LXR and/or RXR/PPARγ, both permissive heterodimers, which suggests that TBT appears to act through RXR, modulating the downstream pathways to increase the adipocyte number (Ouadah-Boussouf and Babin, 2016). However, Riu and co-workers did not observe any increase in lipid accumulation in the zebrafish embryos (from 3dpf to 11dpf) exposed to 100nM and 1µM of Rosi probably because in this early life stage WAT in fish is hardly developed (Flynn et al., 2009; Imrie and Sadler, 2010; Riu et al., 2014). Moreover, 1µM of Rosi failed to transactivate zebrafish PPARγ, supporting the hypotheses that the effect of TBT in the heterodimer RXR/PPARγ occurs through RXR (Riu et al., 2011). Moreover, it was observed that the cysteine present in the position 285 is essential for the activation of human PPARγ by TBT (Harada et al., 2015) and *Danio rerio* PPARγ has a tyrosine in the correspondent position (Den Broeder et al., 2015). This substitution in PPARγ of *Danio rerio* is expected to affect the affinity of TBT to PPARγ. Interestingly, this mutation of cysteine 285 is not common to all members of Actinopterygii, since *Sparus aurata* and *Pleuronectes platessa,* for example, have a cysteine molecule in the homologous position (Leaver et al., 2005).

BPA and BPA analogues

BPA and BPA analogs are ubiquitous chemicals detected in the environment, BPA levels reached 17 mg/L in Japanese landfill leachate and 370 µg/L in paper-mill effluents (Fukazawa et al., 2002; Yamamoto et al., 2001). Moreover, recent data suggests that this group of chemicals is able to impact lipid homeostasis (Birceanu et al., 2015; Riu et al., 2014, 2011). The reported effects of BPA and BPA analogs include increase in lipid accumulation and body mass in *Danio rerio* when exposed to Tetrabromobisphenol A (TBBPA) (5.5 and 55 µg/L) or tetrachlorobisphenol A (TCBPA) (37 µg/L) during 19 days, and in *Sparus aurata* when exposed to BPA (50mg/Kg bw) during 21 days, (Maradonna et al., 2015; Riu et al., 2014, 2011). However, in *Gobiocypris rarus* females a decrease in serum TGA content was observed under BPA (15µg/L) exposure (Guan et al., 2016). Although the transcription patterns of several genes involved in lipid metabolism were altered in *Sparus aurata* (exposed to 50mg/Kg bw of BPA) and in *Gobiocypris rarus* (exposed to 15µg/L BPA), the pattern of expression is not the same, the *fasn* gene appeared up-regulated in *Sparus aurata* and down-regulated in *Gobiocypris rarus* males (Guan et al., 2016; Maradonna et al., 2015). The NRs involved in the lipid homeostasis (PPARα, β, γ and RXR) and lpl genes were up-regulated while hormone sensitive lipase (*hsl*) gene was down-regulated in *Sparus aurata* witch is consistent with the lipid accumulation observed (Maradonna et al., 2015).

In *Gobiocypris rarus* the decrease of *fasn*, acetyl-CoA carboxylase 1 (*acaca*) and acetyl-CoA carboxylase 2 (*acacb*) expression is not consistent with the respective enzymatic activity, but carnitine palmitoyltransferase 1A (*cptla*) expression and enzymatic activity is increased in males and decreased in females (Guan et al., 2016). The disparity between gene expression and enzymatic activity is explained by the several regulatory processes existent post mRNA (Vogel and Marcotte, 2012). The effect of BPA in lipid homeostasis of *Gobiocypris rarus* suggests a gender specificity since the activity of CPT1 is increase in males, but decrease in females similar to *cptla* gene expression. Besides the increase in the TAG content in females serum and the tendency for decrease in males is consistent with the previous observations (Maradonna et al., 2015). The differences in the observations in the *Sparus aurata* study and *Gobiocypris rarus* can be related with the gender since in *Sparus aurata* the observations did not take in account the gender of the specimens tested (Guan et al., 2016; Maradonna et al., 2015).

One possible mechanism for the action of BPA analogs in the lipid metabolism is the modulation of PPARγ. Riu and co-workers (2011) observed that BPA analogs, 10μM TBBPA and 10μM TCBPA, are able of *in vitro* transactivating zebrafish PPARγ with a similar affinity to that observed in human PPARγ (Riu et al., 2011). An alternative hypothesis involves estrogenic pathways (García-Mayor et al., 2012), as BPA has the potential to bind and activate the estrogen receptor (ER), and interact with a variety of other targets in mammalian cells, including thyroid hormone receptors (Bašić et al., 2012; Bonefeld-Jørgensen et al., 2007). Further research is needed to fully understand the underlying mechanisms.

Alkylphenols

Alkylphenols are commonly used in industrial and consumer products (Pereira-Fernandes et al., 2013). Concentrations between 1.1-1347μg/Kg of nonylphenol (NP) and 0.73-54.4μg/Kg of octylphenol (t-OP) were detected in mussels and 5-60.5μg/Kg of NP and 0.2-31.4μg/Kg in fishes (David et al., 2009). The Exposure of *Sparus aurata* to NP (50mg/Kg bw) or t-OP (5 mg/Kg bw and 50mg/Kg bw) caused an increase in the number of specimens with severe lipid accumulation in the liver, more pronounced in the t-OP groups. The transcription patterns of several genes involved in lipid metabolism (i.e. *pparα*, *pparβ*, *pparγ*, *rxr*, *fasn*, *lpl* and *hsl*) were also increased, emphasizing the effect of NP and t-OP in the lipid metabolism (Maradonna et al., 2015).

Is known that alkylphenols, such as NP and t-OP, are able of the activation of the human Estrogen Receptor (ER) and several evidences point to the possible involvement

of ER in the action of specific obesogens, explaining the results observed (Bonefeld-Jørgensen et al., 2007; Kramarova et al., 2009; Yang et al., 2015).

Phthalates

Phthalates are synthetic organic compounds derived from phthalic acid. These compounds are used since the 1930s as plasticizing agents in cosmetics, paints and medicines and were found in the aquatic environment in concentrations as high as 98μg/L (Fromme et al., 2002). Recent evidences shows that these compounds also target the lipid metabolism of fish (García-Mayor et al., 2012; Grün and Blumberg, 2009b, 2007; Pereira-Fernandes et al., 2013). Phthalates were reported to increase the transcription of NR involved in lipid metabolism (*ppaarα*, *pparβ* and *pparγ*) in zebrafish exposed to 39μg/L DEHP and 1.7μg/L – 1.7mg/L phthalic acid (PA) (Maradonna et al., 2013). In *Sparus aurata*, exposure to diisodecyl phthalate (DiDP) (45.1– 450. 7μg/L) not only upregulate the expression of *ppar*s and *rxr*, but also the expression of some downstream genes, *cptIa*, *cptIb*, fatty acid desaturase 2 (*fads2*), stearoyl-CoA desaturase 1A (*scd1a*), *scd1*, *lpl*, hepatic lipase (*hl*), fatty acid binding protein (*fabp*), apolipoprotein A-I (*apoa*-1) and *srebp*) (Cocci et al., 2015). In zebrafish exposed to 5000 mg/Kg di-2-ethylhexylphthalate (DEHP) for 10 days was not observed any alteration in the downstream genes of PPAR pathway (acox and lpl) in the liver, although the hepatosomatic index was increased. The alteration in the hepatosomatic index can be associated with obesity or as a response to the toxicity of the compound (Fabbrini et al., 2010; Milić et al., 2014; Sadekarpawar and Parikh, 2013; Uren-Webster et al., 2010). The interaction of phthalates with the lipid metabolism can occur through the PPARs. DiDPis can bind to *Sparus aurata* PPARα and γ with similar affinity as to the human PPARs, MEHP can transactivate zebrafish PPARγ and the relation between the interaction of phthalates with PPARs and their obesogenic effect was already set for mammals (Cocci et al., 2015; Desvergne et al., 2006; Maradonna et al., 2013; Riu et al., 2014).

Organophosphates

Some organophosphates, such as Fenitrothion and Trichlorfon, are pesticides that have been used extensively in agriculture and can enter the freshwater environments (McCarthy and Fuiman, 2008; Sancho et al., 2009; Xu et al., 2012). Xu and co-workers described an increase in the liver TAG content in the *Carassius auratus gibelio* exposed to trichlorfon (1.0-4.0 mg/L) for 30 days (Xu et al., 2012). However, Sancho and co-workers reported a decrease in liver total lipid content in *Anguilla anguilla* after a short-term exposure (2 and 96 hours) to fenitrothion (0.4mg/L) (Sancho et al.,

1998). The mechanism of action of these compounds in lipid metabolism remains to be elucidated, but similar effects were already reported in mammals (Meggs and Brewer, 2007).

Fibrates

Fibrates are commonly used to control hypercholesterolemia in humans. Given their widespread use, they are frequently detected in the freshwater environment (Velasco-Santamaría et al., 2011). One of the first fibrates to be reported in water samples was clofibric acid (CA), an active and persistent metabolite of clofibrate, a blood lipid lowering agent (Emblidge and DeLorenzo, 2006; Runnalls et al., 2007). Clofibrate was detected in surface water at concentrations of 6-7µg/L, Fenofibrate and Gemfibrozil were detected in much lower concentrations (≈0.05µg/L) (Corcoran et al., 2010). Several studies demonstrated the capacity of these compounds to interact with the lipid metabolism in non-mammalian animal models. A decrease in the weight of mosquito fish (*Gambusia holbrooki*) males when exposed to clofibrate (18.4 to 295 mg/L) and an increase in females exposed to CA (4.03 mg/L) during 28 days have been reported (Nunes et al., 2004). In contrast, an exposure to CA at 10µg/L for 10 days did not alter the levels of lipids and cholesterol in the fish *Fundulus heteroclitus* (Emblidge and DeLorenzo, 2006).

A multigenerational study using zebrafish revealed very informative results on the effect of CA. F0 generation was exposed to 1 mg/g and 10 mg/g CA through the diet and the offspring (F1) were raised with control diet. A decrease in the weight and muscle triglyceride levels was observed in F0, but F1 presented an increase in weight in the offsprings of the highest exposure group. The gene expression pattern for both generations was also different. In male livers of the F0, the expression of *pparα* and *acox1* increased in the animals exposed to the highest concentration, and in F1 although the expression of *pparγ* increased, the expression of *apoa*-1 and *pparβ1* decreased. This study reveals an opposite pattern of response in the descendants when compared with the parent generation (Coimbra et al., 2015). *pparα* expression also increased in *Cyprinus carpio* exposed to 20 mg/L of CA for 4 days and acox1 expression increased not only in the animals exposed to 20 mg/L but also in the animals exposed at a lower concentration (4µg/L). These responses are in line with the ones observed in zebrafish. The expression of other genes, such as Acetyl-CoA Acyltransferase 1 (*acaa1*), cytochrome P450 4 (*cyp4*), sterol 27-hydroxylase (*cyp27a*), *apoA1*, *lpl* and superoxide dismutase (*sod1*) also increased in one or both CA exposures. These observations

support the evidence that these compounds interfere with the lipid metabolism not only in mammals but also in the Actinopterygii group (Corcoran et al., 2015).

The effects of other fibrates, besides CA and clofibrate, have also been reported. A decrease in plasma cholesterol levels was found in zebrafish exposed to 1.7, 33 and 70 mg bezafibrate (BZF)/g food after 2 and 7 days of exposure, along with a decrease in the expression of *pparβ* and *pparγ* after 2 days of exposure and an increase in the expression of *pparβ* and Steroidogenic Acute Regulatory Protein (*star*) after 21 days of exposure (Velasco-Santamaría et al., 2011). The exposure of rainbow trout (*Oncorhychus mykiss*) to 100 mg of Gemfibrozil/Kg during 15 days increased the relative cholesterol levels and caused a decrease in the relative TAG levels and the phospholipids/triacylglycerol ratio. *lpl* gene expression was increased, but no alteration was observed in the *ppar*s genes (Prindiville et al., 2011). These results seem to be in line with data from mammalian models, as fibrates are known PPARα agonists and this NR is associated with fatty acid uptake and oxidation (Coimbra et al., 2015).

Other compounds

Besides the classes of compounds highlighted above, other compounds have also been reported to interfere with fish lipid metabolism. One example is the herbicide atrazine (0.17 mg/L) that caused an increase in hepatic lipid levels in the grey mullet (*Liza ramada*) after an exposure of 21 days (Biagianti-Risbourg and Bastide, 1995). Another example is DDT, a pesticide and well documented persistent organic pollutant (Lyche et al, 2013). Zebrafish exposed to 0.1µg/L and 1µg/L of DDT for 60 days displayed an increase in the saturated long chain fatty acids C16:0 and C18:0 along with monounsaturated C18:1n9 accompanied with a decrease of the levels of polyunsaturated fatty acids C20:3n3, C20:4n6, and C22:6n3 in a DDT-concentration-dependent manner. A decrease in the weight of the females was also observed for the concentration of 0.1µg/L DDT (Zhong et al., 2012).

Environmental mixtures

In the environment compounds are not present alone but in complex mixtures. In a study aiming to evaluate the effects of these complex mixtures, zebrafish was exposed through the diet from day 6 until 5 months post-fertilization to two different mixtures of organic pollutants extracted from fish liver of *Lota lota*, originated from two different lakes in Norway (Lake Mjøsa and Lake Losna). An increase in weight and alterations in the expression of genes related to lipid metabolism were observed. An Ingenuity Pathway

Analysis (IPA) established a relationship between the affected genes and PPARγ, ERα and Hepatocyte Nuclear Factor 4 A (HNF4A), suggesting that these NRs are the key regulators of the genes affected (Lyche et al., 2011; Nourizadeh-Lillabadi et al., 2009). The decedents of F1 were also exposed to the mixtures and presented a decrease in weight and length (Berg et al., 2011). The 3rd generation embryos (4, 7.5, 12 and 24 hpf) also presented alterations in the transcription pattern of the genes related with lipid metabolism (Lyche et al., 2013).

Several additional studies show evidences of changes in lipid homeostasis associated with chemical exposure in the field. Accumulation of lipid droplets and an increase in the *pparγ* and *rxrα* transcription levels was observed in the liver of Atlantic Bluefin tuna (*Thunnus thynnus)* associated with levels of dioxin-like PCBs above the safe limits established by the European commission regulation (EU n. 1259/2011 of 02.12.2011) (Maisano et al., 2015). In *Tilapia guineensis*, *Sarotherodon galileaus* and *Oreochromis niloticus* a correlation between the concentrations of Polycyclic aromatic hydrocarbons (PAHs) and PCBs and the increase of gene transcription of *ppar*s (α, β and γ) was also observed (Adeogun et al., 2016b). *Sarotherodon melanotheron* from two sites in Nigeria with different contamination levels showed alterations in the liver somatic index (LSI), kidney somatic index (KSI) and expression of the genes *pparα*, β and γ. The increase in LSI and in gene transcription was observed in a concentration dependent manner (Adeogun et al., 2016a).

Taking these studies together, it becomes evident that some EDCs can affect fish lipid homeostasis, even though only the sub-class teleost was analysed here. An important remark that should be considered in future studies concerns gender differences; in some studies, males and females show different responses, highlighting the importance of analysing the response in both genders.

Table 2- Summary *of effects of environmental chemicals in fish lipid homeostasis (green: up-regulated; red: down-regulated; n.d – not determined; bold-minimum effect concentration).*

Compound	Exposure *in vivo*	Species	Endpoints			Ref.
			Weight/Lipids alterations	Alteration in gene expression	Biochemical alterations	
TBT	2.8 ng/g and 150 ng/g (diet); 62 days;	*Oncorhynchus tshawytscha*	Total lipid content;	n.d	n.d	(Meador et al., 2011)
	16.3µg/L TBT (diet); 1 day;	*Danio rerio*	Adiposity;	n.d	n.d	(Tingaud-Sequeira et al., 2011)
	27.4-137.10ng/L (water); 9 months;	*Danio rerio*	Male weight. Male CF (27.4ng/L); Females CF; Female LSI;	Tissue: liver Male: *rxrα*/a; *pparγ*; *c/ebpβ*; *srbp1*; *dgat2*; *fasn*; 11β-*hsd2*; *igf*-IIα; Female: *pparγ*; *ifg*-IIα; Tissue: brain Male: *rxrα*/a; *c/ebpα*; *srbp1*; *chrebp*; *dgat2*; *fasn*; *igf*-IIα; Female: *rxrα*/a; *c/ebpβ*; *dgat2*; *accα*; *acoxl*; 11β-*hsd2*; 11β-*hsd3α*;	Male hepatic triglyceride;	(Lyssimachou et al., 2015)
	2.44 ng/L and 24.4 ng/L (water) ; 54 days;	*Carassius auratus*	Body weight; Food intake (only for 2.44 ng/L);	n.d	Metabolic rate indicators (only for 2.44 ng/L);	(Zhang et al., 2016)
	0,75µg/L and 7,5 µg/L (water); 60 days;	*Cyprinus carpio*	n.d	n.d	Enzymes activity: trypsin, lipase and amylase;	(Li et al., 2014)
	0.1 ng/L; 10 ng/L; 100ng/L; 48 days;	*Sebastiscus marmoratus* (females)	Tissue: Ovaries; Total lipid (100 ng/L);	n.d	n.d	(Zhang et al., 2013)
	2.7, 27.4, 274 ng/L (water); 48 days ;	*Sebastiscus marmoratus*	Total lipid content in testes (274ng/L);	Tissue: Testes *rxrβ* (27.4 and 274 ng Sn/L), *rxrγ*, *pparγ*;	n.d	(Zhang et al., 2009)
	3.25, 16.3, 163ng/L, 1.63 and 16.3 µg/L (water);	*Danio rerio*	Adiposity in starvation (163ng/L, 1.63 and 16.3 µg/L).	n.d	n.d	(Ouadah-Boussouf and Babin, 2016)

	1 day;					
	325.5ng/L (water); 3 dpf-11 dpf, followed by 19 days without TBT;	*Danio rerio*	Body mass index;	n.d	n.d	(Riu et al., 2014)
TBBPA	5.5-55µg/L(water); 3 dpf-11 dpf, followed by 19 days without TBT;	*Danio rerio*	Body mass index;	n.d	n.d	(Riu et al., 2014)
TCBPA	37µg/L(water); 3 dpf-11 dpf, followed by 19 days without TBT;	*Danio rerio*	Body mass index;	n.d	n.d	
BPA	15 µg/L (water); 28 days;	*Gobiocypris rarus*	n.d	Females: *acaca; acacb; gpat1;* Males: *fasn; gpat1; cptIα;*	Serum triglyceride content (females); Enzyme activity: FASN, ACC, CPTI (female); CPTI (males); GPAT (males);	(Guan et al., 2016)
NP t-OP	5mg/Kg (1) bw and 50mg/Kg (2) bw (diet); 21 days;	*Sparus aurata*	Liver: Lipid (1, t-OP1 and 2, BPA1); Food intake;	*lpl; hsl; pparα; pparβ; pparγ; rxr* (except t-OP)*; fas;*	n.d	(Maradonna et al., 2015)
DEHP	0.5 – 5000 mg/Kg (injection); 10 days;	*Danio rerio* (males)	HIS (5000 mg/Kg);	Tissue: testis *acox1;*	n.d	(Uren-Webster et al., 2010)
trichlorfon	1.0-4.0 mg/L (water); 30 days;	*Carassius auratus gibelio*	n.d	n.d	Triglyceride content in the liver;	(Xu et al., 2012)
fenitrothion	0.4mg/L (water); 2 - 96 hours;	*Anguilla Anguilla*	Lipid content in liver;	n.d	n.d	(Sancho et al., 1998)
clofibrate	18.4, 37.9, 73.8, 147.5 and 295 µg l^{-1} (water); 28 days;	*Gambusia holbrooki*	Male weight;	n.d	n.d	(Nunes et al., 2004)

Clofibric acid	F0- 1 mg/g and 10 mg/g (diet); F1 - control diet;	*Danio rerio*	Weight (F0); Triglyceride levels (muscle of male fish); Weight (F1) (10mg/g);	F0: *pparα; acox1;* F1: *pparγ; apoa1; pparβ1;*	n.d	(Coimbra et al., 2015)
	4µg/L (water); 4 and 10 days;	*Cyprinus carpio*	n.d	Tissue: liver *acox1; cyp4; lpl;* *acaa1; apoA1; cyp27a;* (only 10 days)	ACOX activity;(only 10 days)	(Corcoran et al., 2015)
	20mg/L (water); 4 and 10 days;			Tissue: liver *acox1; cyp27a;* *pparα;* (only 4 days) *apoa1; sod1;*	ACOX activity;	
BZF	1.7, 33 and 70 mg/g (diet); 2, 7 and 21 days;	*Danio rerio (male)*	n.d	2days: *pparβ; pparγ;* 21days: *pparβ; star; cyp17a1;*	Plasma cholesterol levels (7 and 21 days);	(Velasco-Santamaría et al., 2011)
Gemfibrozil	100 mg/Kg (injection); 15 days;	*Oncorhychus mykiss (female)*	n.d	*lpl;*	Relative cholesterol levels; Relative triacylglycerol levels; Ratio of phospholipids/triacylglycerol;	(Prindiville et al., 2011)
Atrazine	0.17 mg/L (water); 21 days;	*Liza ramada*	Lipid accumulation in the liver.	n.d	n.d	(Biagianti-Risbourg and Bastide, 1995)
DDT	0.1µg/L and 1µg/L (water); 60 days;	*Danio rerio*	Weight of the females (0.1µg/L);	n.d	Saturate long chain fatty acids (C16:0 and C18:0), in monounsaturated long chain fatty acid (C18:1n9); Polyunsaturated fatty acids) C20:3n3, C20:4n6, and C22:6n3);	(Zhong et al., 2012)
PFNA	0.01, 0.1, and 1.0 mg/L (water); 180days;	*Danio rerio*	Weight; Length; HIS (0.01 and 0.1 mg/L);	Males: *fabps; pparαa; pparαb; pparγ; pparβa; pparβb; c/ebps;* Females: *fabps; pparαa; pparαb; pparγ; pparβa; pparβb; c/ebps;*	Tissue: liver Total cholesterol level (0.1 and 1.0 mg/L); Triglyceride content (male); Triglyceride content (female);	(Zhang et al., 2012)

Compound	Exposure *in vitro*	System	Species	Endpoints	Ref.
TBT	32.6µg/L;	Liver cell line (RTL-W1)	Rainbow trout	Genes: *abca1; lpl; fas*	(Dimastrogiovanni et al., 2015)
TPT	36.8µg/L;			Genes: *abca1; fatp1; fas; lxr;* Lipids: TAG;	
4-NP	4.4mg/L;			Genes: *abca1; cd36; lpl; fas; pparβ;* Lipids: TAG;	
DEHP	1.9mg/L;			Genes: *cd36; lpl; fas;* Lipids: TAG;	
BPA	2.28mg/L			Genes: *abca1; cd36; fatp1; lpl; fas; lxr; pparα; pparβ;* Lipids: TAG;	
TBBPA	5.44mg/L	HGELN human cell line; (transfected with PPARγ)	*Danio rerio*	Transactivation;	(Riu et al., 2011)
TCBPA	3.66mg/L				
DEHP	19.53ng/L-39µg/L; 4 days	Hepatocytes	*Danio rerio*	Males genes (390ng/L-39µg/L): *pparα, pparβ, pparγ* (39µg/L); Females genes (39µg/L): *pparα; pparβ; pparγ;*	(Maradonna et al., 2013)
PA	1.66µg/L-1.66mg/L; 4 days;			Males genes: *pparα; pparβ;* Females genes: *pparα; pparβ;*	
Cu	635.5µg/L-6.35mg/L; 24h; 48h; 96h;	Hepatocytes	*Ctenopharyngodon idellus*	Genes: ***srebp-1c; acc; fas; hsl; pparα*** (24h); *pparα* (48h, 96h); ***cptI*** (24h); *cptI* (48h; 96h); Lipids: TAG;Enzyme activity: CPTI;	(Zhu et al., 2014)
DiDP	44.7, 446.7µg/L and 4.5mg/L;	Hepatocytes	*Sparus aurata*	Genes: *pparα, pparβ, pparγ, rxrα, cptIa, cptIb* (446.7µg/L), *fads2, scd1a, scd1b, lpl, hl, fabp, apo-1a* and *srebp* (44.7, 446.7µg/L);	(Cocci et al., 2015)

Non-vertebrates

In comparison with vertebrates, studies addressing the effects of putative obesogens in non-vertebrate *taxa* are scarce. Only a few studies have examined the chemical-induced disruption of lipid metabolism, mostly involving exposure to organotin compounds or complex mixtures.

Tunicata

Organotins

Ciona intestinalis ovaries exposed *in vitro* during 5 hours to 0.33 and 32.6µg/L TBT presented alterations in the lipid content. The increase in phospholipid levels and the decrease in TGA was proposed as a possible adaptive mechanism of resistance to the pollutant. An increase of the long chain fatty acids was also observed and related to an intensification of the membrane fluidity, which is in accordance with the capability of organotins to interact with membranes permeability (Puccia et al., 2005).

Mollusca

Organotins

Organotins also alter the lipid homeostasis in mollusks. TBT (1.4µg/L/L) caused an increase in total lipids of female gastropod *Marisa cornuarietis* and a shift was observed in the proportion of the fatty acids (an increase in the monounsaturated fatty acids and a decrease in the polyunsaturated ones) after 100 days exposure (Janer et al., 2007). In contrast to TBT, a short exposure to TPT caused a decrease in female total lipids (1.5µg/L) and in the total fatty acid levels (93.1ng/L-1.5µg/L) in *Marisa cornuarietis* after 7 days (Lyssimachou et al., 2009). In another gastropod species, *Nucella lapillus*, TBT exposure (100 ng/L – 200 ng/L) caused alterations in the gene expression of key enzymes of fatty acid metabolism, indicated by the up-regulation of Acetyl—CoA synthetase (*acs*) and *cptl* (Pascoal et al., 2013). In vertebrates, both these genes are regulated by the heterodimer PPAR/RXR (Alaynick, 2008; Desvergne et al., 2006; Mello, 2010). The mechanism of action of these compounds in mollusks is still not well understood, although hypotheses involving modulation of NRs have been put forward. PPAR, RXR and ECR are the most likely candidates, although unlike RXR, that is present throughout metazoans, PPAR and ECR are not present in all non-vertebrate lineages (Jordão et al., 2016; Laguerre and Veenstra, 2010; Santos et al., 2012).

Additional functional studies with mollusks PPAR and ECR have yet to be done which limits further interpretations.

Other compounds

The bivalve *Dreissena polymorpha* was exposed to clofibrate (200ng/L – 2mg/L) for 7 days and exhibited a decrease in the total triacylglycerol levels and an increase in the fatty acid concentration in a dose-dependent manner. The increase of fatty acids can be associated with the hydrolysis and consequent decrease of TAGs (Lazzara et al., 2012).

Environmental mixtures

In the environment, animals are not exposed solely to single compounds, but to complex mixtures, so it is essential to determine the effect of these complex mixtures in the metabolism (Lyche et al., 2013). A few studies have already linked oil pollution to lipid metabolic disorders. The mussel *Mytilus edulis* exposed to oil (0.05 – 2.5 ml/L) presented an increase in the gills total lipids (after 10 days) and TG levels (Fokina et al., 2014). An important point-source of xenobiotics to the aquatic ecosystems are municipal wastewaters. In the mussel *Elliptio complanata* exposure to 20% v/v of municipal wastewaters for 2 weeks induced an increase in gonads lipid content (Gagné et al., 2011). A relationship between environmental contamination and lipid metabolism disruption was also established in the field. The clam *Scrobicularia plana* was collected from three locations with different levels of contamination (Bay of St Brieuc (reference site), Goyen and Blavet estuaries), the Goyen and Blavet estuaries presented higher level of compounds with estrogenic activity and PAH. Total lipids and glycolipids were increased in the contaminated sites and the ratio TAG/Phospholipids was also altered, being higher in the Goyan estuary and lower in Blavet estuary (Perrat et al., 2013). The oyster *Strombus gigas* was collected from two sites with a high level of TBT (Road Harbour and Trellis Bay) and two reference sites (Guana Island and Anegada). A microarray analysis showed an alteration in the expression of lipid metabolism-related genes for the Road Harbour and Trellis Bay sites when compared with the reference sites (Titley-O'Neal et al., 2013).

Arthropoda

Organotins

Daphnia magna (class:Branchiopoda) cultured from 4-8 hours old at a high food rate was exposed to 0.1 µg/L and 1 µg/L of TBT during the adolescent instar and produced a progeny with less polyunsaturated fatty acids (PUFA) being the progeny less fit and smaller (Jordão et al., 2015). Additionally, levels of TBT (0.3-4.6 nM) that do not interfere with the feeding rate or the molt process lead to an increase in the lipid droplets of the *D. magna* individuals and an alteration in the expression pattern of several genes, including RXR and ECR. Interestingly, the increase in the lipid droplets was more pronounced in the animals with a higher food input. On the contrary, exposure to TPT (2.6-5.2 nM) decreased lipid accumulation in the animals exposed during the adolescent instar (Jordão et al., 2016, 2015).

It has been demonstrated that Arthropoda, Mollusca and Annelida RXR can be activated by TBT and TPT (Nishikawa et al., 2004; Wang et al., 2011; Wang and LeBlanc, 2009; André, 2016). The knock-down of *Drosophila* ECR caused an increase in lipid accumulation, indicating that this NR may control the lipid accumulation in the fat body of this species (Kamoshida et al., 2012). In addition, in *Daphnia magna* the ECR agonist (20-hydroxyecdysone) caused an increase in lipid accumulation and the antagonist (fenarimol) caused a decrease (Jordão et al., 2016). Furthermore TBT, in addition to the induction of lipid accumulation in *Daphnia magna*, also caused a change in the expression of some ECR downstream genes (Jordão et al., 2015). This suggests that RXR/ECR is involved in the TBT-induced alterations of lipid metabolism in arthropods, possibly through RXR, since a number of studies could not find any transactivation of ECR in the presence of TBT (Verhaegen et al., 2011; Wang et al., 2011; Wang and LeBlanc, 2009). Whether TBT has a synergistic or antagonistic effect in the transactivation of the heterodimer, appears to be species-specific. Wang and colleagues observed that TBT increased the transactivation of the *Daphnia magna* heterodimer RXR/ECR in the presence of the ECR agonist (20-hydroxyecdysone) (Wang et al., 2011), but in the arthropod *Crangon cangron* TBT decreased the transactivation observed when RXR/ECR was exposed to an ECR agonist (Ponasterone A) (Verhaegen et al., 2011).

Other compounds

Three studies used the arthropod *Daphnia magna* to run a screening of the total body lipid content for a wide variety of compounds that were previously identified as obesogens in vertebrates: endogenous compounds, pesticides, NR agonists and pharmaceuticals (Jordão et al., 2016; Sancho et al., 2009; Villarroel et al., 2013). Methylfarnesoate, bisphenol A, pyriproxyfen and 20-hydroxyecdysone in concentration levels that do not affect feeding caused an increase in lipid levels, while fenarimol, fluoxetine, emamectin benzoate, nonylphenol, methoprene and di-2-ethylexyl phthalate, also in concentrations that do not affect feeding, caused a decrease in the lipid content (Jordão et al., 2016). Tebuconazol and propanil decreased lipid accumulation and the feeding rate in *Daphnia magna* (Sancho et al., 2009; Villarroel et al., 2013, 2003). In *Metapenaeus monoceros* (class: Malacostraca), exposure to Endosulfan (40 and 60 ng/L), an insecticide and acaricide, during 23 days also led to a decrease in the total lipid content (Suryavanshi et al., 2009).

Environmental mixtures

Exposure of the arthropod *Macrobrachium borellii* to 0.6 mg/L of water-soluble fraction of crude oil for 7 days caused an increase in the activity of palmitoyl-CoA synthetase, TAG-lipase and β-oxidation, as well as in the TAG stores. The ratio Phospholipids/Triacylglycerol was also altered, being higher in eggs and lower in adults. The results suggest an increase in the energy production through an increment in the fatty acid oxidation (Lavarías et al., 2007, 2006).

Table 3 - Summary of effects of environmental chemicals in non-vertebrate lipid homeostasis (green: up-regulated; red: down-regulated; n.d – not determined; bold-minimum effect concentration).

Compound	Exposure	Species	Endpoints			Ref.
			Weight/Lipids alterations	Alteration in gene expression	Biochemical alterations	
TBT	82 ng/L – 1.4µg/L (water); 100 days;	*Marisa cornuarietis*	Lipids; Fatty acids levels;	n.d	n.d	(Janer et al., 2007)
TBT	100 ng/L – 200 ng/L (water); 3 months;	*Nucella lapillus*	n.d	*acs*; *cptI*;	n.d	(Pascoal et al., 2013)
TPT	93.1 ng/L – 1.5µg/L (water); 7 days;	*Marisa cornuaietis*	Lipids; Fatty acids levels;	n.d	n.d	(Lyssimachou et al., 2009)
Clofibrate	200ng/L – 2mg/L (water); 7 days;	*Dreissena polymorpha*	Triacylglycerol; Fatty acids levels;	n.d	n.d	(Lazzara et al., 2012)
Tebuconazole	0.41-1.14 mg/L (water); 5 days;	*Daphnia magna*	Lipids; Feeding rate;	n.d	n.d	(Sancho et al., 2009)
Propanil	0.07 – 0.55 mg/L (water); 5 days;	*Daphnia magna*	Lipids;	n.d	n.d	(Villarroel et al., 2013)
Endosulfan	40 and 60 ng/L (water); 23 days;	*Metapenaeus monoceros*	Lipids;	n.d	n.d	(Suryavanshi et al., 2009)
Oil pollution	0.05 – 2.5 ml/L; 10 days;	*Mytilus edulis*	Lipid (gills); Triacylglycerol; Phospholipids; Cholesterol (0.05 ml/L); Cholesterol (2.5 ml/L);	n.d	n.d	(Fokina et al., 2014)

Crude oil	0.6mg/L; 7 days;	*Macrobrachium borellii*	Lipid; TAG; Phospholipids/Triacylglycerol (eggs); Phospholipids/Triacylglycerol (adults);	n.d	PCS activity; LPS activity; β-oxidation;	(Lavarías et al., 2007, 2006)
TBT	**0.1** and 1 µg/l (water); Adolescent instar;	*Daphnia magna*	Lipid droplets;	*hr3*; *ecr b*; neverland; *met*; *scr*; *hb2*; *rxr*;	n.d	(Jordão et al., 2015)
TBT	0.98ng/L-1.5µg/L ; Exposure during adolescent instar (water)	*Daphnia magna*	Lipid droplets;	n.d	n.d	(Jordão et al., 2016)
Methyl farnesoate	10 – 250.3 µg/L;		Lipid droplets;	n.d	n.d	
BPA	299µg/L – 10mg/L;		Lipid droplets;	n.d	n.d	
Pyriproxyfen	51ng/L3µg/L;		Lipid droplets;	n.d	n.d	
20-hydroxyecdysone	9 – 480.64 µg/L;		Lipid droplets;	n.d	n.d	
Fenarimol	49.68 – 298 µg/L;		Lipid droplets;	n.d	n.d	
Fluoxetine	15.5 – 278.4 µg/L;		Lipid droplets;	n.d	n.d	
Emamectin benzoate	30.25 – 201.65 ng/L;		Lipid droplets;	n.d	n.d	
Nonylphenol	11.7– 103.35 µg/L;		Lipid droplets;	n.d	n.d	
Methoprene	3 – 100µg/L;		Lipid droplets;	n.d	n.d	
Triphenyltin	221ng/L – 1.9µg/L;		Lipid droplets;	n.d	n.d	
DEHP	9.76 – 199 µg/L;		Lipid droplets;	n.d	n.d	

Compound	Exposure *in vitro*	System	Species	Endpoints	Ref.
TBT	0.33 and 32.6µg/L;	Ovaries	*Ciona intestinalis*	Phospholipid levels; Triglycerides;	(Puccia et al., 2005)

Use of Nuclear Receptors based-assays to improve risk assessment

Approximately 30% of environmental chemicals circumvented detailed toxicity testing. Additionally, for a considerable amount of chemicals toxicity testing was limited to acute toxicity, usually involving a limited number of taxa. Since the production of new chemicals is projected to increase, it becomes evident that classical toxicity screening is inadequate to meet current toxicity assessment requirements, which still rely on extensive animal testing (Castro and Santos, 2014). This approach is time-consuming, expensive and unfeasible for such a number of compounds. Hence, prioritization approaches must be set in place to more accurately select the chemicals that must go through detailed testing and alternative high-throughput approaches should be developed (Celander et al., 2011; Santos et al., 2016).

Among the various processes of chemical interference with animal physiology, the ability of numerous compounds to mimic or block the function of endogenous hormones or signaling molecules represents a major threat to ecosystem health. These compounds display a wide array of structures and are bioactive at rather low levels, particularly in sensitive time-windows.

In addition to EDCs, there is also increased concern that emerging pollutants (EPs) may pose a considerable health and environmental risk (Ahmed et al., 2016; Noguera-oviedo and Aga, 2016). Many of these chemicals are not new, but the knowledge on their behavior and toxicological/ecological risk is extremely insufficient. Given that many classes of EPs, such as pharmaceuticals, are designed to be biologically active molecules, these compounds may behave as EDCs and putative obesogens and therefore environmental leakages are likely to produce physiological disruptions in non-target organisms.

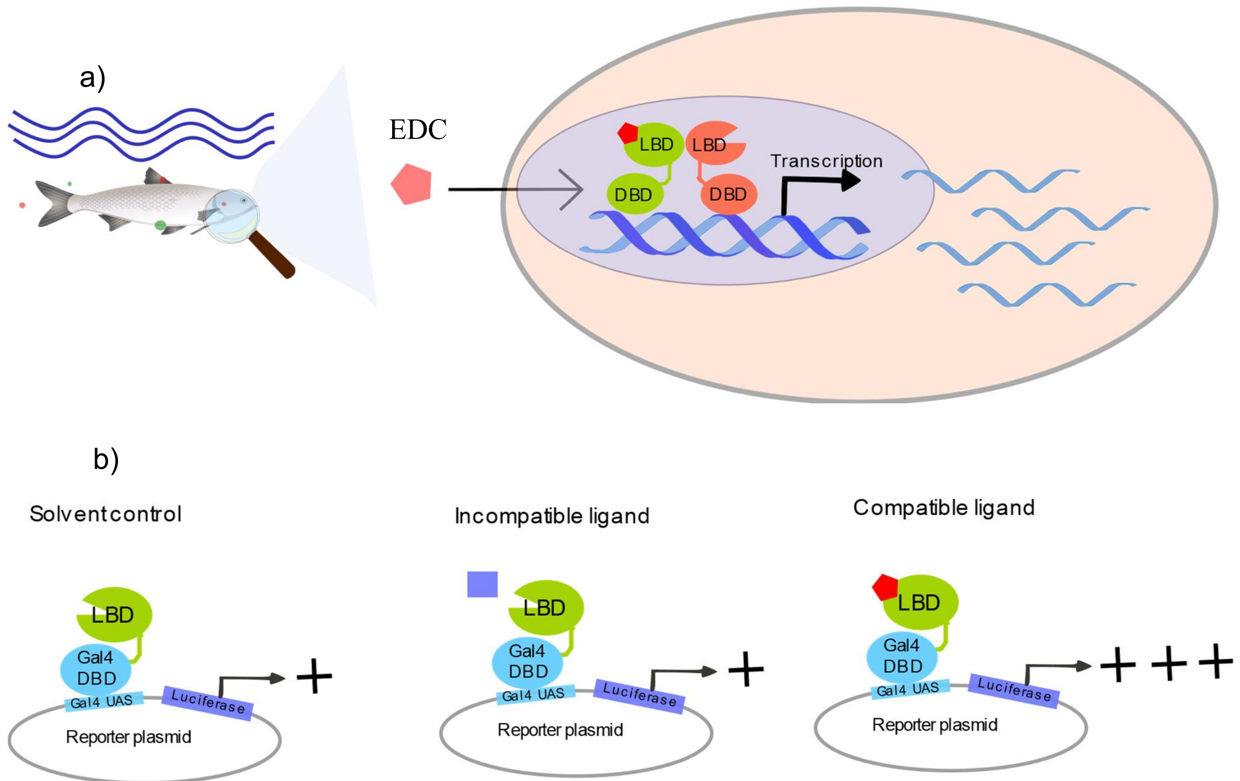

Fig. 5– a) Interaction of EDCs (for example: obesogens) with NRs in vivo. In the environment, EDCs can act as ligands of some NRs leading to alterations in the expression of the target genes. b) In vitro transactivation assay to evaluate the ability of a specific compound to activate or represses a NR (assay used in the experimental part of this thesis). In this example, the LBD of a NR is cloned into the pBind plasmid and produce a phusion protein with the GAL4 DBD. This plasmid is then co-transfected with the reporter plasmid, which contain the GAL4 Upstream Activation Sequence (UAS). The activation of the Phusion protein by the compatible ligand leads to an increase in the transcription of luciferase, that can be measured by luminescence.

Considering the limitations of classical toxicity testing outputs for wildlife protection from EDCs and PPCPs (Pharmaceuticals and personal care products), environmental agencies such as Organization for Economic Co-operation and Development (OECD) and Environmental Protection Agency (EPA) suggest a tiered approach, with a step-wise procedure, to prioritize and select chemicals to assess the potential risk to Humans and wildlife. The OECD Conceptual Framework for Testing and Assessment of Endocrine Disruptors considers five levels. Level 1 relies on existing data and non-test information such as QSARs and other in silico tools; Level 2 suggests the use of in vitro assays providing data on the endocrine mechanisms/pathways; Level 3 suggests the use of in vivo assays providing data on the endocrine mechanisms/pathways; Level 4 suggests the use of partial life-cycle tests focusing on

adverse effects endpoints and; Level 5 anticipates the use of comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism, including multigenerational studies.

Over the past decades, a flurry of studies has disclosed that many of the harmful effects resulting from the in vivo exposure to EDCs is modulated by Nuclear Receptors (NRs) (Fig. 5). In this context, both the EPA and OECD guidelines suggest a key role for NR screening in this framework. Although NRs are present in all Metazoans, the OECD guidelines for testing and assessment of EDCs is at present strongly skewed towards mammalian models due to the lack of validated in vitro assays with non-vertebrate NRs that can integrate tier 2 of this conceptual framework.

In addition to the need of high-throughput testing approaches that can be met by the use of in silico tools (tier 1) and NR-based ligand-binding/transactivation assays (tier 2), if we aim to protect biodiversity at an ecosystem scale, the understanding of the EDCs mode of action (MOA), including of putative obesogens, in disparate taxa should be a central piece of toxicity testing and risk assessment. Hence, to full implement the framework envisaged by EPA and OECD, tools should be developed in a representative sample of metazoans, and should not be limited to vertebrates and arthropods.

Current challenges

Today it is well established that the adverse effects of EDCs on living organisms go beyond the interaction with the reproductive system and are not limited to mammals. Considering the crucial role of lipids in biological processes across metazoans, if we aim to protect biodiversity at an ecosystem scale, it is important to understand how chemicals affect metabolic pathways across different lineages, the interplay with the genetic repertoire and evolutionary histories and the significance of these alterations at the population level (Castro and Santos, 2014; Thornton, 2003; Adeogun et al., 2016a). Although our knowledge on the full spectra of *taxa* that can experience disruption of lipid homeostasis following chemical exposure is still limited, it is now recognized that a great variety of metazoans are impacted. The limited number of studies available indicate that the effects on lipid homeostasis manifest at different levels, i.e, alterations in the weight, fat content, total lipids content, fatty acids levels, enzyme activity and gene expression (Corcoran et al., 2015; Jordão et al., 2016; Lyssimachou et al., 2015; Riu et al., 2014). Alterations in the lipid homeostasis can cause major adverse effects in the organisms, since lipids have important roles in the overall metabolism and reproduction (Birsoy et al., 2013; Lyssimachou et al., 2015).

The assessment of the effects of obesogens at an ecosystem level is challenging. In fact, there are still major gaps in our knowledge of lipid homeostasis across lineages, despite the many shared features in the lipid metabolism of metazoans. The various NRs involved in lipid homeostasis are not transversally present in all animals and therefore, obesogens may interact differently across animal species. For example, RXR is present in most metazoans and is a common denominator of the NRs that can be implicated in lipid homeostasis, as it is the heterodimeric partner of PPAR, LXR, FXR and ECR, which are all involved, to a certain extent, in lipid regulation. While ECR has been characterized and genome predicted in some protostome *taxa*, LXR and FXR are deuterostomes novelties and PPAR is present in deuterostomes and in some protostomes lineages, i.e., mollusks (Fig. 6) (Bertrand et al., 2004; Fonseca et al., 2017; Laguerre and Veenstra, 2010; Santos et al., 2012; Sladek, 2011; Vogeler et al., 2014).

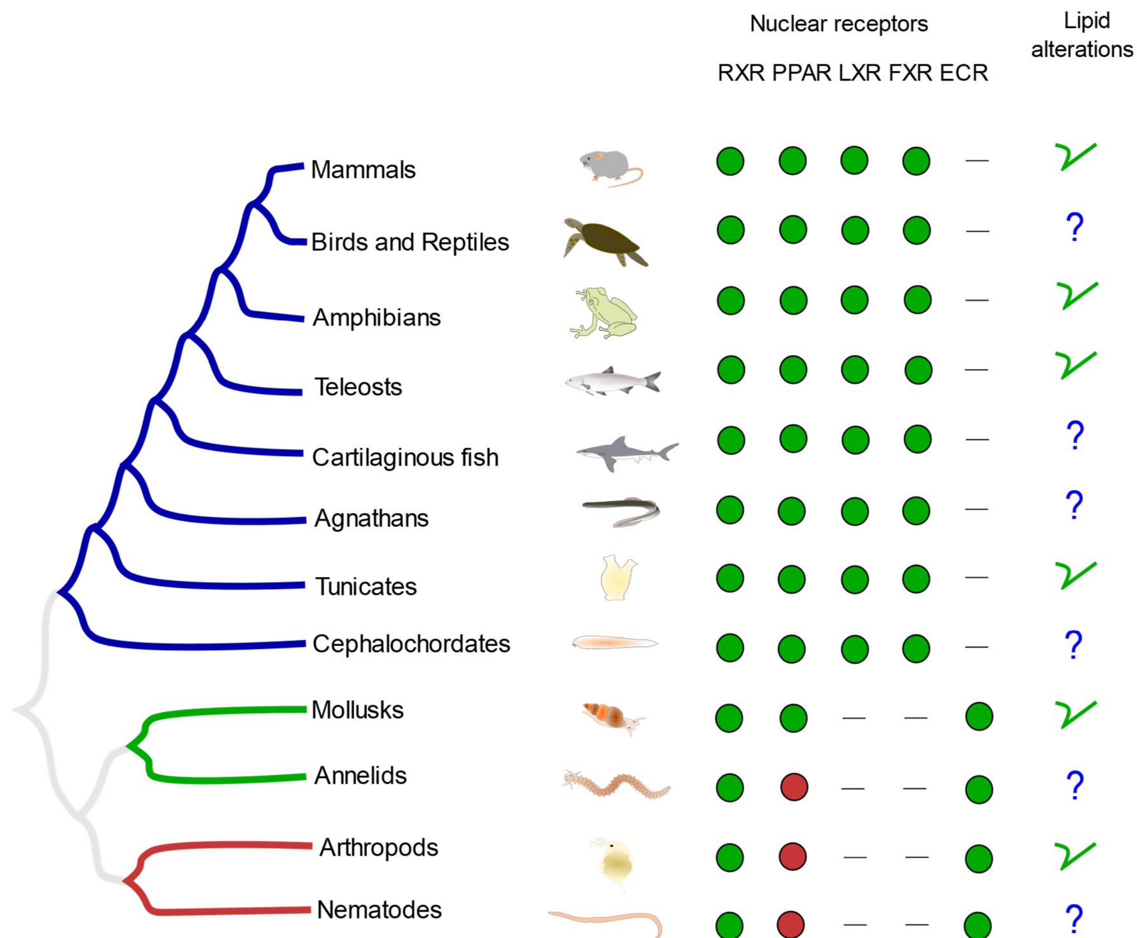

Fig. 6 – Presence (green), absence (red) or inexistence (-) of nuclear receptors involved in lipid metabolism in different phylogenetic groups. Reported lipid alterations due to putative obesogens in the different phylogenetic groups (√-lipid alterations reported; ?-lipid alterations unknown).

The diversity of compounds able to bind and/or activate NRs is vast. In the NRs reported to regulate lipid metabolism, PPARs have been reported to bind to many

different chemicals, probably due to the size of their binding pocket (Casals-Casas et al., 2008). LXR and FXR also interact with EDCs, such as phthalates and alkylphenols. For ECR, several insecticides (tebufenozide, halofenozide, methoxyfenozide, chromafenozide and fufenozide) were synthesized to exert their toxic action through this NR in arthropods (Dhadialla et al., 1998; Fahrbach et al., 2012). Whether ECR from *taxa* other than arthropods are also modulated by these insecticides remains to be investigated.

Although it is not possible to point to a single pathway for the adverse action of EDCs on lipid metabolism, several hypotheses are under scrutiny. Most known examples seem to implicate disruption of NRs signaling pathways. The modulation of NRs such as PPARs, LXR, FXR and ECR or their heterodimeric partner RXR can lead to a deregulation of the gene transcription and protein levels causing the disruption of the lipid homeostasis in a large range of analyzed *taxa* (Jordão et al., 2016; Ouadah-Boussouf and Babin, 2016; Riu et al., 2011, Santos et al., 2012). The presence of background genomic data of a large Metazoan sample is key to illuminate NR distribution, variability and function (e.g. Vogeler et al., 2014; Cruzeiro, et al., 2016; Fonseca et al., 2017), but also to isolate and characterize NRs in un-sampled phyla, and therefore to develop tools for the implementation of high-throughput testing approaches for identifying putative obesogens and chemicals targeting other pathways. It is also essential as a proxy to identify the affected signaling pathways. As a consequence of new *omics* advances (e.g. various Next Generation Sequencing platforms) transcriptomes from representative species of all Metazoan clades are now publically available. Likewise, genomes are also available for most Metazoan lineages. It is expectable that this background information boost the implementation of risk assessment of EDCs (including obesogens) and EPs (emerging pollutants) in a large sampling of animals, using a mechanistic based-approach. This should be a key aspect of toxicity testing and assessment, since we cannot expect that the extrapolation of data from a few laboratory model species, such as arthropods, that show very derived genomes with extensive gene loss, can be representative at an ecosystem scale.

The lack of systematic approaches and the poor taxonomic sampling are still problematic with respect to NRs diversity in Metazoans. NRs have been characterized in a modest number of groups, mostly Chordates, Arthropods, Nematodes, Molluscs, Annelids and Porifera. Also, for most non-vertebrates where data on NRs is available, only a limited number of the predicted NRs has been functionally characterized. For all other groups, we still lack a detailed functional analysis. Hence, given the availability of genomes/transcriptomes of representatives of major metazoan clades, functional

characterization of NRs gene repertoire should be carry out, thus allowing the implementation of a high-through approach in a large array of species.

Disclosing the molecular kernel of a given species is fundamental to understand the MOA (mode of action) of environmental pollutants and should be a central aim in toxicity testing and risk assessment. Understanding the MOA of environmental chemicals allows the building of a toxicant response and to anticipate the effects of novel chemicals (such as obesogens and EPs) targeting conserved signaling pathways. The combination of bioinformatics and both *in vitro* and *in vivo* studies will help to assess the potential of the numerous environmental contaminants to bind and activate/antagonize different NRs, the levels of gene transcription and protein activity and the physiological significance of these effects at the organism level. Considering lipid homeostasis, these approaches need to include a broad group of animal lineages, since some of the NRs involved in the lipid signaling and metabolism are group-specific and the differences in the NR structure may determine distinct chemical binding and activation outcomes. Overall, a combination of approaches is fundamental to understand the involvement of NRs in the action of obesogens across the different animal phyla and to estimate the ecological consequences. This first requires functional characterization of NRs in representative *taxa*, then it will be important to disentangle the downstream signaling pathways which is today possible due to *omics* advances (e.g. various Next Generation Sequencing platforms and the advances in mass spectrometry in proteomics and metabolomics), thus identifying the genes and proteins regulated by each NR to expand our knowledge in the lipid homeostasis outside mammals. In addition, both laboratory and field studies are needed to evaluate the effects of suspected obesogens in different *taxa* given that for several lineages we still lack information (Fig. 6). This will establish the foundations to explore the obesogenic potential of suspected chemicals across different lineages.

Aims and thesis organization

This work aims not only to increase the current knowledge on the targets of putative obesogenic compounds, but also provide a deeper knowledge on the evolution and functional characterization of PPARs. To accomplish this task, we set here the following main goals:

1) To functionally characterize PPAR orthologues in aquatic molluscs, echinoderms and chordates;

2) To evaluate the effects of the model obesogens, organotins, in the lipid metabolism of phylogenetic distinct aquatic animals;

3) To dissect the molecular pathways involved in lipid metabolism in basal deuterostomes and their modulation by EDCs;

4) To improve environmental risk assessment of chemicals affecting lipid homeostasis.

The present thesis is organized in four parts: I - Introduction; II – Obesogens in non-chordates; III – Obesogens in chordates; IV – General discussion and conclusions. Each part is composed by chapters, making a total of five chapters.

Part I – Introduction. This part is composed by a single chapter, providing an overview of current knowledge into the problematic associated with the environmental presence of obesogens. We carried out a systematization of knowledge in the field, with a special focus in aquatic organisms and nuclear receptors. This chapter originated the following publication: Capitão, A., Lyssimachou, A., Filipe, L., Castro, C., Santos, M.M., 2017. Obesogens in the aquatic environment : an evolutionary and toxicological perspective. Environ. Int. 106, 153–169. doi:10.1016/j.envint.2017.06.003.

Part II – Obesogens in non-chordates. This part is composed by two different chapters organized in scientific papers.

- Chapter 2 makes use of transactivation assays to functionally characterize the PPAR/RXR signalling module from the mollusc *Patella depressa*. Additionally, an *in vivo* study was carried out to evaluate the ability of TBT to disrupt limpet lipid metabolism through fatty acid analysis.
- Chapter 3 uses transactivation assays to functionally characterize the PPAR/RXR signalling module from the echinoderm *Paracentrotus lividus* in parallel with an *in vivo* approach to evaluate the ability of TBT to disrupt the lipid metabolism through fatty acid profile and gene expression analysis.

Part III – Obesogens in chordates. This part is composed by a single chapter organized in scientific paper format.

- Chapter 4 explores the evolution of the nuclear receptor PPAR within chordates and uses comparative genomics, phylogeny and in *vitro* transactivation assays to functionally characterize PPAR in key groups.

Additionally, we evaluate the ability of TBT to disrupt the lipid metabolism through fatty acid analysis, cholesterol and triglycerides quantification in a cartilaginous fish, *Scyliorhinus canicula*.

Part IV – General discussion and conclusions. This part is composed by one chapter that includes a general discussion about the current gaps of knowledge and set research priorities.

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Chapter 2 - The molluscan PPAR is implicated in lipid metabolism and is exploited by organotins

The molluscan PPAR is implicated in lipid metabolism and is exploited by organotins

Abstract

The importance of the heterodimer PPAR/RXR (peroxisome proliferator-activated receptor/retinoid X receptor) in the regulation of the lipid metabolism in vertebrates is well established. Recently, genome sequencing of non-vertebrates shows that *PPAR* is not a deuterostome novelty, being present in molluscs. Yet, functional characterization is lacking in lineages other than vertebrates. Therefore, we aimed at examining the repertoire of *PPAR*-like sequences and to provide the first functional characterization of the non-deuterostome PPAR and evaluate its response towards the model obesogen, tributyltin (TBT). In the present work, we isolated a mollusc *ppar* orthologue of *Patella depressa* and functionally characterized the receptor and its heterodimer in the presence of a putative ligand, TBT. In order to get further insight, we used site-directed mutagenesis to replace the tyrosine 277 by a cysteine (the human homologous amino acid) and an alanine and evaluated the response produced. Additionally, we explore the alterations in the fatty acid profiles after an exposure to the model obesogen TBT, *in vivo*. Our results show that TBT behaves as an antagonist of *Patella sp.* PPAR/RXR and the tyrosine 277 is important, but not essential in the repression of the PPAR by TBT. Moreover, TBT exposure altered the fatty acid profile of *P. depressa.* Our work demonstrates for the first time the role of the heterodimer PPAR/RXR in the action of these compounds and a clear gender specificity in the response of the mollusc *P. depressa* to the model obesogen TBT.

Introduction

Pollutants can affect the ecosystems in a variety of ways, including the disruption of gene expression through nuclear receptors (NR) (Lima et al., 2011; Vogeler et al., 2017). NRs are a vast family of transcriptions factors that are essential in metabolic regulation and homeostasis. NRs are mostly ligand-activated and regulate the expression of specific genes; they show two conserved domains, the DNA binding domain (DBD) and, to a lesser extent, the ligand binding domain (LBD) (Alaynick, 2008; Evans and Mangelsdorf, 2014; Gronemeyer et al., 2004; Mello, 2010; Thornton, 2003). The NR peroxisome proliferator-activated receptor (PPAR) form a heterodimer with the promiscuous retinoid X receptor (RXR) and respond to small lipophilic ligands, such as

fatty acids and fatty acids derivates (Casals-Casas et al., 2008; Tyagi and Gupta, 2011). The heterodimers PPAR/RXR are important regulators in lipid homeostasis in mammals (Ahmadian et al., 2013; Berkenstam and Gustafsson, 2005; Janesick and Blumberg, 2011). The number of *PPAR* genes in metazoans varies extensively between phylogenetic groups. While mammals possess three distinct *PPARs* (α, β and γ), (Santos et al., 2012), in the molluscs *Biomphalaria glabrata* and *Lottia gigantea* were found only two *ppar*s (*ppar*1 and *ppar*2) (Kaur et al., 2015) and in the mollusc *Crassostrea gigas ppar*2 seems apparently absent (Vogeler et al., 2017, 2014). Molluscs *ppar*2 has been recognized as a direct orthologue of the vertebrate PPARs (Kaur et al., 2015). While RXR is present in most metazoans, PPAR has only been reported in deuterostomes and molluscs (Sladek, 2011; Vogeler et al., 2014). In mammals, the heterodimer PPAR/RXR is permissive, being activated in the presence of PPAR and/or RXR ligands (Harada et al., 2015; Ouadah-Boussouf and Babin, 2016; Santos et al., 2012).

Several chemicals display endocrine disrupting proprieties, altering the normal function of the endocrine system of several metazoans (Bergman et al., 2013; Castro and Santos, 2014; Diamanti-Kandarakis et al., 2009; Kabir et al., 2015; Schug et al., 2011). The effects of endocrine disrupting chemicals (EDCs) include alterations in the reproductive system (Abidli et al., 2009; Bhandari and Tillitt, 2015; Jordão et al., 2015; Lima et al., 2011; Rodrigues et al., 2006), embryonic development (Bhandari and Tillitt, 2015; Guillette et al., 1995; Soares et al., 2009) and immune system (Jin et al., 2010; Kuo et al., 2012; Schug et al., 2011). More recently, lipid metabolism has been identified as a new target of EDCs (Grün and Blumberg, 2007, 2006; Jordão et al., 2015; Lyssimachou et al., 2015; Nappi et al., 2016; Riu et al., 2014). PPAR/RXR is a key regulator of the lipid homeostasis and several studies link the action of EDCs, suchs as obesogens, to the improper modulation of these receptors (Desvergne et al., 2009; Harada et al., 2015; Hurst and Waxman, 2003; Janesick and Blumberg, 2011; le Maire et al., 2009; Nishikawa et al., 2004; Riu et al., 2011).

Lipid storages in molluscs are mainly used in gametogenesis and growth (Morais et al., 2003a, 2003b). The interference of the model obesogens, TBT and TPT, with the lipid metabolism in molluscs has previously been reported (Janer et al., 2007; Lyssimachou et al., 2009; Pascoal et al., 2013), and PPAR/RXR was suggested as a possible intermediary in this response (Janer et al., 2007; Pascoal et al., 2013).

Here we isolated and functionally characterize for the first time a PPAR from a non-vertebrate, the mollusc *P. depressa.* We show that this PPAR heterodimerize with RXR and the transactivation assays reveal that the mollusc PPAR/RXR can be modulated by

organotins and potential natural ligands, fatty acids. Additionally, *in vivo P. depressa* exposed to TBT displays gender specific alterations in the fatty acid profile.

Methods

Gene isolation and cloning

P. vulgata rxr (ALQ43971.1) was obtained from a previous study (Gesto et al., 2016). Full *P. depressa ppar* sequence was obtained using a combination of PCR-based approaches. First, degenerate PCR primers were designed from conserved regions of related sequences using CODEHOPE (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) program to obtain the initial fragment. 3' and 5' ends were further extended using the SMARTer™ RACE cDNA Amplification Kit (Clontech) following the manufacturer's instructions. The complete Open Reading Frame (ORF) was obtained using specific primers for the 3' and 5' ends, designed in Primer-BLAST (NCBI) (primer sequences can be found in the supplementary material – table 1). Gene sequences were verified using Sanger sequencing (GATC). Two PPAR mutant constructs in which Y277 was mutated to cysteine (Y277C PPAR) and alanine (Y277A PPAR) were purchase from Nzytech.

The hinge and ligand-binding domains of *P. depressa ppar*, the two mutants (Y277C PPAR, Y277A PPAR) and *P. vulgata rxr* were cloned into the pBIND vector to produce an NR LBD-Gal4 hybrid protein. This hybrid protein, containing the DNA binding domain of Gal4, acts on a UAS response element (Primer sequences in the supplementary material – table 1). For *in vitro* assays with the PPAR/RXR heterodimer, hinge and ligand-binding domains of *P. vulgata ppar* was further sub-cloned into the pACT vector to produce a PPAR LBD-VP16 hybrid protein. VP16, which acts on proximal downstream promoters further amplifies transcription and is used as a two-hybrid system to assess protein-protein interaction. Plasmid sequences were verified using Sanger sequencing (GATC).

Sequence analysis

The amino acid sequences of the BDB, LBD and hinge of the receptors were predicted using the Conserved Domain Database at NCBI. The sequence of each domain was then compared with *Biomphalaria glabrate* and human homologs. The LBD amino acids identified as interacting with TBT and/or TPT were predicted based on previously published works (Harada et al., 2015; Hiromori et al., 2015; le Maire et al., 2009).

Phylogenetic analysis

Tblastn and blastp searches using human *PPARα* (Q07869), *PPARβ* (Q03181) and *PPARγ* (P37231) as query were performed in the available databases NCBI (https://www.ncbi.nlm.nih.gov/), Ensembl (http://www.ensembl.org/) and JGI (https://genome.jgi.doe.gov/). *PPAR* sequences were retrieved for the major vertebrate lineages: mammals (*Homo sapiens*, *Mus musculus*); birds (*Gallus gallus*) reptiles (*Anolis carolinesis*); amphibians (*Xenopus tropicalis*); coelacanths (*Latimeria chalumnae*); Lepisosteiformes (*Lepisosteus oculatus*) Osteoglossomorpha (*Pantodon buchholzi*), Cypriniforme (*Danio rerio*), Chondrichthyes (*Callorhinchus milii*) and for the following invertebrates, tunicates (*Ciona intestinalis*) cephalochordates (*Branchiostoma floridae, Branchiostoma lanceolatum, Branchiostoma belcheri*), hemichordate (*Saccoglossus kowalevskii*), Molluscs (*Biomphalaria glabrata, Lottia gigantea, Crassostrea gigas*) and Echinodermes (*Strongylocentrotus purpuratus*) (See supplementary table 2 for accession number). Phylogenetic analysis was performed using the set of sequences through database search as well as the isolated sequence. A total of 47 sequences were aligned in MAFFT (Katoh et al., 2005; Katoh and Toh, 2008) with L-INS-I method. The resulting sequence alignment was stripped of all columns containing 90% gaps leaving a total of 545 positions for phylogenetic analysis. Maximum likelihood phylogenetic analysis was performed in PhyML V3.0 (Guindon et al., 2010) and the evolutionary model was determined using the smart model selection (SMS) option resulting in a JTT +G+I+F. The branch support was calculated using aBayes. The resulting tree was analysed in Fig Tree V1.3.1 available at http://tree.bio.ed.ac.uk/software/figtree/ and rooted with the PPAR1 mollusc sequences.

Chemicals and solutions

All chemicals were obtained from Sigma-Aldrich unless stated otherwise in the text. For the *in vitro* experiment three TBT-Cl and three TPT-Cl (Triphenyltin chloride) stock solutions were prepared: 250 µM (29.7 mg Sn/L), 100 µM (11,87 ng Sn/L) and 10 µM (1,187 ng Sn/L); three ARA (Arachidonic acid) and three EPA (cis-5,8,11,14,17-Eicosapentaenoic acid) solutions with the concentrations of 200 mM, 100 mM and 50 mM; one mixture of 6 fatty acids (lipid mix) containing 50 mM of ARA, 50 mM of EPA, 50 mM of oleic acid, 50 mM of linoleic acid, 50 mM of γ-linolenic acid and 13.3mM of palmitic acid and two dilutions were made, 50% and 25%. The final concentration of solvent in the multi-plate wells was 0,1%. All stock solutions were prepared in DMSO. For the *in vivo*

experiment, the concentrations of TBT stock solutions were 250 mg Sn/L and 100 mg Sn/L. The final concentration of solvent (DMSO) in the aquariums was 0,00025%.

Cell culture and *in vitro* assays

COS-1 cells were grown in a humidified chamber containing 5% CO2 at 37ºC in Dulbecco's modified Eagles medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Cells were seeded at a density of 2×10^5 live cells/ well in 24-well culture plates. After 24 hours cells were transfected using lipofectamine 2000 reagent (Invitrogen), in Opti-MEM reduced serum medium (Gibco, Thermo Fisher), according manufacturer's indications and 0.5 µg of pBind PPAR LBD-Gal4 or RXR LBD-Gal4 and 1µg of pGL4.31 luciferase reporter vector, containing five UAS elements upstream the firefly luciferase reporter gene. For heterodimer transfection assays 0.5 µg of pBind PPAR LBD-Gal4 and 0.5 µg of pACT and 1µg of pGL4.31, were used. After 5 hours of incubation the medium was replaced with phenol red-free DMEM supplemented with 10% dextran-coated charcoal-treated serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) containing the test compounds (TBT-10nM; 100nM; 250nM; TPT-10nM; 100nM; 250nM; ARA-50µM; 100µM; 250µM; EPA-50µM; 100µM; 250µM and a mixture of lipids) dissolved in DMSO (0.1%). 24 hours later cells were lysed and Firefly luciferase (pGL4.31) and Renilla luciferase (pBIND) luminescent activities were determined using the Dual luciferase assay system kit (Promega) according to manufacturer's instructions using a Synergy HT Multi-Mode Microplate reader (BioTek). Renilla Luciferase, co-expressed with the NR LBD-Gal4 hybrid proteins, served as an internal control for transfection efficiency. All assays were performed, independently, three times with two technical replicates per condition.

Experiments *in vivo*

The limpets *P. depressa* habite the intertidal zone from the north of Wales to the south of Portugal (Silva et al., 2003). Three hundred animals (with similar sizes) from *P. depressa,* collected at the Mindelo beach, Portugal, were randomly distributed by 8 aquaria (30/aquarium). Each aquarium contained rocks covered with biofilm (collected at Viana do Castelo, Portugal) and 25L of filtrated salt water at 16,3ºC±0,4 and a 16/8 light/dark cycle. After approximately 72 hours of acclimatization the animals were exposed to DMSO (0.00025%), TBT1 (100 ngSn/L) and TBT2 (250ngSn/L), respectively. Each treatment was applied to two aquaria. The TBT concentration was established every other day following previous protocols from our group (Lima et al., 2011). After 3 weeks, the

animals were anesthetized in a solution of 7% $MgCl_2.6H_2O$ prior to sacrifice. Gender, length, whole weight, tissue weight (weight after removing the shell), gonad weight and gonad stage were recorded. Gonads, viscera and the rest of the body were frozen in liquid nitrogen and subsequently stored at -80ºC until analysis. Gonad index (GSI) was calculated using:

$$GSI = \frac{Wet\ weight\ of\ gonads}{Wet\ weight\ of\ soft\ tissues} \times 100$$

(Morais et al., 2003a).

Biochemical analyses

For fatty acid quantification, pools of three samples of males and pools of three samples of females of each group were extracted and analysed. Fatty acid extraction was performed according to the Folch method with minor adjustments (FOLCH et al., 1957). Briefly, the tissue was homogenized with chloroform/methanol (2:1) to a final volume of 20ml/gr of tissue. After homogenization tricosanoic acid was added as internal standard the mixture was agitated during 3 hours in an orbital shaker at room temperature protected from the light. The sample was then centrifuged for 30 minutes at 2000 rpms. The supernatant was recovered for a new tube and vortexed for 1 minute with 0.25 volume of a NaCl (9%) solution. The mixture was then centrifuged for 10 min at 2000 rpm for phase separation, the upper phase was discarded. A final wash with methanol/water (1:1) was performed previous to the centrifugation. The upper phase was discarded and the lower phase was evaporated under a nitrogen stream. The samples were kept at -20ºC until derivatization. Fatty acid derivatization was performed as described by Cohen et al., 1988 using acetyl chloride as catalyst and the detection was performed according to Guedes et al., 2011 (quantification limits for the fatty acids analysed are presented in the supplementary table 3). Fatty acids were quantified in µg/mg of tissue as done in Morais et al., 2003.

Statistical analysis

Transactivation results were expressed as fold-induction using the ratio between luciferase and renilla luminescence. Differences between groups were analyzed using one-way ANOVA followed by Fisher LSD post hoc test when parametric criteria were achieved, if not non-parametric Kruskal-wallis ANOVA followed by a Games-Howel test was used. $P < 0.05$ was considered statistically significant in all analyses. All analyses

were performed using IBM SPSS Statistics 24. Outliers were verified by the outlier labelling rule (Hoaglin and Iglewicz, 1987).

Results

P. depressa PPAR isolation and sequence analysis

Alignment of molluscs PPARs, from *P. depressa, B. glabrata* PPAR2 (XP_013069840.1) and PPAR1 (XP_013072795.1) and human PPARγ (BAH02283.1) showed that *P. depressa* PPAR DBD exhibit 88% similarity when compared to PPAR2 of *B. glabrata, 68% with PPAR1 of B. glabrata* and 61% with the human PPAR (Table 4). The hinge region, on the other hand, has no similarity with human and *B. glabrata* PPAR1, but is 74% similar to *B. glabrata* PPAR2. The LBD of *P. depressa* exhibited 54% similarity with *B. glabrata* PPAR2, 28% with *B. glabrata* PPAR1 and 31% with human (Table 4). The conservation status of residues involved in TBT and/or TPT binding to human PPARγ was also listed (Fig. 7). The cysteine anchor residue from the human PPARγ, shown to interact with the tin atom, of both TBT and TPT (Harada et al., 2015), is not conserved in the mollusc sequence. Three residues that interact exclusively with TPT and two that interact with TBT and TPT in the human PPARγ are conserved in *P. depresa* PPAR.

Fig. 7– PPAR sequence alignment. *P. depressa* (PPAR_Pd) Human PPARγ (Hs_LBD) amino acid sequences. Red- anchor; Blue- interact with TBT and TPT; Yellow-interact only with TPT; Green – interact only with TBT. Based on (Harada et al., 2015)

Table 4 – Similarity between *P. depressa* PPAR domains and Homo sapiens.

	DBD	H	LBD
***Biomphalaria glabrata* PPAR2**	88%	74%	54%
***Biomphalaria glabrata* PPAR1**	68%	0%	28%
***Homo sapiens* PPARγ**	61%	0%	31%

***P. depressa* PPAR phylogenetic analysis**

To elucidate the orthology of the *PPAR* isolated from *P. depressa* a phylogenetic and synteny analysis was performed. The Maximum Likelihood revealed that invertebrate sequences outgroup vertebrate PPARα, PPARβ and PPARγ. Placed accordingly at the base of the vertebrate sequences, we find single PPAR sequences from the urochordate *C. intestinalis* and from the cephalochordate *B. floridea*, *B. lanceolatum* and *B. belcheri.* The *P. depressa PPAR* groups with other gastropods *PPARs*, namely *B. glabrata PPAR2* and *L. gigantea PPAR2* (Fig.8). Interestingly, a second group of PPAR sequences previously identified in *Lottia gigantea, Biomphalaria glabrata* and *Crassostrea gigas* named *PPAR1*, falls outside this larger PPAR clade and placed basely to all previously analysed sequences. To further clarify the orthology of isolated *P. depressa* PPAR shown in the phlyogenetic analysis we next analysed the genomic *locus* of *H. sapiens* PPARα, PPARβ, and PPARγ, *B. floridae* PPAR and *Biomphalaria glabrata PPAR2* and *PPAR1* (supplementary Fig. 1). Here we find that *B. floridae PPAR* and *Biomphalaria glabrata PPAR2* gene are neighboured by genes with orthologues that locate to the corresponding genomic loci of *H. sapiens* PPARα, PPARβ and PPARγ chromosomes 2 (2.6), 3 (3.2), 12 (12.5 and 12.9) and 17 (17.6) in regions linked to a four-fold paralogy namely LG16 (Putnam et al., 2008) or ancestral chromosome E (Nakatani et al., 2007).

Both synteny and phylogenetic analysis support the isolated PPAR from *P. depressa* corresponds to a direct orthologue of the vertebrate PPAR, while the orthology of the second group of PPAR sequences found in mollusc should be interpreted with caution.

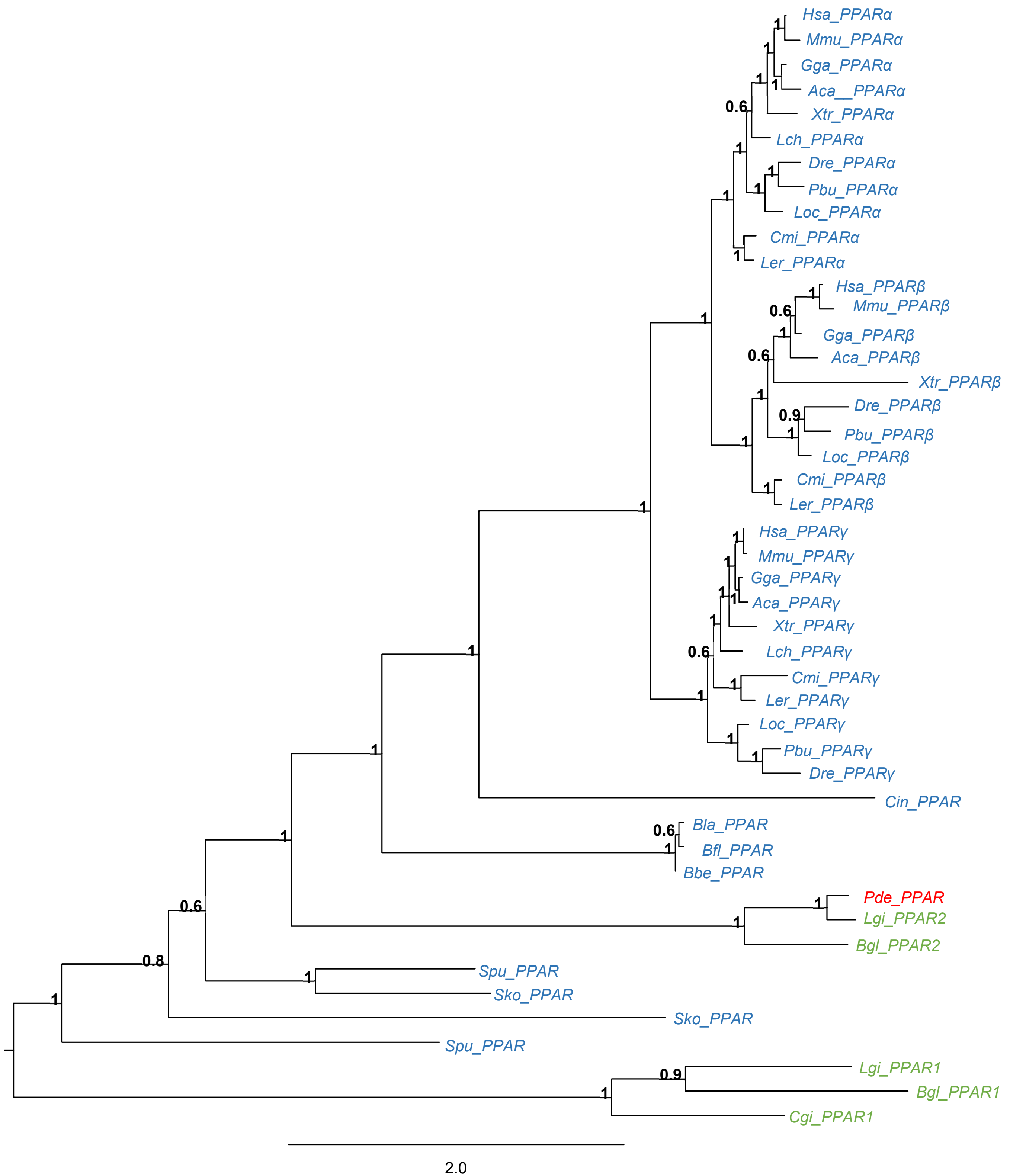

Fig. 8 – Bayesian phylogenetic analysis of PPAR amino acid sequences, values at nodes indicate posterior probabilities. Hsa: *Homo sapiens;* Mmu: *Mus musculus;* Gga: *Gallus gallus;* Aca: *Anolis carolinesis;* Xtr: *Xenopus tropicalis*; Lch: *Latimeria chalumnae;* Dre: *Danio rerio;* Pbu: *Pantodon buchholzi;* Loc: *Lepisosteus oculatus;* Cmi: *Callorhincus milii;* Ler:*Leucoraja erinacea;* Bbe: *Branchiostoma belcheri;* Bla: *Branchiostoma lanceolatum;* Bfl: *Branchiostoma floridae;* Sko: *Saccoglossus kowalevskii;* Spu: *Strongylocentrotus purpuratus;* Bgl: *Biomphalaria glabrata;* Pde: *Patella depressa;* Lgi: *Lottia gigantea;* Cgi: *Crassostrea gigas.* Red: *P. depressa* PPAR; Blue: Deuterostomia; Green: Mollusca.

P. vulgata RXR sequence analysis

Regarding RXR, alignment of *P. vulgata* RXR and *B. glabrata* RXR showed a 90% similarity while when compared with the human RXRα showed 85% similarity in the DBD, 74 % similarity was observed in the hinge region when compared with *B. glabrata* and 67% with human. The LBD was 95% similar to *B. glabrata* and 83% to human (Table 5). The key amino acid residue involved in TBT and TPT binding to human RXRs, cysteine in the position 432, is conserved in *P. vulgata* RXR (Fig.9) (Hiromori et al., 2015; le Maire et al., 2009).

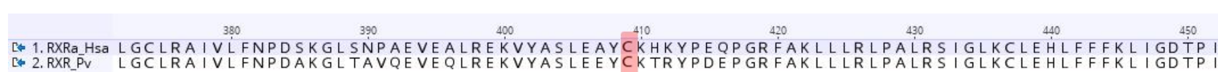

Fig. 9 -RXR sequence alignment. P. vulgata (RXR_Pv) and Human (RXRa_Hsa) amino acid sequence. Red- TBT anchor. Based on (Hiromori et al., 2015; le Maire et al., 2009)

Table 5- Similarity between *P. vulgata RXR* domains, *B. glabrata* and *H. sapiens.*

RXR	**DBD**	**H**	**LBD**
***Biomphalaria glabrata* RXR**	90%	74%	95%
***Homo sapiens* RXRα**	85%	67%	83%

Transactivation responses of PPAR, RXR and PPAR/RXR

The ability of putative natural ligands – fatty acids (ARA, EPA, lipid mix and lipid extracts) and synthetic agonist - organotins (TBT, TPT) to activate the NRs PPAR, RXR and the heterodimer PPAR/RXR were evaluated using a luciferase reporter gene assay. To evaluate if *P. depressa* PPAR form a heterodimer with RXR was used a two-hybrid reporter cell system without potential ligands. The interaction between PPAR phusion protein and RXR phusion protein lead to an increase in the production of luciferase that was measure afterwards. We observe a significant increase in the luciferase levels when the potential heterodimeric partner (RXR) was present (Fig.10).

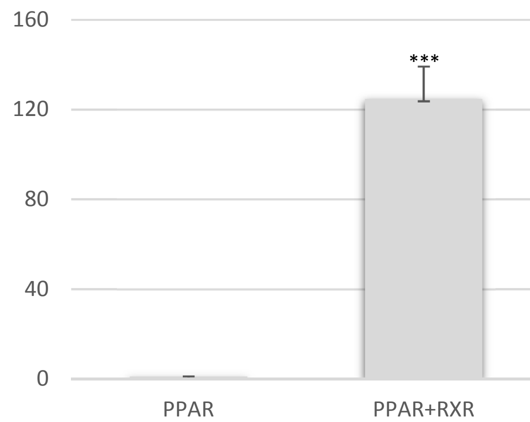

Fig. 10 – Luciferase luminescence upon transfection and co-transfection of PPAR and co-transfection of PPAR and RXR in the absence of ligands.

In the figure 11 we can observe that both fatty acids ARA and EPA and the mixture of lipids were able to transactivate the human PPARγ. *P. depressa* PPAR was transactivated by ARA and EPA. *P. vulgata* RXR was only activated by the mixture of lipids. ARA was not able to activate the heterodimer PPAR/RXR.

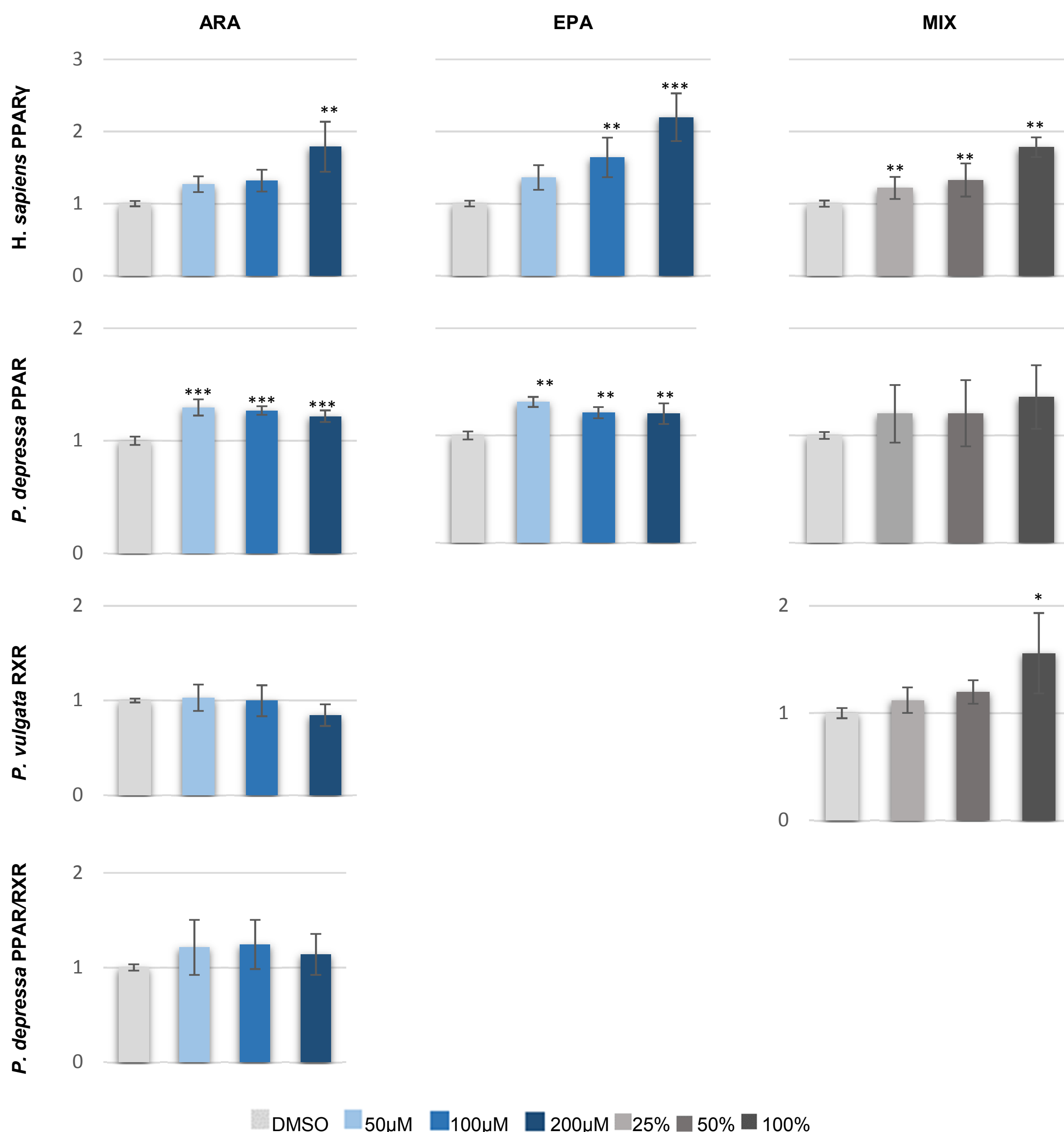

Fig. 11– *H. sapiens* PPARγ, *P. depressa* PPAR, *P. vulgata* RXR and heterodimer PPAR/RXR transactivation in the presence of the fatty acids ARA and EPA and the lipid mixture containing 50 μM of ARA, 50 μM of EPA, 50 μM of oleic acid, 50 μM of linoleic acid, 50 μM of γ-linolenic acid and 13.3μM of palmitic acid). Values are presented as mean± standard deviation of the normalized fold-induction (*-$p<0.05$; ** - $p<0.01$; *** - $p<0.001$).

P. depressa PPAR was repressed by TBT, while RXR was significantly induced only by 100nM TBT (Fig.12). The heterodimer PPAR/RXR exhibited a repression in the presence of TBT, in a concentration-dependent manner. Similarly, TPT also repressed the NR heterodimer, and PPAR alone, in a concentration-dependent manner. Yet, RXR was significantly induced by 100nM and 250nM TPT (Fig.12).

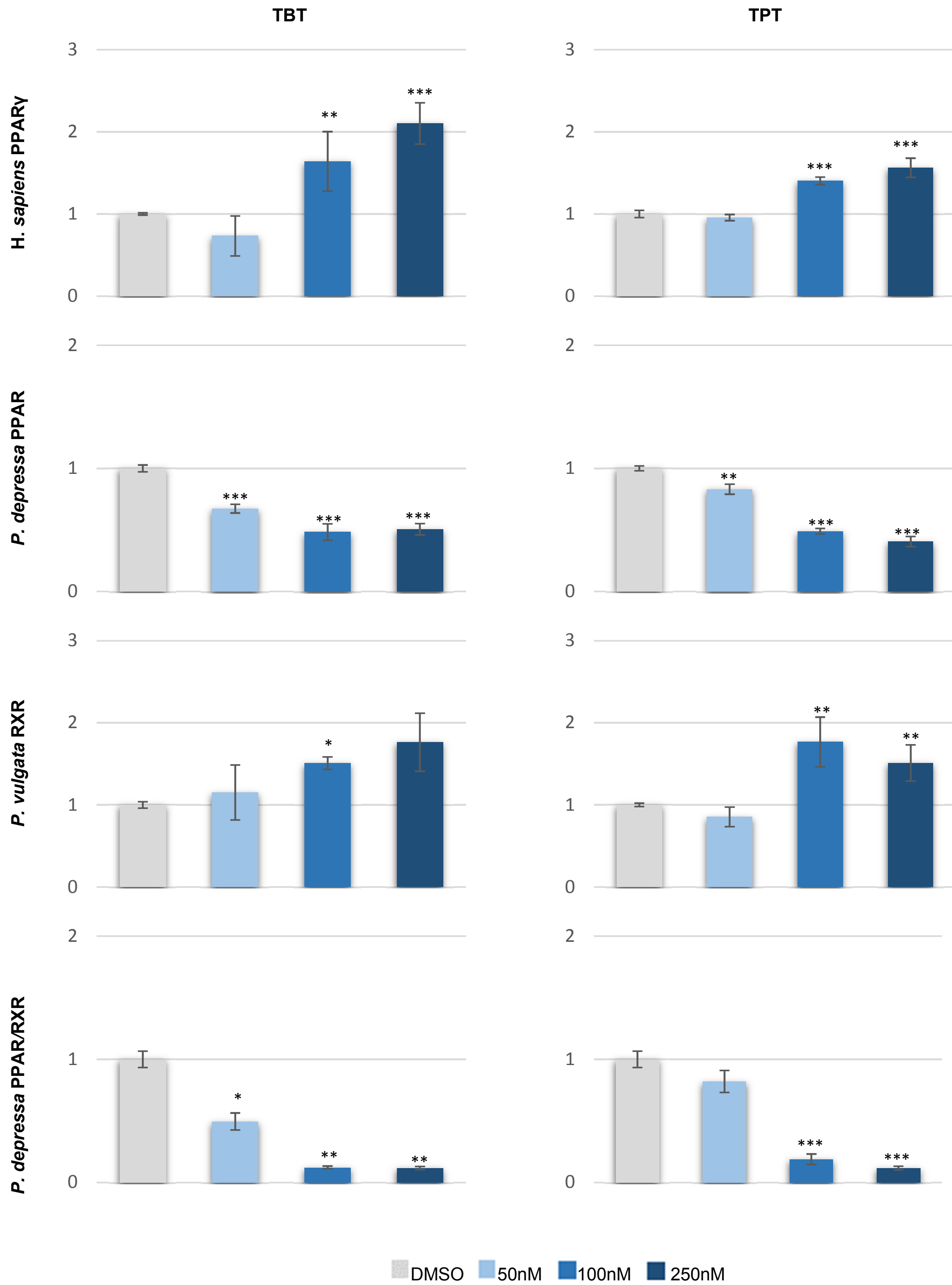

Fig. 12– H. sapiens *PPAR,* P. depressa PPAR, P. vulgata RXR and heterodimer PPAR/RXR transactivation in the presence of TBT and TPT. Values are presented as mean± standard deviation of the normalized fold-induction (*-p<0.05; ** - p<0.01; *** - p<0.001).

The *P. depressa* PPAR mutant, Y277A, was not repressed by TBT; although in another mutant, Y277C, a slight repression was observed at the higher concentration of TBT (Fig.13).

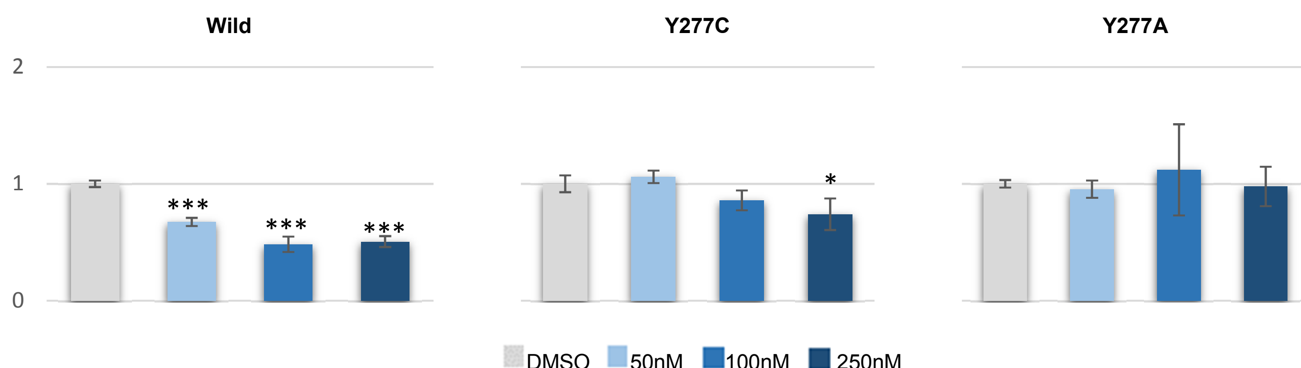

Fig. 13 – *P. depressa* PPAR, Y277C PPAR and Y277A PPAR transactivation in the presence of TBT. Y277C: *P. depressa* PPAR with the tyrosine 277 mutated to a cysteine; Y277A: *P. depressa* PPAR with the tyrosine 277 mutated to a alanine. Values are presented as mean± standard deviation of the normalized fold-induction (*-p<0.05; ** - p<0.01; *** - p<0.001).

Morphological parameters

Several morphological parameters (length, whole weight, gonad stage and developmental index) were recorded or calculated for each sampling time and treatments (table 6). Mortality in control, solvent control and TBT1 was under 20%, while in TBT2 the mortality was 30%. Was found food in the digestive system of all animals. In the several parameters analyzed, no alterations were observed between the different treatments.

Table 6 - Morphological parameters of *P. depressa* male and female in the end of the experiment. TBT1-100 ng Sn/L; TBT2-250 ng Sn/L. Values are the Mean±STD.

Gender	Treatment	Length (mm)	Whole Weight (gr)	Gonad stage	Gonad Index
Females	Control	2.807±0.287	2.560±0.780	2.773±0.915	18%±20%
	DMSO	2.876±0.183	2.694±0.676	2.900±0.829	11%±4%
	TBT1	2.874±0.255	2.688±0.789	2.647±0.786	11%±6%
	TBT2	2.971±0.383	3.059±0.710	3.071±1.134	17%±8%
Males	Control	2.790±0.258	2.606±0.811	3.500±1.000	12%±5%
	DMSO	2.857±0.284	2.747±0.837	2.727±1.182	8%±5%
	TBT1	2.817±0.188	2.578±0.654	3.030±1.075	10%±3%
	TBT2	2.781±0.227	2.551±0.563	2.892±1.141	12%±4%

P. depressa fatty acid profile

The fatty acid composition of *P. depressa* gonads is presented in the tables 7 and 8. Since control and solvent control samples did not present differences in females, control and solvent control samples were grouped (Control/DMSO) in females. From the 36 fatty acids analysed 13 were detected and quantified in female gonads and 5 in the male gonads. Fatty acids with less than 14 or more than 20 carbon atoms were not detected. Most of the detected fatty acids in females were saturated corresponding to 50.63% of the total fatty acids content in Control/DMSO. Regarding the unsaturated fatty acids in females, polyunsaturated fatty acids (PUFA) were present in higher amounts, 27.86%, with monounsaturated fatty acids (MUFA) representing 21.55% in control/DMSO females. The most abundant fatty acid detected in Control/DMSO females and Control males was the saturated palmitic acid followed by the PUFA arachidonic acid (ARA). In females exposed to 100 ng Sn/L of TBT a significant decreased was observed in palmitic acid, linolelaidic acid and PUFA. In males exposed to the higher concentration of TBT was observed significant increases in Oleic acid, ARA, total PUFA and total UFA. Differences between control and solvent control were also found in ARA, PUFA and total unsaturated fatty acids (UFA). No comparation was performed in the females exposed to the higher concentration of TBT due to the low number of samples.

Table 7– Fatty acid profile of *P. depressa* female gonads. Control and exposed animals (TBT1 – 100 ng Sn/L; TBT2 – 250 ng Sn/L 2). Values are expressed as µg/mg of tissue (*-p<0.05; ** - p<0.01; *** - p<0.001; ∆-mean difference from control in percentage).

	Control/DMSO		TBT1			TBT2	
Name	*Mean*	*STD*	*Mean*	*STD*	*Δ*	*Mean*	*STD*
Miristic (14:0)	2.80	±1.24	1.45	±0.67	-48.0%	0.67	
Pentadecanoic (15:0)	0.24	±0.09	0.15	±0.03	-37.5%	0.15	
Palmitic (16:0)	12.36	±3.14	**7.84***	±1.59	-36.6%	6.68	±2.09
Heptadecanoic (17:0)	0.19	±0.05	0.13	±0.02	-31.6%	0.12	±0.01
Stearic (18:0)	1.91	±0.51	1.26	±0.10	-34.0%	1.19	±0.03
Σ SFA	15.79	±6.98	10.88	±1.73	-31.1%	8.40	±2.71
Palmitoleic acid (16:1)	1.50	±0.38	1.08	±0.22	-28.0%	1.15	
Oleic Acid (18:1 n9 c)	4.93	±0.98	3.59	±1.08	-27.2%	3.22	±0.51
Eicosanoic acid (20:1)	0.62	±0.17	0.86	±0.84	38.7%	0.37	±0.05
Σ MUFA	6.72	±1.62	5.53	±2.08	-17.7%	4.17	±1.37
Linolelaidic (18:2 n6 t)	0.30	±0.07	**0.19***	±0.02	-36.7%	0.21	
Linolenic acid (18:3 n3)	0.39	±0.13	0.23	±0.05	-14.0%	0.16	±0.01
11,14-Eicosadienoic acid (20:2)	0.91	±0.22	0.66	±0.08	-27.5%	0.58	±0.06
Arachidonic (20:4 n6)	6.17	±1.16	4.63	±0.38	-24.9%	4.98	±1.09
EPA (20:5 n3)	1.03	±0.29	0.74	±0.09	-28.2%	0.41	±0.03
Σ PUFA	8.69	±1.61	**6.45***	±0.39	-25.8%	6.22	±1.27
Σ UFA	15.41	±3.08	11.98	±2.46	-22.3%	10.40	±2.64
Σ FA	31.19	±9.91	22.86	±3.69	-26.7%	18.80	±5.35

Table 8– Fatty acid profile of *P. depressa* male gonads. Control, Solvent control (DMSO) and exposed animals (TBT1 – 100 ng Sn/L; TBT2 – 250 ng Sn/L 2). Values are expressed as µg/mg of tissue (*-p<0.05; ** - p<0.01; *** - p<0.001; ∆-mean difference from control in percentage).

	Control		DMSO		TBT1			TBT2		
Name	Mean	STD	Mean	STD	Mean	STD	Δ	Mean	STD	Δ
Palmitic (16:0)	1.91	±0.83	1.45	±0.51	1.94	±0.37	33.8%	2.37	±0.99	63.4%
Stearic (18:0)	1.21	±0.40	0.78	±0.14	1.08	±0.19	38.5%	1.24	±0.56	59.0%
Σ SFA	3.12	±1.16	2.23	±0.65	3.02	±0.55	35.4%	3.13	±1.87	40.4%
Oleic Acid (18:1 n9 c)	0.48	±0.26	0.31	±0.08	0.40	±0.07	29.0%	**0.68***	±0.35	119.4%
Σ MUFA	-	-	-	-	-	-	-	-	-	-
Arachidonic (20:4 n6)	**1.45***	±0.58	0.71	±0.02	1.01	±0.19	42.3%	**1.45***	±0.33	104.2%
EPA (20:5 n3)	0.48	±0.09	0.48	±0.21	0.55	±0.08	14.6%	0.53	±0.16	10.4%
Σ PUFA	**1.93***	±0.59	1.18	±0.22	1.56	±0.26	32.0%	**1.98***	±0.35	67.8%
Σ UFA	**2.42***	±0.77	1.50	±0.30	1.96	±0.34	30.7%	**2.65****	±0.27	76.7%
Σ FA	5.53	±1.81	3.73	±0.94	4.99	±0.87	33.8%	5.79	±2.10	55.2%

Discussion

Organotins are known endocrine disrupters of molluscs (Lima et al., 2011; Pascoal et al., 2013; Santos et al., 2005), however their mode of action and effects is not completely elucidated. A limited number of reports indicate alterations in molluscs lipid metabolism when exposed to organotins (Janer et al., 2007; Lyssimachou et al., 2009; Pascoal et al., 2013). The recent discover of *ppar* in molluscs (Kaur et al., 2015; Vogeler et al., 2017, 2014) contributed to the hypothesis that TBT impair the lipid homeostasis through PPAR/RXR also in molluscs (Pascoal et al., 2013; Vogeler et al., 2017). We use *in vitro* approaches to characterize functionally for the first time one of the most basal PPARs and an *in vivo* experiment to assess the effect of TBT in the lipid metabolism.

PPAR/RXR is a known regulator of lipid metabolism in mammals (Bašić et al., 2012; Berkenstam and Gustafsson, 2005; Grün and Blumberg, 2007; Reilly and Lee, 2007). We successfully isolate a *ppar* from *P. depressa* (sequence in the supplementary material). The most conserved region of *P. depressa* PPAR is the DBD, similar to the observations in *C. gigas* (Vogeler et al., 2017). Our phylogenetic analysis demonstrated that the PPAR isolated in this work group with *B. glabrata* PPAR2 and *L. gigantea* PPAR2, and other gastropods. Moreover, the synteny analysis support the claim that the NR isolated is a true orthologue of the vertebrate PPARγ. Using an *in vitro* approach, we demonstrated that *P. depressa* PPAR is capable of heterodimerizing with RXR supporting the *in silico* observations in *C. gigas* (Vogeler et al., 2017).

To assess the conservation of PPAR response towards putative natural ligands, we tested the ability of two fatty acids (ARA and EPA) and a mixture of six fatty acids

(present in *P. depressa* gonads (Morais et al., 2003a) known mammalian PPAR ligands (Kliewer et al., 1997; Krey et al., 1997; Xu et al., 1999)) to transactivate both the receptors alone and in heterodimer. ARA and EPA were able to induce transactivation, similar to that observed in the mammalian PPAR orthologue. However, no transactivation was observed when the heterodimer PPAR/RXR was exposed to ARA. The lipid mixture transactivated the human PPARγ, but not the orthologue in *P. depressa* indicating that although ARA and EPA, present in the mixture, behave as agonists of *P. depressa* PPAR the other fatty acids present in the mixture are probably very weak agonists, the concentrations present of the individual fatty acids in the mixture can be too low to detect a response. The mixture of fatty acids was able of activate RXR, which is consistent with the high degree of conservation in the ligand and DNA binding domains of the receptor through the metazoans (André et al., 2017; Nishikawa, 2006; Urushitani et al., 2011; Vogeler et al., 2017), since the transactivation of RXR in the presence of fatty acids was previously reported in other species (Bordoni et al., 2006; Bouton et al., 2005; Egea et al., 2002; Lengqvist et al., 2004). The results indicate that some fatty acids naturally present in the gonads of *P.* depressa are able to modulate the PPAR found in this organism similar to what is observed in mammals pointing to a conservation in the function of the receptor.

Since the human heterodimer PPARγ/RXR was identified as targets of the obesogens TBT and TPT (Hiromori et al., 2015, 2009; le Maire et al., 2009) and the organotins are known EDCs in molluscs (Lima et al., 2011; Lyssimachou et al., 2009; Nishikawa et al., 2004) we choose these compounds to assess the vulnerability of the PPAR/RXR pathway to EDCs. In the human PPARγ the amino acid residues responsible for the binding of TBT and TPT have been previously identified (Harada et al., 2015). When we compare PPARγ sequence with the sequence isolated from *P. depressa* (Fig.7) we verify that the cysteine considered essential for the binding of TBT and TPT to the human PPARγ (Harada et al., 2015) is not conserved in *P. depressa*. We used an *in vitro* luciferase-based assay to evaluate if TBT and TPT were able of modulate the isolated receptors (PPAR and RXR) and the heterodimer PPAR/RXR (Fig.12). In humans, TBT and TPT are able to transactivate the individual units PPARγ and RXR and the functional unit the heterodimer PPARγ/RXR (Harada et al., 2015; Hiromori et al., 2015, 2009; le Maire et al., 2009). Our work demonstrates that in the mollusc *P. depressa* the response of the individual units to organotins is opposite, organotins transactivate *P. vulgata* RXR but represses *P. depressa* PPAR. The transactivation of RXR by organotins is in accordance with previous observations in molluscs (Nishikawa, 2006; Nishikawa et al., 2004; Urushitani et al., 2011). In mammals, PPAR/RXR is a permissive heterodimer being activated by PPAR and RXR ligands (Hiromori et al., 2015; Kojetin et al., 2015; le Maire

et al., 2009). Since in this work we found an oposite response in both receptors, it was necessary to retrive the response of the functional unity. The heterodimer in the presence of organotins is repressed, similar to what is observed in *P. depressa* PPAR. These results suggest that in the presence of TBT this heterodimer behaves according to PPAR. The repression observed in PPAR and in the heterodimer might be explained by the differences in the LBD of *P. depressa* PPAR when compared with the human PPARγ. Further studies are necessary to verify if the molluscs PPAR/RXR is permissive or not.

To determine the structural importance of the homologous amino acid at position 277 – cysteine in mammals (activation) and tyrosine in *P. depressa* (repression), in the binding to organotins we conducted some mutational analysis (Harada et al., 2015; Hiromori et al., 2009). We observed that mutations in the amino acid residue tyrosine 277 in *P. depressa* PPAR to cysteine leads to a weaker response in the presence of TBT, but is not enough to recover the activation phenotype observed with mammalian PPAR. Both amino acids (cysteine and tyrosine) are able of establish non-covalent bonds, and that has been reported to be the type of interaction between the tin moyet of TBT and cysteine 285 of human PPARγ (Harada et al., 2015). When we compare the similarity found in RXR LBD and PPAR LBD of the limpets with human RXRα and PPARγ, we observe a much higher conservation in RXR than in PPAR. The poor conservation found in PPAR can possibly explain why a single mutation is not capable to fully restore an activation similar to that observed in human PPARγ. We also observed that the substitution of tyrosine 277 to an alanine in *P. depressa* PPAR abolished the response to TBT (Fig. 13), showing that the position 277 (tyrosine) is important in the response of the molluscan PPAR to TBT. In *C. gigas in silico* observations suggested that TBT could use another cysteine present in the helix 3 to modulate this mollusc PPAR (Vogeler et al., 2017), although in the former study no functional characterization was done. Further studies are essential to reveal the structural basis for the TBT action in molluscs PPAR.

The responses obtained from the *in vitro* assays indicate a modulation of PPAR/RXR by organotins also in molluscs. Although the most explored effect of organotins in gastropods has been imposex induction (imposition of male secondary sexual characteristics in females) (Abidli et al., 2009; Castro et al., 2007; Lima et al., 2011; Nishikawa, 2006; Nishikawa et al., 2004, 2004; Santos et al., 2005; Sternberg et al., 2010), previous studies with gastropods suggest that lipid metabolism can also be disrupted in this *taxa* in the presence of TBT and TPT (Janer et al., 2007; Lyssimachou et al., 2009; Pascoal et al., 2013). To evaluate the possible interference of TBT with *P. depressa* lipid metabolism we analyse the fatty acid profile in the gonads of *P. depressa* exposed to environmentally relevant concentrations of TBT (100ng Sn/L (TBT1) and 250ng Sn/L

(TBT2)). In molluscs the main lipid reserve is found mostly in the gonads (Morais et al., 2003a). As observed in previous studies, males of *P. depressa* present a lower quantity of fatty acids than females (Morais et al., 2003a, 2003b). The lower quantity of fatty acids in male gonads impaired the identification and quantification of some fatty acids and in females the low survival observed in the animals exposed to the higher concentration of TBT impaired the evaluation of effects in this group. From the identified fatty acids the most abundant in females and males was palmitic acid, that is in accordance with previous studies (Morais et al., 2003a, 2003b). The effect of TBT was distinct in females and males, revealing a gender-specific response towards TBT. In females, the concentrations of some fatty acids decrease (Palmitic acid, Linolelaidic acid and PUFAs), while in males others increased (ARA, PUFAs and UFAs). In *Marisa cornuarietis* females, although a decrease in polyunsaturated fatty acids was observe after 100 days exposer to 500ng TBT Sn/L, the overall fatty acid content increased (Janer et al., 2007). On the other hand, TPT (30-500ng Sn/L) caused a general decrease in the fatty acids of *M. cornuarietis* females after 7 days of exposure (Lyssimachou et al., 2009). In the studies with *M. cornuarietis* males and females were analysed, but only females showed alterations in the fatty acid profile (Janer et al., 2007; Lyssimachou et al., 2009). Lipids have a major importance in the reproduction (Morais et al., 2003a, 2003b), also providing energy for growth when carbohydrates are not available (Morais et al., 2003a). It is possible that the decrease in the female fatty acids affect the reproduction and/or embryos survival, although more studies are necessary to fully disclosure the exact mechanisms.

This work demonstrates for the first time that the molluscs PPAR respond to some of the natural agonists of vertebrates PPARs strengthening the hypothesis that the function of PPAR shares some similarities with their vertebrate counterparts. Although, an opposite effect was observed regarding organotins and mollusc PPAR, we show that a clear modulation is present. We demonstrate that TBT modulate the mollusc PPAR and RXR individually and as heterodimer, although if TBT modulates the heterodimer through PPAR or RXR is still not clear. The *in vivo* and *in vitro* observations point to PPAR/RXR as a mediator of the interference observed in the lipid homeostasis of molluscs when exposed to organotins. Future work should focus in understanding how the NR PPAR regulates the lipid homeostasis in molluscs.

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Supplementary material

Table 1- Primer list

Target	Primer name	Primer sequence	Direction	Aim
RXR	P.vulg_RXR_pbind	GTCAGGATCCAAAGGCAGAGAACTAAGGAAAA	Foward	Pbind
RXR	P.vulg_RXR_pbind_R 2	TATAGCGGCCGCAACAGCTACGTCTGTGGGCT	Reverse	
PPAR	Pd_PPAR_Xbal_F	ACTGTCTAGAGCGGAGCGAGAAAAATTGTTG	Foward	
PPAR	Pd_PPAR_Knp1_R1	TATAGGTACCTCACATCTGTCGGATTTAAGC	Reverse	

P. depressa PPAR sequence

MLLADEYHPASGPQSHHFEIFADPYEGYYDSNQLMPTMCFDVFGGSTMMEDARLAVAFSQEESSQSSSVLYPHMTTSS
PSQHEALSRSPSVSSDGGKPLFKPQYTAELDVLCRICGDRASGFHYGVHSCEGCKGFFRRTLKKSLVYKPCQMGSQCK
IDMGTRNKCQYCRYQRCLNAGMSQDAVRFGRCRSGREKLLADKEELMTTCGKRIVELRSLTDLIKAAFRDIFINTIFFARE
NSRAMIFNQSEGQCVNYDDLIVVECLSSEEFFEKGIFTQYQELIVPVLEASVKFAKKLPGFTNMSMADQICLMKTNGFMV
VQIALHMLIDKDYINFVSRRNAFRLSRLSPYLCTEIQKLLERTICVGDRLHNMQLTAGEIALFCGILLTSECPGLQNVHQAE
QVQSDLIDALRLELKHNHPKDKLMLPKLLVLIPDLCQIVDEFSRNLQNHLFDHSHNFSSVQPLLQEIFDIEAVKNKNA

Table 2 - Accession numbers of the *PPAR* sequences used in the phylogenetic analysis * indicates sequences isolated in the current work see primer table 1 for more details.

Vertebrates	PPARα	PPARβ	PPARγ
Homo sapiens	Q07869	Q03181	P37231
Mus musculus	P23204	P35396	P37238
Gallus gallus	NP_001001464.1	NP_990059.1	NP_001001460.1
Anolis carolinesis	XP_003221452.1	*	XP_003220387.1
Xenopus tropicalis	XP_002940784.2	XP_012826243.1	*
Danio rerio	ABI30003.1	NP_571543	NP_571542.1
Pantodon buchholzi	Ctg24603	Ctg21483	Ctg18143
Lepisosteus oculatus	W5NK92*	ENSLACG00000015749	XP_006631094.2*
Latimeria chalumnae	XP_005991369.1	Nf	XP_006004475.1
Callorhinchus milii	SINCAMG00000010348	SINCAMP00000016425	XP_007901520
Leucoraja erinacea	*	*	*
Invertebrates	***Accession number***		
Ciona intestinalis	NP_001071801.1		
Branchiostoma floridae	jgi|Brafl1|174611		
Branchiostoma lanceolatum	*		
Branchiostoma belcheri	XP_019618227.1_		
Biomphalaria glabrata	XP_013069840.1, XP_013069841.1		
Patella depressa	*		
Lottia gigantea	jgi1|Lotgi1|174409, jgi2|Lotgi1|238472		
Strongylocentrotus purpuratus	XP_781750.1 XP_784429.3		
Saccoglossus kowalevskii	XP_006819446.1, NP_001164713		
Crassostrea gigas	XP_011412920.1		

Table 3– Fatty acids limits of quantification with the methodology used.

Fatty acid		LOQ (µg/ml)
Butiric	4:0	65,840
Hexanoic	6:0	53,890
Octanoic	8:0	36,207

Decanoic	10:0	35,948
Undecanoic	11:0	20,067
Lauric	12:0	48,223
Tridecanoic	13:0	28,980
Miristic	14:0	62,327
Myristoleic acid	**14:1**	30,236
Petadecanoic	15:0	35,570
Pentadecanoic cis	15:1	35,512
Palmitic	16:0	122,593
Palmitoleic acid	**16:1**	40,004
Heptadecanoic	17:0	46,833
Heptadecanoic	17:1	46,454
Stearic	18:0	96,904
Oleic Acid	**18:1 n9 c**	144,446
Elaidic	**18:1 n9 t**	
Linolelaidic	**18:2 n6 t**	44,549
Linoleic acid	**18:2 n6 c**	53,191
gamma- linoleic	18:3n6	43,696
Linolenic acid	**18:3 n3**	45,512
Arachidic	20:0	95,937
Eicosanoic acid	**20:1**	49,438
11,14-Eicosadienoic acid	20:2	50,209
11,14,17-eicotrienoic	20:3 n3	48,835
8,11,14- eicosatrienoic	20:3 n7	46,591
Arachidonic	**20:4 n6**	46,811
EPA	20:5 n3	46,327
Heneicosanoic	21:00	43,687
Docosanoic	22:0	177,982
Erucic acid	**22:1 n9**	77,250
13,16-docosadienoic	22:2	48,964

Tricosanoic	23:0	45,077
Lignoceric	24:0	94,636
DHA	**22:6 n3**	51,653
Nervoic acid	**24:1**	48,986

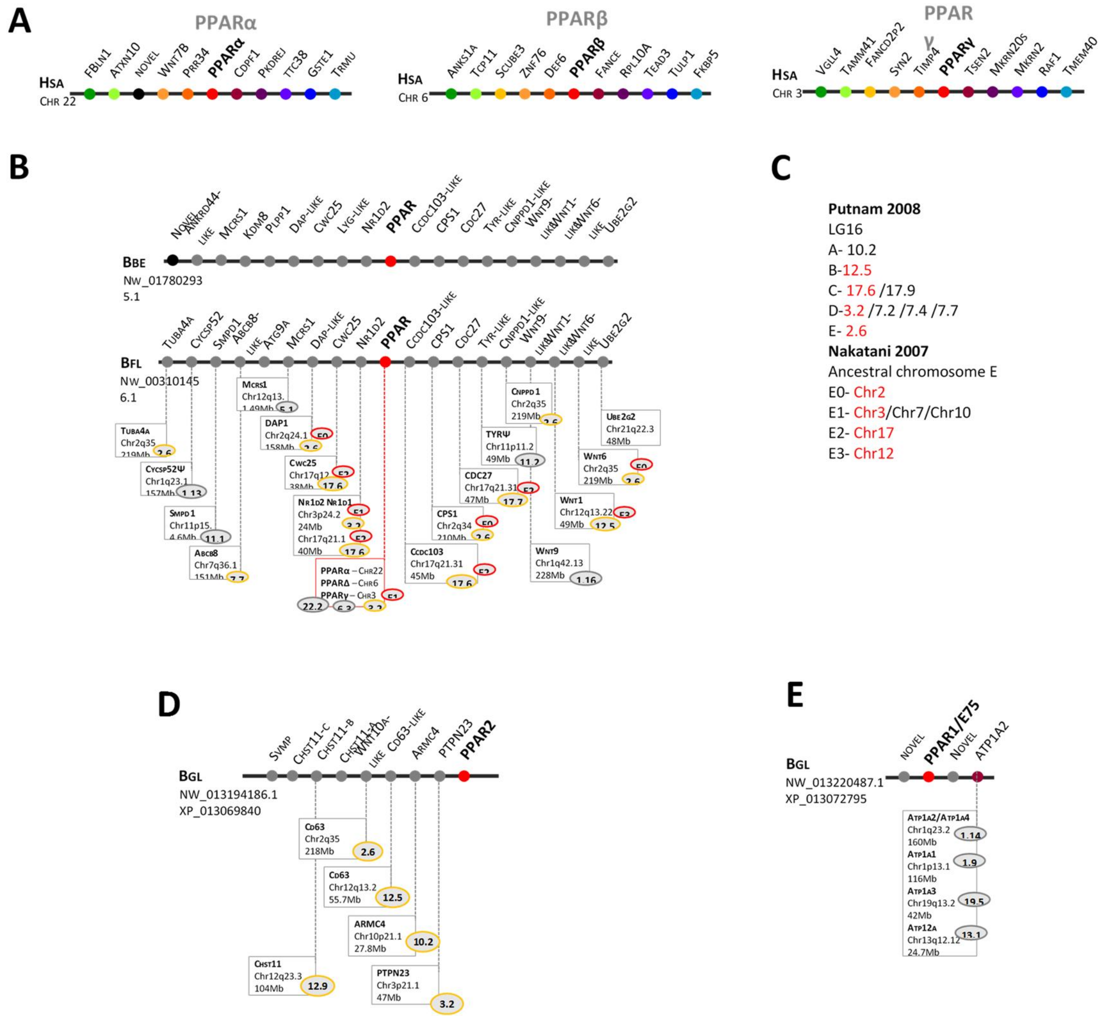

Fig. 1 - **A**: Synteny maps of the genomic location of *PPARα, PPARβ* and *PPARγ* in HSA-*Homo sapiens* **B**: Synteny maps of the genomic location of *PPAR* in BFL-*Branchiostoma floridae* and BBE- *Branchiostoma belcheri,* localization of the orthologous genes in *H. sapiens* is indicated below the synteny map of BFL, as well as the localization to the corresponding ancestral linkage groups or chromosomes in circles. **C** – Reconstruction of linkage group 16 and ancestral chromosome E according to Nakatani *et al*. 2007 and Putnam *et al.* 2008. **D** and **E**: Synteny maps of the genomic location of *PPAR2* and *PPAR1,* respectively*,* in BGL- *Biomphalaria glabrata* localization of the orthologous genes in *H. sapiens* is indicated below the synteny map of BFL, as well as the localization to the corresponding ancestral linkage groups or chromosomes in circles.

Chapter 3 - The sea urchin PPAR/RXR heterodimer is a target of the model obesogen TBT

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The sea urchin PPAR/RXR heterodimer is a target of the model obesogen TBT

Abstract

The wide ecological reach of obesogens has been increasingly acknowledged. Tributyltin (TBT), for instance, was shown to cause lipid accumulation, not only in mammals, but also in fish, molluscs and arthropods. In vertebrates, TBT interacts with a nuclear receptor heterodimer, formed by the retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor (PPAR), which modulates the expression of genes involved in lipid homeostasis. In the present work, we isolated for the first time an Echinodermata (*Paracentroctus lividus*) PPAR and RXR orthologue and evaluated the ability of a model obesogen, TBT, to interfere with the lipid metabolism in this species. Our results demonstrate that TBT alters the gonadal fatty acid composition. Gene expression patterns also denoted an impact of the model obesogen; an upregulation of long-chain acyl-CoA synthetase (*acsl*) and the retinoid X receptor (*rxr*) was observed. The former is required to convert long-chain fatty acids into acyl-CoA esters for downstream storage, in the form of triglycerides, or oxidation. Furthermore, organotins (TBT and triphenyltin (TPT)) repressed the heterodimer PPAR/RXR, *in vitro*, in a concentration-dependent manner. Together, these results suggest that TBT acts as an obesogen in Echinodermata and highlight a possible conserved mode of action via the PPAR/RXR heterodimer.

Introduction

Sea urchins, members of the Echinodermata phylum, play important roles in the ecosystem and display increasing economic interest (González-Irusta et al., 2010; Ribeiro et al., 2015; Romero et al., 2016). In the ecosystem they are one of the key groups regulating seaweed communities, due to their feeding behaviour (González-Irusta et al., 2010; Romero et al., 2016), and serve as prey for large crustaceans and fishes (Guidetti, 2004). In addition, their embryos provide an important tool for environmental monitoring and risk assessment (Khosrovyan et al., 2013; Repolho et al., 2011; Ribeiro et al., 2015). From an economic standpoint, these species hold a high commercial value, with their gonads considered a gastronomic delicacy (Guidetti, 2004; Guidetti et al., 2004; Shpigel et al., 2005). *Paracentrotus lividus* is an herbivorous sea urchin widely distributed in the

Atlantic and Mediterranean coasts (Arafa et al., 2012; Carboni et al., 2012; Kabeya et al., 2017). This species is subjected to intensive fishing because of the high value of their gonads (Arafa et al., 2012).

Lipids are important energy reserves as their energetic value per volume can be higher than proteins or carbohydrates (Castell et al., 2004). Besides the energetic value, lipids play structural roles (i.e. phospholipids and cholesterol) and serve as signalling molecules and enzyme cofactors, among other roles (Birsoy et al., 2013; Goel and Mushegian, 2006). In sea urchins, fatty acids are essential for gonad maturation and larvae development (Carboni et al., 2013). Before reaching the feeding stage, sea urchin embryos use nutrients provided by the egg. This makes maternal provisions, including essential fatty acids, crucial for embryo development and offspring success (Carboni et al., 2013). In agreement, previous studies found that total lipid levels were maintained constant prior to hatching; yet, decreased after the digestion of the envelope, between free-swimming blastula and the first feeding stages (Sewell, 2005; Smith et al., 2008). Among fatty acids, several long-chain are known to play a role in larvae development: docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA) (Carboni et al., 2013).

Animal lipid composition is not static and can be altered by diet, life-cycle stage or external stimuli (Arafa et al., 2012). Recently, several environmental chemicals have been shown to modulate lipid homeostasis (Castro and Santos, 2014; De Cock and Van de Bor, 2014; Diamanti-Kandarakis et al., 2009; Grün and Blumberg, 2009; Jordão et al., 2016b; Lyssimachou et al., 2015; Ouadah-Boussouf and Babin, 2016; Santos et al., 2012). Those compounds, able to interfere with lipid homeostasis, are known as obesogens and were primarily found to modulate lipid homeostasis in mammals; yet, recent studies have shown that their scope of action transcends mammals, or even vertebrates (Capitão et al., 2017; Janer et al., 2007; Jordão et al., 2016b, 2015; Lyssimachou et al., 2009). Tributyltin (TBT) is a recognised model obesogen. Although TBT is no longer used as biocide in anti-fouling paint for boats, its levels are still alarmingly high in some areas: reaching 241,8 µg Sn/Kg, as reported in biological samples from one of the largest harbour regions in China (Chen et al., 2017). TBT disrupts mammalian lipid homeostasis through the interaction with the nuclear receptors peroxisome proliferator-activated receptor γ (PPARγ) and retinoid X receptor (RXR) (Harada et al., 2015; Hiromori et al., 2009; le Maire et al., 2009). Nuclear receptors (NR) are a large family of transcription factors, mostly ligand activated, which modulate the expression of genes engaged in a myriad of biological processes (Escriva et al., 2004). PPARγ and RXR cooperate in the form of an heterodimer (PPARγ/RXR) that

is considered a master regulator of lipid homeostasis in vertebrates (Ahmadian et al., 2013; Berkenstam and Gustafsson, 2005; Janesick and Blumberg, 2011). Given that both units can elicit a response upon activation, the PPARγ/RXR heterodimer is considered permissive and thus able to accommodate PPARγ and RXR ligands (Hiromori et al., 2015; Ouadah-Boussouf and Babin, 2016; Santos et al., 2012). TBT has been identified and shown to modulate RXR transactivation in several invertebrate groups, including annelids (André et al., 2017), gastropods (Nishikawa et al., 2004) and crustaceans (Wang and LeBlanc, 2009). Outside deuterostomes, PPAR has only been reported in mollusks (Vogeler et al., 2014). Yet, in vertebrates, TBT, and the related organotin, triphenyltin (TPT), were also shown to interact with PPARγ exhibiting a similar binding mode as that described for RXR: through the interaction of the tin atom and a cysteine residue (Harada et al., 2015; Hiromori et al., 2009; le Maire et al., 2009). In the arthropod *Daphnia magna*, TBT induced an increase in lipid storage. Yet, this effect was shown to be mediated by RXR in partnership with the ecdysteroid receptor (EcR), and not PPAR, which has not been found in this group (Jordão et al., 2016a). Although NRs are present in the major metazoan groups (Bridgham et al., 2010), the data available focus in a restrict number of groups (Castro and Santos, 2014; Tan and Palli, 2008; Thornton, 2003; Vogeler et al., 2017). PPAR was identified outside vertebrates, but the only characterization existent is from the mollusk *Crassostrea gigas* and through *in silico* assay (Vogeler et al., 2017). Since echinoderms are a basal group of deuterostomes (Lavado et al., 2006), the characterizations of their NRs involved in the lipid homeostasis is essential to better understand the evolutionary response to obesogen compounds.

In this work, we isolated and functionally characterize *in vitro* for the first time the PPAR orthologue of a non-chordate using transactivation assays. We also evaluated the ability of the model obesogen, TBT, to alter lipid homeostasis in the echinoderm *Paracentrotus lividus* (*P. lividus*), following a 3 weeks exposure to environmentally relevant concentrations (100 and 250ng Sn/L) assessing the fatty acid profile and gene expression. Since echinoderms are deuterostomes, and thus more related to chordates than any other invertebrate group (Lavado et al., 2006), our central aim was to get insights into the adverse outcomes of model obesogens in basal deuterostomes.

Methods

Gene isolation and cloning

Full sequence of the gene rxr and partial sequences of the genes ppar, rxr, acc (*acetyl-CoA carboxylase*), acsl (*long-chain acyl-CoA synthetase*), fas (fatty acid synthetase) and cpt1 (*carnitine palmitoyltransferase 1*) were obtained using a combination of PCR-based approaches. First, degenerate PCR primers were designed from conserved regions of related sequences using CODEHOPE (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) program to obtain the initial fragment or fragments. For PPAR and RXR, 3' and 5' ends were further extended using the SMARTer™ RACE cDNA Amplification Kit (Clontech) following the manufacturer's instructions. The complete Open Reading Frame (ORF) was obtained using specific primers for the 3' and 5' ends, designed in Primer-BLAST (NCBI) (primer sequences can be found in the supplementary material – table 4). Gene sequences were verified using Sanger sequencing (GATC).

The hinge and ligand-binding domains of *P. lividus* PPAR and RXR were cloned into the pBIND vector to produce an NR LBD-Gal4 hybrid protein. This hybrid protein, containing the DNA binding domain of Gal4, acts on a UAS response element (Primer sequences in the supplementary material – table 4). For *in vitro* assays with the PPAR/RXR heterodimer, hinge and ligand-binding domains of *P. lividus* RXR was further sub-cloned into the pACT vector to produce a RXR LBD-VP16 hybrid protein. VP16, which acts on proximal downstream promoters further amplifies transcription and is used as a two-hybrid system to assess protein-protein interaction. Plasmid sequences were verified using Sanger sequencing (GATC).

Sequence search

Tblastn and blastp searches using human PPARα (Q07869), PPARβ (Q03181) and PPARγ (P37231) as query were performed in the available databases NCBI Ensemble and JGI. PPAR sequences were retrieved for the major vertebrate lineages: mammals (*Homo sapiens*, *Mus musculus*); birds (*Gallus gallus*) reptiles (*Anolis carolinesis*); amphibians (*Xenopus tropicalis*); coelacanths (*Latimeria chalumnae*); Lepisosteiformes (*Lepisosteus oculatus*) Osteoglossomorpha (*Pantodon buchholzi*) Cyprinipforme (*Danio rerio*), Chondrichthyes (*Callorhinchus milii*) and for the following invertebrates, urochoradate (*Ciona intestinalis*) cephalochordate (*Branchiostoma floridae, Branchiostoma lanceolatum, Branchiostoma belcheri*), hemichordate (*Saccoglossus

kowalevskii), Molluscs (*Biomphalaria glabrata, Lottia gigantea, Crassostrea gigas*), Echinodermes (*Strongylocentrotus purpuratus*). (See supplementary table 6 for accession number).

Tblastn and blastp searches using human RXRα (NP_002948.1) as query were performed in the available databases NCBI Ensemble and JGI. RXR sequences were retrieved for the major vertebrate lineages: mammals (*Homo sapiens*); amphibians (*Xenopus laevis*); Cyprinipforme (*Danio rerio*), Chondrichthyes (*Callorhinchus milii*) and for the following invertebrates, urochoradates (*Ciona intestinalis, Polyandrocarpa misakiensis*) cephalochordate (*Branchiostoma floridaei*), hemichordate (*Saccoglossus kowalevskii*), Molluscs (*Biomphalaria glabrata, Lottia gigantea, Crassostrea gigas, Nucella lapillus, Thais clavigera, Lymnaea stagnalis),* brachiopoda (*Lingula anatina*), Echinodermes (*Strongylocentrotus purpuratus, Mesocentrotus nudus, Acanthaster planci*), arthropods (*Daphnia magna, Locusta migratoria, Tribolium castaneum, Leptuca pugilator, Marsupenaeus japonicus, Drosophila melanogaster, Bombyx mori)*, cnidaria (*Aurelia aurita, Tripedalia cystophora)*. (See supplementary table 6 for accession number).

Phylogenetic analysis

Phylogenetic analysis were performed using the set of sequences through database search as well as the isolated sequences. A total of 48 sequences for PPAR and 26 for RXR were aligned in MAFFT (Katoh et al., 2005; Katoh and Toh, 2008) with L-INS-I method. The resulting sequence alignment was stripped of all columns containing 90% gaps leaving a total of 544 positions for PPAR and 576 for RXR for phylogenetic analysis. Maximum likelihood phylogenetic analysis was performed in PhyML V3.0 (Guindon et al., 2010) and the evolutionary model was determined using the smart model selection (SMS) option resulting in a JTT +G+I+F. The branch support was calculated using aBayes. The resulting tree was analysed in Fig Tree V1.3.1 available at http://tree.bio.ed.ac.uk/software/figtree/ and rooted with the invertebrate sequences.

Cell culture and *in vitro* assays

COS-1 cells were grown at 37ºC in a humidified chamber containing 5% CO2 in Dulbecco's modified Eagles medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Cells were seeded in 24-well culture plates at a density of 2 x 10^5 live cells/ well 24 hours prior to

transfection. Cells were transfected using lipofectamine 2000 reagent (Invitrogen), in Opti-MEM reduced serum medium (Gibco, Thermo Fisher), according manufacturer's indications and 0.5 µg of pBind PPAR LBD-Gal4 or RXR LBD-Gal4 and 1µg of pGL4.31 luciferase reporter vector, containing five UAS elements upstream the firefly luciferase reporter gene. For heterodimer transfection assays 0.5 µg of pBind PPAR LBD-Gal4 and 0.5 µg of pACT and 1µg of pGL4.31, were used. After 5 hours of incubation the medium was replaced with phenol red-free DMEM supplemented with 10% dextran-coated charcoal-treated serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) containing the test compounds dissolved in DMSO (0.1%). 24 hours later cells were lysed and Firefly luciferase (pGL4.31) and Renilla luciferase (pBIND) luminescent 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). Renilla Luciferase, co-expressed with the NR LBD-Gal4 hybrid proteins, served as an internal control for transfection efficiency. All assays were performed, independently, three times. In each experiment, two technical replicates per condition were performed to validate the results.

Sequence analysis

The amino acid sequences of the BDB, LBD and hinge of the receptors were predicted using the Conserved Domain Database at NCBI. The sequence of each domain of RXR was then compared with *Mesocentrotus nudus* and human homologs. The LBD amino acids identified as interacting with TBT and/or TPT were predicted based on previously published works (Harada et al., 2015; Hiromori et al., 2015; le Maire et al., 2009).

Chemicals and solutions

All chemicals and reagents were obtained from Sigma-Aldrich unless stated otherwise in the text. Two TBT-Cl (Tributyltin chloride) stock solutions were prepared in DMSO (Dimethyl sulfoxide) for the in the *in vivo* experiment, with the concentrations of 250 mg Sn/L and 100 mg Sn/L. The final concentration of DMSO in the aquaria was 0.0001%. Three TBT-Cl and three TPT-Cl (Triphenyltin chloride) stock solutions were prepared in DMSO for the in vitro experiment: 250 µM (29.7 mg Sn/L), 100 µM (11,87 ng Sn/L) and 10 µM (1,187 ng Sn/L). Three ARA (Arachidonic acid) and three EPA (cis-5,8,11,14,17-Eicosapentaenoic acid) solutions were prepared in DMSO with the concentrations of 200 mM, 100 mM and 50 mM. One mixture of 6 fatty acids (lipid mix)

containing 50 mM of ARA, 50 mM of EPA, 50 mM of oleic acid, 50 mM of linoleic acid, 50 mM of γ-linolenic acid and 13.3mM of palmitic acid was prepared in DMSO, two dilutions were made in DMSO, 50% and 25%.

Animal maintenance, experimental setup and sampling

To evaluate changes on lipid profile and gene transcription in the presence of a putative PPPAR/RXR ligands, we carried out an *in vivo* exposure using the same dosing set up of our previous experiments with TBT (Lima et al., 2011). Approximately 150 *P. lividus* specimens, with homogenous sizes, were collected at São Bartolomeu do Mar beach (Portugal) and transported to CIIMAR's (Interdisciplinary Centre of Marine and Environmental Research) aquatic animal facilities. In CIIMAR the animals were randomly distributed in 12 aquaria (13 animals/aquarium) containing 20L of filtrated salt water at 16 ± 1°C, a 16/8 light/dark cycle and continuous aeration. During all experimental period, the animals were feed with broken maize (bought in a local store), *Ulva sp.* and *Palmaria palmata* (collected at Granja beach, Portugal) *ad libitum* (Cook et al., 2007; Repolho et al., 2011; Shpigel et al., 2006, 2005)**.** Temperature was monitored daily and the water physicochemical parameters (34,2‰ of salinity, 0.68 ± 0.31 mg/L of ammonium and 0.1 ± 0.06 mg/L of nitrite) were monitored weekly. After 7 days of acclimatization the following experimental treatments were set: control; solvent control (DMSO at 0.0001%), TBT1 (100 ngSn/L), TBT2 (250ngSn/L), with three replicate aquaria per condition. Chemical dosing (as in Lima et al., 2011) and feeding was performed 3 times per week during water renew. After acclimatization, at least 6 males and 6 females were randomly chosen and sacrificed (time 0). After 48 hours and after 3 weeks at least 6 males and 6 females from each treatment were randomly chosen and sacrificed (48h and final) (scheme in supplementary material Fig.2). Gender, length, whole weight, gonad weight and maturation stage were recorded. Gonads were frozen in liquid nitrogen and subsequently stored at -80°C until further analysis. Gonads and gut (divided in three parts) were stored in RNA later (Sigma) according to manufacturer's recommendations. Gonad index (GSI) was calculated using:

$$GSI = \frac{Wet\ weight\ of\ gonads}{Wet\ weight\ of\ whole\ animal} \times 100$$

(Sánchez et al., 2004)

Fatty acid quantification

For fatty acid quantification, six samples of males and six samples of females of each group were extracted and analysed. Fatty acid extraction was performed according to the Folch method with minor adjustments (FOLCH et al., 1957). Briefly, the tissue was homogenized with chloroform/methanol (2:1) to a final volume of 20ml/gr of tissue. After homogenization tricosanoic acid was added as internal standard the mixture was agitated during 3 hours in an orbital shaker at room temperature protected from the light. The sample was then centrifuged for 30 minutes at 2000 rpms. The supernatant was recovered for a new tube and vortexed for 1 minute with 0.25 volume of a NaCl (9%) solution. The mixture was then centrifuged for 10 min at 2000 rpm for phase separation, the upper phase was discarded. A final wash with methanol/water (1:1) was performed previous to the centrifugation. The upper phase was discarded and the lower phase was evaporated under a nitrogen stream. The samples were kept at -20ºC until derivatization. Fatty acid derivatization was performed as described by Cohen et al., 1988 using acetyl chloride as catalyst and the detection was performed according to Guedes et al., 2011. Fatty acids were quantified in µg/mg of tissue as done in Morais et al., 2003 (quantification limits for the fatty acids analysed are presented in the supplementary table 7).

Tissue RNA extraction and cDNA synthesis

Total RNA was purified from approximately 12,5 mg of gonad tissue using a combination of methods. First, tissues were homogenized with Purezol RNA isolation reagent (Biorad) 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 verified by electrophoresis in a 1,5% agarose gel and quantification was performed using the Take3 microplate spectrophotometer (Synergy HT Multi-Mode Microplate Reader, Biotek) and the Gen5 software for nucleic acid quantification. First strand cDNA was generated from 1 µg of total RNA using the NZY First-Strand cDNA Synthesis Kit (Nzytech). All assays were carried out following the manufacturer's recommendations.

Real-time quantitative PCR

Gonadal gene expression profile was evaluated using fluorescence-base quantitative (real-time) PCR. The genes selected code for proteins involved in fatty acid synthesis (*acc*), catabolism (*acsl* and *cpt1*) and regulation (*ppar* and *rxr*). For each treatment at time 0 and 48 hours six samples of male and six samples of female gonads were used to assess the expression of selected genes using the Mastercycle ep realplex system (Eppendorf). Forward and Reverse primers were designed using Primer-BLAST (NCBI) and verified for secondary structure using Beacon Designer™ Free Edition. In 96 well plates, 2µl of each cDNA was added to a mixture containing 1x iQ™ SYBR® Green Supermix (Biorad) and 300 nM of primers (primer sequences and amplification efficiency can be found in the supplementary material – table 5). Samples were run in duplicate and a no template control was included in each run. A melting curve was generated in every run and random samples were further inspected by gel electrophoresis (2%) to confirm the specificity of the assays. *Tubulin 1a* was used as reference gene and relative gene expression was calculated using the Livak method (Livak and Schmittgen, 2001). Amplified sequences were confirmed using Sanger sequencing (GATC).

Statistical analysis

One-way ANOVA followed by Fisher LSD post hoc test was used to analyze differences when parametric criteria was achieved, otherwise a non-parametric Kruskal-wallis ANOVA followed by a Games-Howel test was used. All analyses were performed using IBM SPSS Statistics 24. $P < 0.05$ was considered statistically significant in all analyses. Outliers were verified by the outlier labelling rule (Hoaglin and Iglewicz, 1987).

Results

***P. lividus* PPAR and RXR isolation and sequence analysis**

The partial alignment of echinoderm PPARs, from *P. lividus* and *Strongylocentrotus purpuratus*, and human PPARγ showed that the residues involved in TBT and/or TPT binding to human PPARγ are partially conserved in *P. lividus* (Fig.14). The cysteine anchor residue, shown to interact with the tin atom, of both TBT and TPT, is conserved in both echinoderm sequences (Harada et al., 2015). Yet, other residues, interacting with TBT and/or TPT, are marginally conserved in *P. lividus* PPAR: only two

residues interacting with both receptors, and one exclusive of TPT, are conserved (phenylalanine, histidine and lysine, respectively).

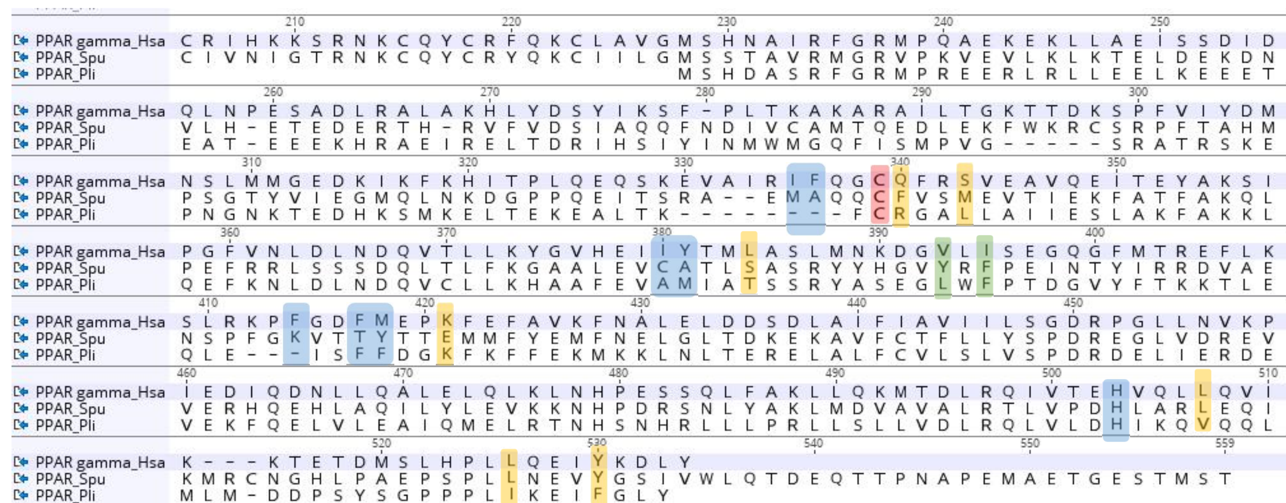

Fig. 14 – PPAR sequence alignment. Amino acid marked in: red- anchor; blue- interact with TBT and TPT; yellow-interact only with TPT; green – interact only with TBT. Based on (Harada et al., 2015).

P. lividus PPAR phylogenetic analysis

To infer about the orthology of the *PPAR* isolated from *P. lividus* a phylogenetic and analysis was performed. The phylogenetic analysis (maximum likelihood) shows that invertebrate sequences outgroup vertebrate PPARα, PPARβ and PPARγ. Two *PPAR* sequences are genome predicted for echinoderms. The sequence of *PPAR* isolated in this work groups with another echinoderm, *Strongylocentrotus purpuratus,* and a hemichordate, *Saccoglossus kowalevskii.* The second sequence predicted for echinoderms outgroup the previous ones (Fig.15).

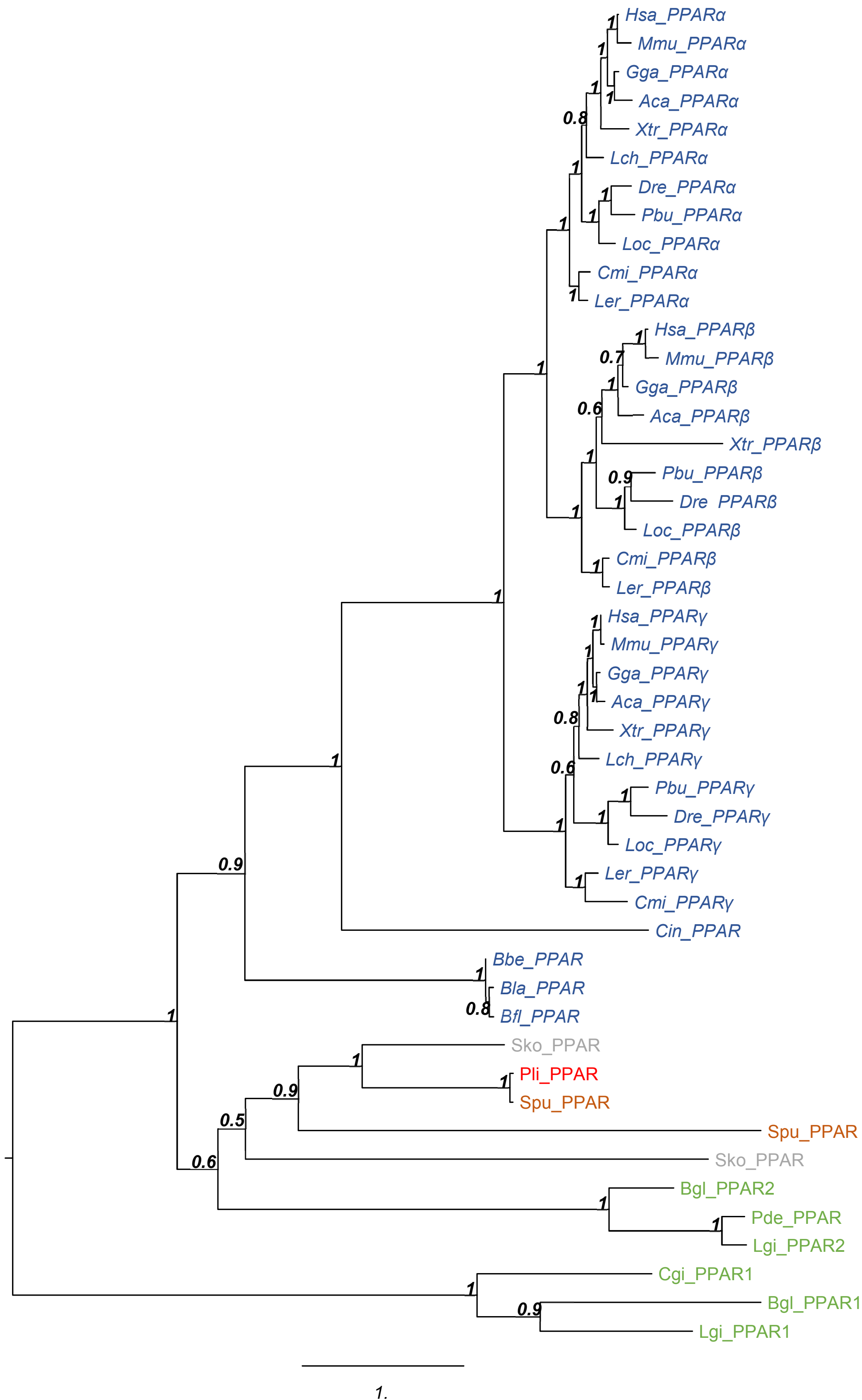

Fig. 15 - Bayesian phylogenetic analysis of PPAR amino acid sequences, values at nodes indicate posterior probabilities. Hsa*: Homo sapiens*; Mmu: *Mus musculus*; Gga*: Gallus gallus*; Aca*: Anolis carolinesis*; Xtr: *Xenopus tropicalis;* Lch: *Latimeria*

chalumnae; Dre*: Danio rerio*; Pbu*: Pantodon buchholzi*; Loc*: Lepisosteus oculatus*; Cmi*: Callorhincus milii*; Ler:*Leucoraja erinacea*; Bbe*: Branchiostoma belcheri*; Bla: *Branchiostoma lanceolatum*; Bfl: *Branchiostoma floridae*; Sko: *Saccoglossus kowalevskii*; Pli: *Paracentrotus lividus*; Spu: *Strongylocentrotus purpuratus*; Bgl*: Biomphalaria glabrata*; Pde*: Patella depressa*; Lgi*: Lottia gigantea*; Cgi: *Crassostrea gigas.* Red*: P. lividus* PPAR*;* Blue: Chordatha; Orange: Echinodermata; Green: Mollusca; Grey: other Metazoans.

RXR sequence alignment

Regarding RXR, alignment of echinoderm RXRs, from *P. lividus* and *Mesocentrotus nudus* (AEL87703.1), and human RXRα (NP_002948.1) showed that *P. lividus* RXR DBD exhibited 99% similarity when compared to *M. nudus* RXR and 88% when compared to the human homologue (Fig.16 and Table 9). The hinge region exhibited a 95% similarity with *M. nudus* and 53% similarity with human RXR. Finally, the LBD of *P. lividus* RXR presented 99% and 82% similarity when compared to *M. nudus* and to the human receptors, respectively. The key amino acid residue involved in TBT and TPT binding to human RXRs, a cysteine residue, is conserved in *P. lividus* as well as in *M. nudus* RXR (Hiromori et al., 2015; le Maire et al., 2009).

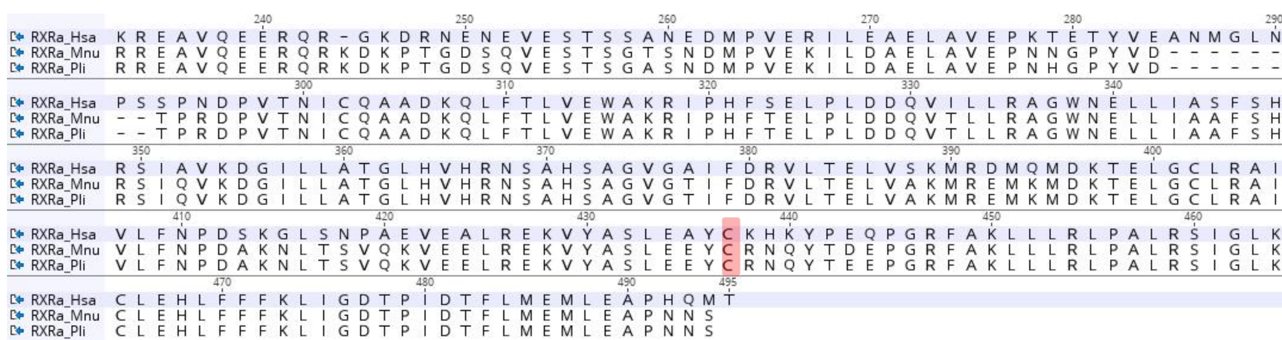

Fig. 16 -*RXR* sequence alignment. Amino acid marked in red- anchor cysteine. Based on (Hiromori et al., 2015; le Maire et al., 2009)

Table 9 - Similarity between *P. lividus* RXR domains and *M. nudus* and *H. sapiens*.

RXR	DBD	H	LBD
Mesocentrotus nudus	99%	95%	99%
Homo sapiens	88%	53%	82%

P. lividus RXR phylogenetic analysis

The phylogenetic analysis (maximum likelihood) performed using several *RXR* sequences demonstrate that *P. lividus RXR* isolated in this work group with other

echinoderm sequences (Fig.17). Chordates and non-chordates *RXR* form are two distinct groups that are outgroup by the cnidarian *RXRs*.

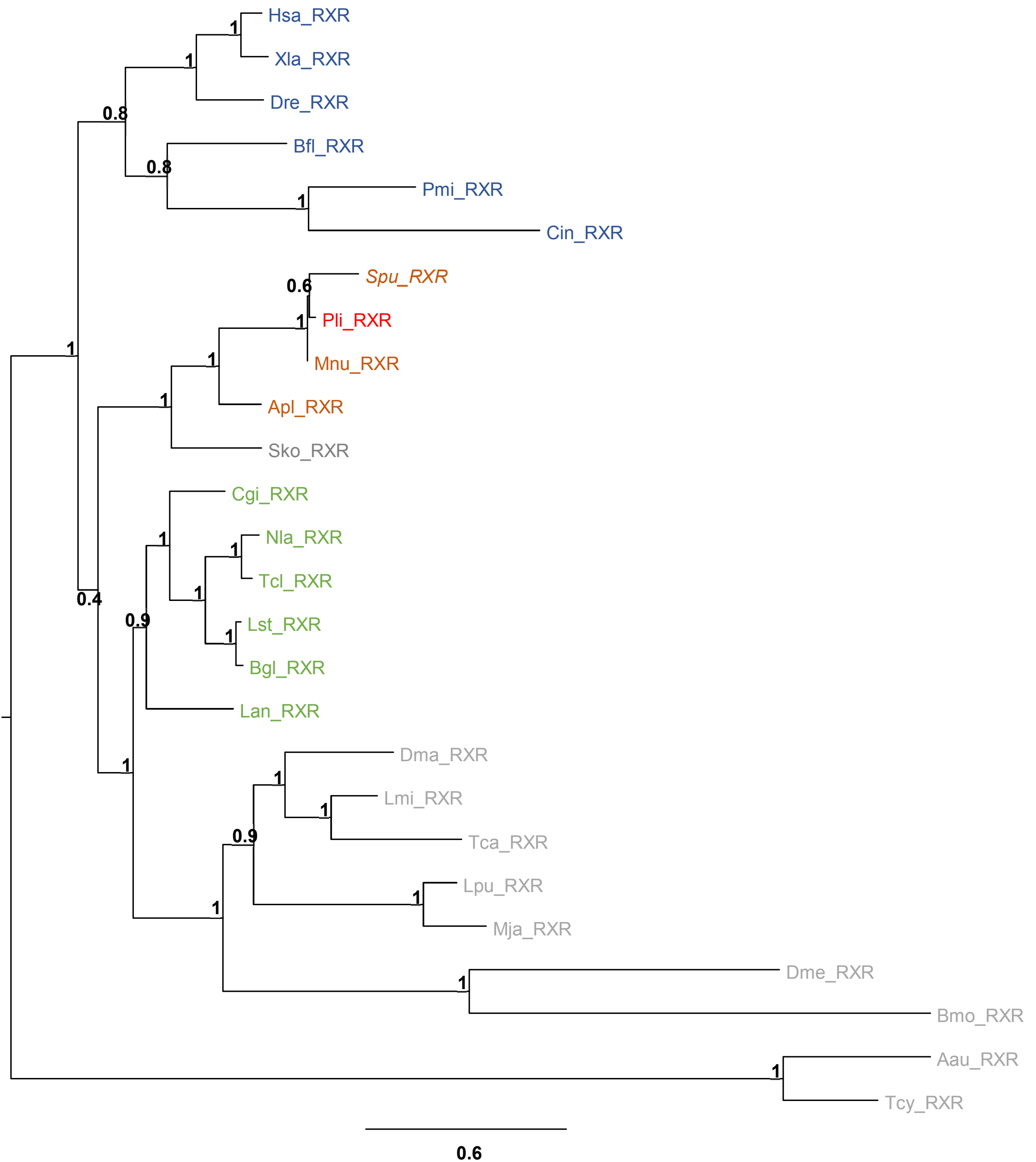

Fig. 17- Bayesian phylogenetic analysis of RXR amino acid sequences, values at nodes indicate posterior probabilities. Hsa: *Homo sapiens*; Xla: *Xenopus laevis*; Dre: *Danio rerio*, Cmi: *Callorhinchus milii*, Cin: *Ciona intestinalis*, Pmi: *Polyandrocarpa misakiensis*, Bfl: *Branchiostoma floridaei*, Sko: *Saccoglossus kowalevskii*, Bgl: *Biomphalaria glabrata*, Lgi: *Lottia gigantea*, Cgi: *Crassostrea gigas*, Nla: *Nucella lapillus*, Tcl: *Thais clavigera*, Lst: *Lymnaea stagnalis*, Lan: *Lingula anatina*, Spu: *Strongylocentrotus purpuratus*, Mnu: *Mesocentrotus nudus*, Apl: *Acanthaster planci*, Dma: *Daphnia magna*, Lmi: *Locusta migratoria*, Tca: *Tribolium castaneum*, Lpu: *Leptuca pugilator*, Mja: *Marsupenaeus japonicus*, Dme: *Drosophila melanogaster*, Bmo: *Bombyx mori*, Aau: *Aurelia aurita*, Tcy: *Tripedalia cystophora*. Red: *P. lividus* PPAR; Blue: Chordatha; Orange: Echinodermata; Green: Mollusca; Grey: other Metazoans.

Transcriptional activation of PPAR, RXR and PPAR/RXR

The ability of several compounds (TBT, TPT, ARA, EPA and lipid mix) to activate the nuclear receptors PPAR, RXR and the heterodimer PPAR/RXR were evaluated using a luciferase reporter gene assay. To evaluate if *P. lividus* PPAR form a heterodimer with RXR a two-hybrid reporter cell system was used. When PPAR phusion protein interacts with RXR phusion protein cause an mesurable increase in the luciferase production. A significant increase in the luciferase levels was observed when the potential heterodimeric partner (RXR) was present (Fig.18).

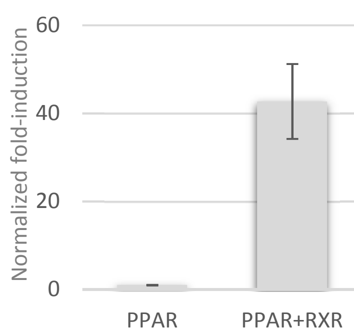

Fig. 18 - Luciferase luminescence upon transfection of PPAR and co-transfection of PPAR and RXR in the absence of ligands.

The ability of two different fatty acids (ARA and EPA) and a mixture of 6 different fatty acids, also including ARA and EPA among others, to transactivate *P. lividus* receptors was tested (Fig.19). The EPA concentrations tested, ranging from 50µM to 200µM, were not able to transactivate neither PPAR nor RXR. Thus, exposures to both receptors, in the heterodimeric form, were not performed. ARA, on the other hand, induced significant NR-dependent transactivation with RXR alone, but not with PPAR nor PPAR/RXR. RXR was also activated by the fatty acid mix, at the three concentrations tested (25%, 50% and 100%), as well as PPAR/RXR, when exposed to lipid mix at 50% or 100%, but not PPAR alone. When exposed to organotins the tested receptors present distinct responses (Fig.20). *P. lividus* PPAR was not transactivated by TBT, while RXR was significantly transactivated by the three different concentrations of TBT tested (10nM, 100nM and 250nM). Inversely, the heterodimer PPAR/RXR exhibited a repression in the presence of TBT, in a concentration-dependent manner. Similarly, TPT also repressed the NR heterodimer, and PPAR alone, in a concentration-dependent manner. Yet, RXR was significantly induced by 100nM and 250nM TPT.

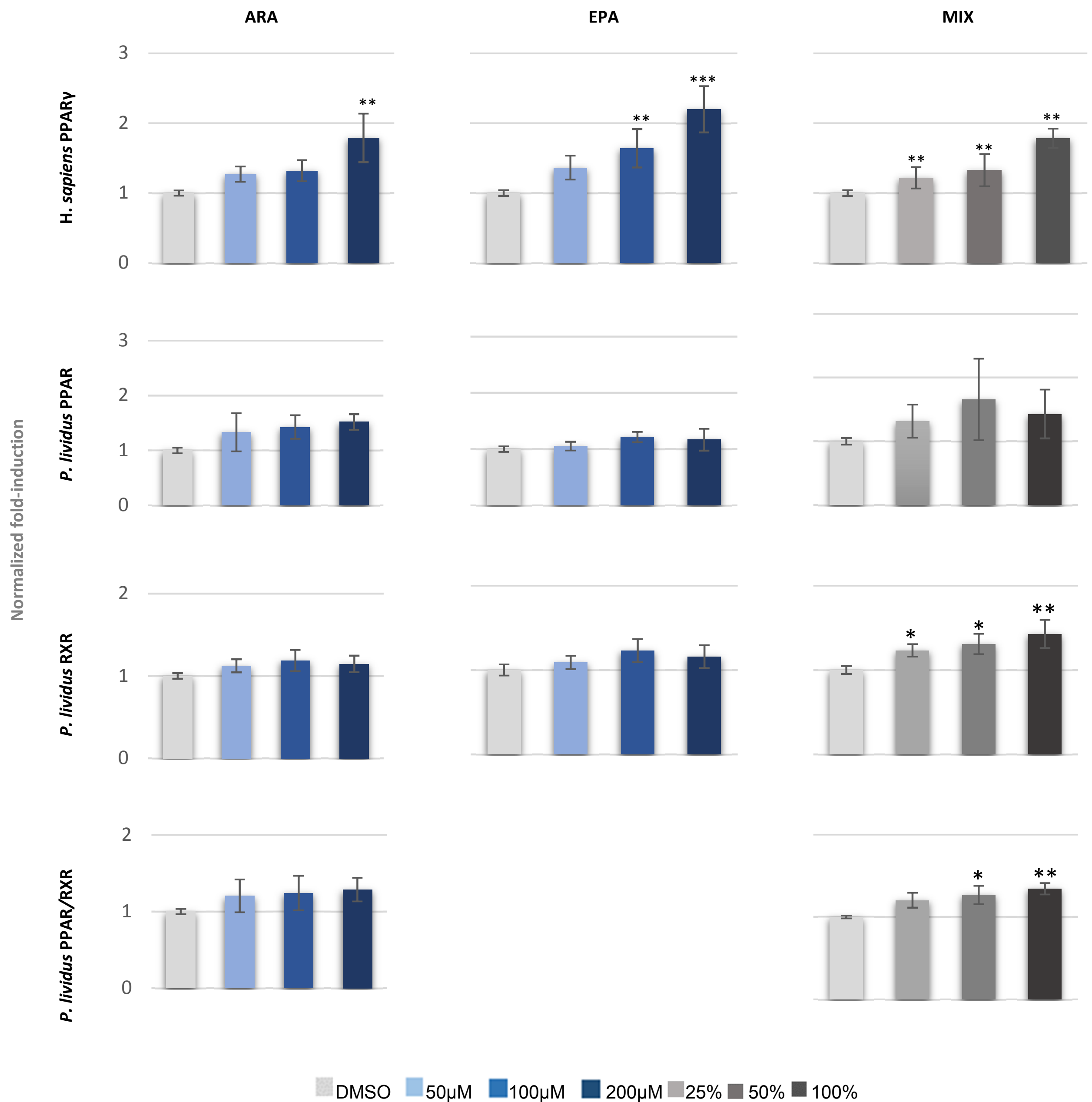

Fig. 19 - *H. sapiens* PPARγ, *P. lividus* PPAR, RXR and heterodimer PPAR/RXR transactivation in the presence of the fatty acids ARA and EPA and the lipid mixture containing 50 µM of ARA, 50 µM of EPA, 50 µM of oleic acid, 50 µM of linoleic acid, 50 µM of γ-linolenic acid and 13.3µM of palmitic acid). *Data are presented as mean± standard deviation of the normalized fold-induction (*-p<0.05; ** - p<0.01; *** - p<0.001).*

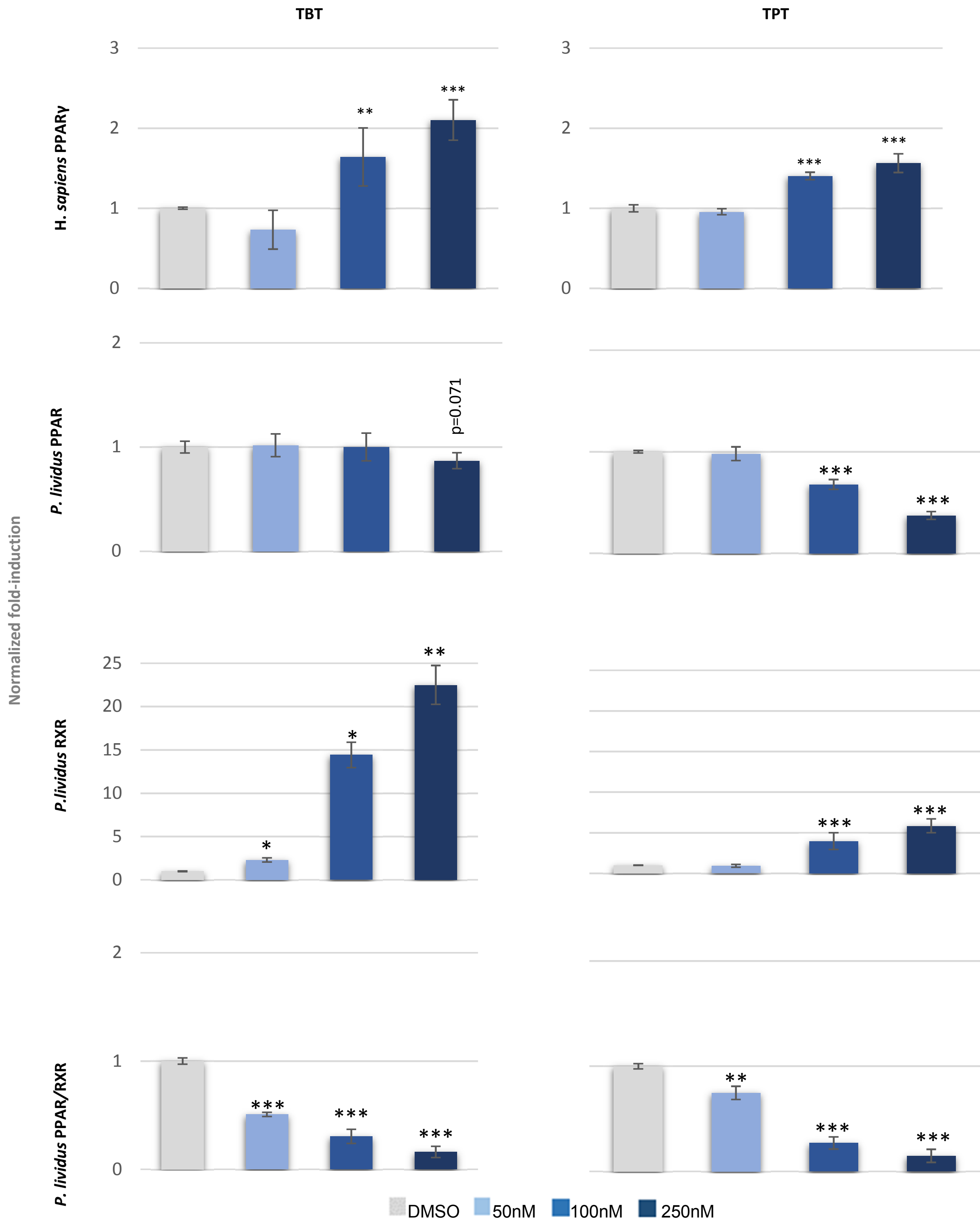

Fig. 20 – *H. sapiens* PPAR, *P. lividus* PPAR, RXR and heterodimer PPAR/RXR transactivation in the presence of TBT and TPT. Data are presented as mean± standard deviation of the normalized fold-induction (*-p<0.05; ** - p<0.01; *** - p<0.001).

Morphological parameters

Several morphological parameters (length, whole weight, gonad maturation stage and index) were recorded or calculated for each sampling time and treatments (table 10). In the several parameters analysed, no alterations were observed between the sampling times (0, 24 hours and 3 weeks) or between the different treatments.

Table 10- Morphological parameters of *P. lividus* male and female in the beginning of the experiment (0h), 48 hours later (48h) and in the end of the experiment (final). Values are the Mean±STD.

	Gender	Treatment	Length (mm)	Whole Weight (gr)	Gonad stage	Gonad Index
0h	Females	n.a.	43.20±4.13	31.45±8.75	2.89±0.42	6.2%±3.6
	Males	n.a.	46.28±7.38	39.73±17.62	2.93±0.73	9.6%±8.0
48 h	Females	Control	47.85±4.58	43.25±10.52	3.2±0.41	8.17%±3.9
		DMSO	47.30±6.19	44.93±16.89	3.3±0.52	9.12%±4.11
		TBT1	47.41±6.78	44.96±18.42	2.9±1.02	8.03%±5.31
		TBT2	45.26±3.86	38.73±8.77	3.0±1.04	5.90%±3.29
	Males	Control	43.80±4.56	36.42±10.52	2.9±0.64	6.74%±1.46
		DMSO	43.52±3.17	40.52±9.97	2.8±0.48	6.77%±2.33
		TBT1	45.99±3.30	42.00±7.93	2.8±1.03	4.92%±1.80
		TBT2	43.98±5.07	39.45±13.48	2.9±0.89	7.42%±3.00
Final	Females	Control	46.56±2.62	39.72±6.06	2.6±0.5	5.85%±0.02
		DMSO	44.92±3.40	38.31±8.04	3.0±0.7	6.49%±0.02
		TBT1	46.33±3.93	42.21±12.15	3.0±0.7	7.03%±0.03
		TBT2	44.75±5.61	36.87±13.95	2.5±0.8	5.09%±0.02
	Males	Control	44.60±5.35	36.92±14.02	3.0±0.5	5.92%±0.02
		DMSO	46.96±4.70	42.78±14.37	2.9±0.6	5.68%±0.02
		TBT1	48.90±5.80	47.63±18.64	2.7±0.8	5.05%±0.02
		TBT2	46.83±7.84	43.30±20.44	2.6±1.1	5.13%±0.02

P. lividus fatty acid profile

The fatty acid composition of *P. lividus* gonads is presented in the tables 11 and 12: quantitative data is provided for time 0 and final sampling. Since control and solvent samples didn't present differences, control and solvent control samples were grouped (Control/DMSO). From the 36 fatty acids analysed only 17 were detected in female gonads and 16 in the male gonads. Fatty acids with less than 14 or more than 22 carbon atoms were not detected. Most of the detected fatty acids were unsaturated corresponding to 50.45% and 56.98% of the total fatty acids content in Control/DMSO females and males, respectively. From those, the polyunsaturated fatty acids (PUFA) were present in higher amounts, 34.70% and 43.80%, with monounsaturated fatty acids (MUFA) representing 15.75% and 13.18 % in control females and males, respectively. Yet, the single most abundant fatty acid in females was the saturated palmitic acid followed by the PUFA arachidonic acid (ARA); in males, the relative abundance of palmitic and arachidonic acids was reversed.

Significant variations in the fatty acid profile of non-exposed organisms (females) sampled in the beginning (time 0) and end of the experiment (3 weeks) were observed: namely pentadecanoic, palmitic and palmitoleic acids. The exposure to TBT yielded to some variations in gonadal fatty acid content in both genders. In females, pentadecanoic acid levels are similar to time 0 in the animals exposed to 100 ng Sn/L of TBT ($p=0.011$) and 250 ng Sn/L of TBT ($p=0.006$) and higher in comparison to controls at the end of the assay. An increase in stearic ($p=0.007$) and arachidonic (ARA, $p=0.004$) acid levels was also observed in females exposed to 100 ng Sn/L of TBT. In males exposed to 100 ng Sn/L of TBT a decrease in the levels of stearic ($p=0.044$) and eicosapentaenoic (EPA, $p=0.021$) acids and an increase in the levels of linolelaidic acid ($p=0.039$) were observed.

Although not significant, in both males and females several fatty acids exhibited fluctuations. In females, palmitic acid ($p=0.077$) and, the overall saturated fatty acids ($p=0.055$), exhibited a rising trend in animals exposed to 100 ng Sn/L of TBT. Erucic acid decreased in a time-dependent manner throughout the experiment in control/DMSO animals. In males, linolenic acid exhibited a tendency to reduction in control/DMSO animals ($p=0.053$) and pentadecanoic acid when exposed showed a tendency to decrease upon exposure to 100 ng Sn/L of TBT ($p=0.070$).

Table 11– Females fatty acid profile of *P. lividus* gonads in the beginning of the experiment (Time 0) and in the end of the experiment of the control and exposed animals (TBT1 – 100 ng Sn/L; TBT2 – 250 ng Sn/L). Values are expressed as µg/mg of tissue (*-p<0.05; ** - p<0.01; *** - p<0.001; ∆-mean difference from control in percentage).

Name	Time 0		Control/DMSO		TBT1			TBT2		
	Mean	**Std**	**Mean**	**Std**	**Mean**	**Std**	**∆**	**Mean**	**Std**	**∆**
Miristic (14:0)	**1.58**	±0.61	**1.18**	±0.31	**1.49**	±0.26	26%	**1.38**	±0.35	17%
Pentadecanoic (15:0)	**0.09***	±0.03	**0.06**	±0.02	**0.09***	±0.02	49%	**0.09****	±0.02	50%
Palmitic (16:0)	**2.66***	±0.84	**2.02**	±0.56	**2.51**	±0.34	25%	**2.36**	±0.48	17%
Stearic (18:0)	**0.67**	±0.15	**0.56**	±0.12	**0.74****	±0.14	31%	**0.61**	±0.09	8%
Arachidic (20:0)	**0.07**	±0.03	**0.05**	±0.03	**0.06**	±0.03	22%	**0.06**	±0.03	6%
Σ SFA	**5.07***	±2.35	**3.87**	±1.31	**4.89**	±0.96	26%	**4.487**	±0.91	12%
Myristoleic acid (14:1)	**0.08**	±0.09	**0.04**	±0.04	**0.05**	±0.03	21%	**0.03**	±0.03	-10%
Palmitoleic acid (16:1)	**0.35***	±0.23	**0.19**	±0.12	**0.27**	±0.09	39%	**0.22**	±0.06	11%
Oleic Acid (18:1 n9 c)	**0.16**	±0.11	**0.27**	±0.21	**0.20**	±0.05	-27%	**0.33**	±0.29	23%
Eicosanoic acid (20:1)	**0.53**	±0.16	**0.43**	±0.16	**0.51**	±0.04	20%	**0.49**	±0.14	14%
Erucic acid (22:1 n9)	**0.47**	±0.17	**0.36**	±0.10	**0.44**	±0.12	24%	**0.39**	±0.08	11%
Σ MUFA	**1.57**	±0.68	**1.24**	±0.57	**1.44**	±0.29	16%	**1.46**	±0.57	18%
Linolelaidic (18:2 n6 t)	**0.20**	±0.20	**0.54**	±0.53	**0.31**	±0.17	-42%	**0.75**	±0.75	40%
Linolenic acid (18:3 n3)	**0.45**	±0.17	**0.30**	±0.11	**0.41**	±0.09	34%	**0.36**	±0.05	19%
11,14-Eicosadienoic acid (20:2)	**0.19**	±0.06	**0.22**	±0.12	**0.20**	±0.06	-6%	**0.24**	±0.12	13%
11,14,17-eicotrienoic (20:3 n3)	**0.08**	±0.04	**0.12**	±0.07	**0.08**	±0.03	-35%	**0.10**	±0.06	-16%
Arachidonic (20:4 n6)	**1.48**	±0.34	**1.24**	±0.30	**1.68* ***	±0.33	36%	**1.36**	±0.16	10%
Eicosapentaenoic (20:5 n3)	**0.46**	±0.16	**0.30**	±0.09	**0.38**	±0.10	26%	**0.34**	±0.07	12%
Σ PUFA	**2.88**	±0.74	**2.71**	±0.95	**3.06**	±0.21	13%	**3.16**	±0.80	17%
Σ UFA	**4.45**	±1.34	**3.94**	±1.48	**4.50**	±0.36	14%	**4.62**	±1.36	17%
Σ FA	**9.52**	±3.65	**7.81**	±2.64	**9.39**	±1.26	21%	**9.11**	±1.84	14%

Table 12– Male fatty acid profile of *P. lividus* gonads in the beginning of the experiment (Time 0) and in the end of the experiment of the control and exposed animals (TBT1 – 100 ng Sn/L; TBT2 – 250 ng Sn/L 2). Values are expressed as µg/mg of tissue (Mean±STD; *-p<0.05; ** - p<0.01; *** - p<0.001; ∆-mean difference from control in percentage). (n.d.- not detected)

Name	Time 0		Control/DMSO		TBT1			TBT2		
	Mean	**Std**	**Mean**	**Std**	**Mean**	**Std**	**Δ**	**Mean**	**Std**	**Δ**
Miristic (14:0)	**0.44**	±0.14	**0.50**	±0.13	**0.52**	±0.18	4%	**0.55**	±0.26	10%
Pentadecanoic (15:0)	**0.03**	±0.01	**0.05**	±0.03	**0.03**	±0.01	-43%	**0.03**	±0.02	-36%
Palmitic (16:0)	**1.14**	±0.27	**1.13**	±0.27	**1.18**	±0.29	4%	**1.25**	±0.53	11%
Stearic (18:0)	**0.51**	±0.08	**0.52**	±0.06	**0.40***	±0.09	-22%	**0.48**	±0.05	-8%
Arachidic (20:0)	**0.03**	±0.02	**0.02**	±0.01	**0.02**	±0.01	-4%	**0.02**	±0.01	-18%
Σ SFA	**2.15**	±0.80	**2.22**	±0.72	**2.15**	±0.65	-7%	**2.33**	±0.95	-6%
Myristoleic acid (14:1)	**n.d.**		**n.d.**		**n.d.**			**n.d.**		
Palmitoleic acid (16:1)	**0.04**	±0.02	**0.04**	±0.04	**0.06**	±0.04	61%	**0.07**	±0.09	89%
Oleic Acid (18:1 n9 c)	**0.06**	±0.07	**0.06**	±0.07	**0.19**	±0.10	205%	**0.12**	±0.18	90%
Eicosanoic acid (20:1)	**0.32**	±0.11	**0.38**	±0.10	**0.40**	±0.11	4%	**0.38**	±0.13	-1%
Erucic acid (22:1 n9)	**0.21**	±0.06	**0.21**	±0.08	**0.18**	±0.06	-17%	**0.17**	±0.09	-18%
Σ MUFA	**0.60**	±0.18	**0.68**	±0.24	**0.76**	±0.33	13%	**0.67**	±0.44	-1%
Linolelaidic (18:2 n6 t)	**0.08**	±0.09	**0.18**	±0.16	**0.45***	±0.34	151%	**0.32**	±0.40	76%
Linolenic acid (18:3 n3)	**0.08**	±0.04	**0.04**	±0.02	**0.05**	±0.03	20%	**0.04**	±0.04	0%
11,14-Eicosadienoic acid (20:2)	**0.20**	±0.06	**0.24**	±0.07	**0.25**	±0.12	6%	**0.23**	±0.07	-2%
11,14,17-eicotrienoic (20:3 n3)	**0.06**	±0.03	**0.09**	±0.05	**0.12**	±0.06	40%	**0.10**	±0.08	20%
Arachidonic (20:4 n6)	**1.28**	±0.39	**1.37**	±0.29	**1.18**	±0.31	-14%	**1.19**	±0.11	-13%
Eicosapentaenoic (20:5 n3)	**0.38**	±0.13	**0.35**	±0.08	**0.27***	±0.04	-24%	**0.29**	±0.06	-18%
Σ PUFA	**2.04**	±0.57	**2.26**	±0.51	**2.31**	±0.59	2%	**2.17**	±0.52	-4%
Σ UFA	**2.65**	±0.74	**2.94**	±0.73	**3.08**	±0.86	5%	**2.84**	±0.94	-3%
Σ FA	**4.80**	±1.52	**5.16**	±1.41	**5.23**	±1.31	-2%	**5.17**	±1.76	-5%

P. lividus gene expression

The mammalian PPAR/RXR heterodimer is responsible for the transcriptional regulation of several modules in the lipid metabolism (Alaynick, 2008; Berkenstam and Gustafsson, 2005; Desvergne et al., 2006; Mello, 2010). Thus, we evaluated the expression of selected genes in male and female gonads 48 hours after the exposure to TBT (Fig.21). In males, no alteration was observed in the expression of acetyl-CoA carboxylase (*acc*), long-chain acyl-CoA synthetase (*acsl*), carnitine palmitoyltransferase 1 (*cptl*), *rxr* or *ppar* in the individuals exposed to TBT when compared to the control. In females, *acsl* and *rxr* were significantly downregulated in animals exposed to the highest concentration of TBT (250 ng Sn/L). *Acc* expression, on the other hand, was significantly higher in unexposed animals, at time 0, than control, unexposed, animals 48 hours after exposure debut. The reference gene, *tubulin1a*, remained unchanged across treatments.

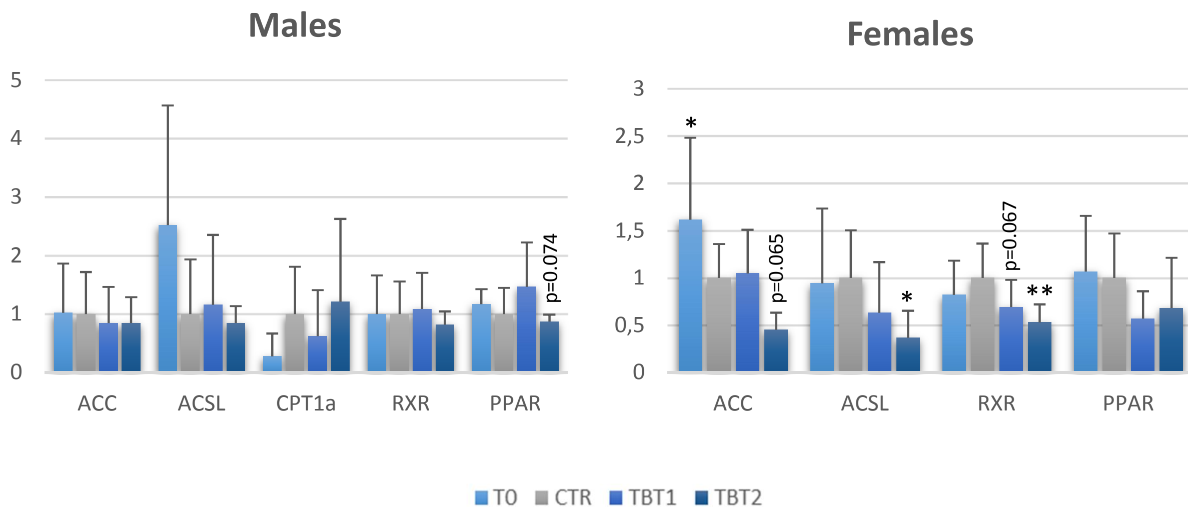

Fig. 21– TBT effect in the expression of genes involved in the lipid synthesis and catabolic pathways. qRT-PCR analysis of selected genes in the gonads of *P. lividus* males and females 48 hours following a TBT exposure (T0 – Time 0; CTR – Control and solvent control; TBT1 – 100 ng Sn/L; TBT2 – 250 ng Sn/L). Values were normalized to tuba1a and expressed as the average fold changes ±STD (n=6) of the CTR group. *-p<0,05; ** - p<0,01; *** - p<0,001 compared to CTR.

Discussion

Although the ability of different EDCs to interfere with the lipid metabolism of mammals is well established, data on the effect of those compounds in invertebrates is still very scarce. Yet, recent research sustains the hypothesis that obesogenic outcomes transcend mammals (Capitão et al., 2017; Janer et al., 2007; Jordão et al., 2016b, 2015, Lyssimachou et al., 2015, 2009; Maradonna et al., 2015). For instance, the obesogenic effect of TBT, a known vertebrate obesogen (Grün et al., 2006; Grün and Blumberg, 2006; Lyssimachou et al., 2015), was previously addressed in other non-vertebrate groups: such as molluscs (Janer et al., 2007), crustaceans (Jordão et al., 2016a, 2016b, 2015) and tunicates (Puccia et al., 2005). In agreement, the present work provides (1) a possible mechanism for organotin-induced disruption of lipid homeostasis in echinoderms and (2) *in vivo* evidence for the wider range of obesogen action, namely TBT. Previous studies, had highlighted deleterious effects of organotin exposure in *P. lividus* larvae: when exposed to 0.1 µg/L of TBT larval length decreased (Marin et al., 2000) and 0.5 µg/L of TBT was considered toxic (His et al., 1999).

In mammals, TBT disrupts lipid metabolism acting through the heterodimer PPARγ/RXR (Harada et al., 2015; Kanayama et al., 2005). To assess the conservation status of this mechanism we isolated for the first time the sequences of both *ppar* and *rxr* from *P. lividus*. In the sea urchin *S. purpuratus* two *ppar*s were found (Goldstone et al., 2006), however, in *P. lividus* only one receptor was retrieved with a PCR-based methodology. Phylogenetic analysis shows that the NRs isolated group with other Echinodermata PPAR and RXR. Further sequence analysis showed that, for PPAR, the anchor cysteine, forming an ionic bond with the tin atoms of TBT and TPT (Harada et al., 2015), was conserved in our sequence. Despite the similar anchoring mode, human PPARγ displays distinct selectivities towards TBT and TPT, due to additional, organotin-specific interactions (Harada et al., 2015). Thus, TPT is defined as a full agonist, given its ability to stabilize helix 12 allowing a higher activation. TBT, on the other hand, is displaced from helix 12 inducing a lower activation of PPARγ (Harada et al., 2015). While the anchor cysteine is conserved in *P. lividus* PPAR, the additional pocket residues interacting with TBT and TPT are not fully conserved. To experimentally assess the ability of organotins to modulate this receptor we used a chimeric *P. lividus* PPAR receptor in combination with an *in vitro* luciferase-based reporter assay. While TBT did not cause a significant response, TPT, in his turn, repressed transactivation at the higher concentrations. Although reversed, the observed response is in agreement with the differential potency of TBT and TPT proposed for PPARγ (Harada et al., 2015).

Regarding RXR, sequence analysis and the conservation status of the key cysteine, crucial for TBT and TPT binding (Hiromori et al., 2015; le Maire et al., 2009), suggested a conserved response in *P. lividus*. In agreement, subsequent *in vitro* assays confirmed TBT and TPT as high-affinity ligands of *P. lividus* RXR, with an apparently higher potency, when compared to that of human RXR. In the heterodimeric form, PPAR/RXR were significantly repressed by both TBT and TPT in a concentration-dependent manner. Given that TBT serves as ligand to both receptors, it is challenging to fully grasp the activation mode of the heterodimer. In humans, using saturating concentrations of receptor-specific ligands, RXR was shown to account for the activation of the PPARγ/RXRα couple upon exposure to TBT (le Maire et al., 2009). However, when RXR was mutationally silenced, PPARγ/RXRα was still activated by TBT (Hiromori et al., 2015). Thus, the mechanisms behind these observations remain elusive. Nonetheless, our results suggest that TBT and TPT interact with the sea urchin PPAR/RXR heterodimer yielding an opposite response when compared to the human counterpart (Hiromori et al., 2015; le Maire et al., 2009).

Besides TBT, fatty acid, such as ARA and EPA, also serve as ligands to mouse PPARs (Takeuchi et al., 2006). Fatty acids were also shown to activate mammalian RXRs (Georgiadi and Kersten, 2012; Lengqvist et al., 2004; Steineger et al., 1998); the long-chain DHA was suggested to induce human adipocyte differentiation through RXR (Suzuki et al., 2009). To better understand the role of PPAR and RXR in *P. lividus* lipid metabolism regulation, we tested the ability of fatty acids, individually or in mixtures to activate the nuclear receptors. The heterodimer PPAR/RXR positively responded to increasing concentrations of fatty acid mixtures, suggesting a similar activation mechanism to those described for mammals.

Due to the crucial importance of lipids in the gonads and eggs of *P. lividus* (Carboni et al., 2013), we selected this tissue to evaluate the fatty acid profile. The fatty acid composition of the analysed gonads is in agreement with previous studies, with palmitic acid and ARA as the most abundant fatty acids; EPA, detected in lower amounts than previously reported, is nonetheless one of the major polyunsaturated fatty acids present in *P. lividus* gonads (Arafa et al., 2012; Carboni et al., 2013; Hughes et al., 2011). In non-exposed females, sampled in the beginning (time 0) and at the end of the experiment (3 weeks), the concentration of some fatty acids displayed significant fluctuations. Those differences probably derive from the dietary changes in captivity and were only detected in females, due to their higher fatty acid content (Arafa et al., 2012;

Carboni et al., 2013, 2012; Cook et al., 2007; Repolho et al., 2011; Shpigel et al., 2006, 2005).

Three-week TBT exposure elicited significant effects on fatty acid content, in both *P. lividus* female and male gonads. The alterations observed were gender specific. This duality of results, between males and females, demonstrates the importance of independent evaluations of gender-specific responses, and were previously reported in zebrafish (Lyssimachou et al., 2015). Changes in lipid storage and/or fatty acid profile of invertebrates upon organotin exposure were already reported in invertebrate species: namely, the mollusc *Marisa cornuarietis* (Janer et al., 2007; Lyssimachou et al., 2009), the crustacean *D. magna* (Jordão et al., 2016a, 2016b, 2015) and the tunicate *Ciona intestinalis* (Puccia et al., 2005). For *M. cornuarietis* and *D. magna* both TBT and TPT were tested (Jordão et al., 2016b; Lyssimachou et al., 2009). Exposure to TBT (36,5 and 365 ng Sn/l), during *D. magna* adolescent instar, lead to an increase in lipid droplets while TPT (64,5 - 613 ng Sn/L) produced an opposite effect (Jordão et al., 2016b). In *M. cornuarietis*, similar results were obtained in females exposed to 500 ng Sn/L TBT for 100 days (Janer et al., 2007) and in both males and females exposed to 30-500 ng Sn/L TPT for 7 days (Lyssimachou et al., 2009).

In the developing *P. lividus*, embryos consume mostly ARA, EPA and DHA as energy source (Carboni et al., 2012). High EPA/ARA, or EPA/DHA, enhance the performance of the larvae, improving survival (Carboni et al., 2012). In the gonads of females ARA increased and in males EPA decreased due to TBT exposure. Additionally, the linolelaidic acid increase observed in exposed males could further promote ARA synthesis, given that sea urchins can synthetize ARA and EPA from linolelaidic acid and linolenic acid (Carboni et al., 2013; Kabeya et al., 2017). Thus, by disrupting the EPA and ARA ratios, these variations could promote disturbances affecting growth, development, and survival of *P. lividus* larvae. Furthermore, fatty acids, maternally-derived and acquired during the free-swimming larval stages, were suggested to fuel the metamorphic transition to the sessile juvenile stage, which entails a non-feeding period (Sewell, 2005). It would be relevant to evaluate the consequences on TBT-induced fatty acid variations on this developmental process. Despite the necessity of further studies to assess the impact of this obesogen in sea urchin populations, we should keep in mind that, while our study spans three weeks, in the wild sea urchins can be exposed to similar concentrations during the full life cycle (Dowson et al., 1992; Harino et al., 1999). Similar to other invertebrate herbivores, sea urchins occupy an important position in the LC-PUFA supply chain, from primary production to higher levels (Hughes et al., 2011). When

mature, the gonads represent the major tissue in sea urchins (Chen et al., 2010), so gonad composition may affect FA intake in higher levels of the food chain.

Finally, to further investigate the effect of TBT exposure, we isolated for the first time several genes involved in fatty acid β-oxidation and synthesis and evaluated any alterations in the expression levels: *fas, acc, cpt1 and acls* (Shi and Burn, 2004). FAS synthetizes *de novo* saturated fatty acids, ACC catalyses the carboxylation of acetyl-CoA to malonyl-CoA, a CPT1 inhibitor, CPT1 in turn is essential for the transport of long-chain fatty acyl-CoA from the cytosol to the mitochondria and ACSL converts long-chain fatty acids into acyl-CoA esters for downstream storage, in the form of triglycerides, or oxidation (Shi and Burn, 2004). No differences in gene expression were detected in males. In females, *acc* expression decreased during the first 48hours in control but was insensitive to TBT exposure. *Acsl* and *rxr* were downregulated in females exposed to the higher concentration of TBT (250 ng Sn/L), in agreement with previous reports in vertebrates (Lyssimachou et al., 2015). In fact, mammalian *ACSL* is a known target of PPARγ (Desvergne et al., 2006). Although, a downregulation of *rxr* was also observed in *Danio rerio* upon exposure to 50 ng Sn/L of TBT for 9 months (Lyssimachou et al., 2009), an opposite response was detected in the invertebrates *Nucella lapillus* and *D. magna* (Jordão et al., 2015; Pascoal et al., 2013).

However, the present study is, to our knowledge, the first to isolate and functionally characterize the NRs PPAR and RXR from an echinoderm. Additionally, the observations *in vivo* show the effects of TBT as an obesogen in this group. In conclusion, the present work provides robust evidence for the ability of TBT to interfere with sea urchin lipid metabolism and reveal the modulation of PPAR/RXR as a potential mechanism. The developmental and full life cycle studies should further unravel the full extent of obesogenic responses in sea urchin populations and at an ecosystem scale (Ribeiro et al., 2015).

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Supplementary material

Table 4 – Primer list

Target	Primer name	Primer sequence	Direction	Aim
ACC	Inv_ACAC_Deg4_F	5'-ACATCACCTCCGTGCTGtgycarttycc-3'	Forward	First fragment
ACC	Inv_ACAC_Deg42_R	5'-CACGATGGGCTGGGTGktcatrwaraa-3'	Reverse	
ACC	Inv_ACAC_Deg43_R	5'-AGCACGCAGGCCttcatrtrncc-3'	Reverse	
ACC	Inv_ACAC_Deg5_F	5' – CGGCTTCCCCGTGatgathaargc - 3'	Forward	Second fragment
ACC	Inv_ACAC_Deg51_R	5' – GCCAGGGTGACGGCGkyyttytccat – 3'	Reverse	
ACC	Inv_ACAC_Deg52_R	5' – TCGGCGATCATCTCGgtrcanggrtg – 3'	Reverse	
ACC	Inv_ACAC_Deg6_F	5'-TCCGGCGGCatgaargayatg-3'	Forward	Third fragment
ACC	Inv_ACAC_Deg6_R	5'-CAGCTGGTCGGCGTacatytccatrt-3'	Reverse	
RXR	P1	5'-CGGGCCTCCGGCaarcaytaygg-3'	Forward	First fragment (André et al., 2017)
RXR	P2	5'-CAGCCCAGCTCGGTCttrtccatntt-3'	Reverse	
RXR	PL_RXR_RACE_F	5'- ACGCAAGGACAAACCCACCGGAGACA-3'	Forward	3' end
RXR	PL_RXR_RACE_R	5'-TGTCTCCGGTGGGTTTGTCCTTGCGT-3'	Reverse	5' end
RXR	P.lividus_RXR_ORF_F	5'-CCTGAAGATGGATACTATGCCA-3'	Forward	ORF
RXR	P.lividus_RXR_ORF_R	5'-ATCTCTCAACTGTTATTCGGCG-3'	Reverse	
RXR	P.lividus_RXR_pbind_F	5' - GTTAGGATCCAA CGC AAG GAC AAA CCC ACC GG -3'	Forward	Pbind
RXR	P.lividus_RXR_pbind_R	5' - GCCA TCTAGA TCAACTGTTATTCGGCGCTT -3'	Reverse	
CPT I	Inv_CPT1_Deg3_F	5'-CCCTGTGCTCCTGGcartaygarmg-3'	Forward	First fragment
CPT I	Inv_CPT1_Deg3_R	5'-TGTGGGCCATGATGggngcrtcngc-3'	Reverse	
ACS	INV_ACSL_Deg4_F	5'-TGCTGAACCGGTTCTACgayaarrtnat-3'	Forward	First fragment
ACS	INV_ACSL_Deg4_R	5'-CAAGGTGGGGGTCAGCadnccrttytc-3'	Reverse	
ACS	INV_ACSL_Deg5_F	5'-CCAAACGGCACGCTGaarathathga-3'	Forward	
Fas	Inv_Fasn_Deg5_F	5'-GCAGATGCCCGTGGTGttyrarrayrt-3'	Forward	First fragment
Fas	Inv_Fasn_Deg5_R	5'-CCTGGAAGGTGGGGccrtantcrta-3'	Reverse	
Fas	Inv_Fasn_Deg6_F	5'-GGGACGGCATGTTCgaraaycarac-3'	Forward	Second fragment
Fas	Inv_Fasn_Deg6_R	5'-CCACGGAGGAGAACACCacraaccantc-3'	Reverse	
PPAR	PlividusPPAR_ORF_F	5'-AAACCATGTCCATTTGTCAAGA-3'	Forward	ORF
PPAR	PlividusPPAR_ORF_R	5'-ATCAGGTAGTCCAGTTCTACAAGTC-3'	Reverse	
PPAR	PlividuspBIND_PPAR_BAMHI_F	5'-GCTGCT GGATCC TT ATGTCACACGATGCCTCCAGG-3'	Forward	Pbind
PPAR	PlividuspBIND_PPAR_KPNI_R	5'-ATA GGTACC GGCCAATCAATAGAGTCCAAAT-3'	Reverse	

Table 5 – Real time primer conditions

Gene	Primer name	Primer sequence	Direction	Temp.	Efficiency	Fragment Size
PPAR	P.lividus_PPAR_RT_F	ACCAAGAAGACGCTGGAACAG	Forward	62ºC	90%	145 bp
PPAR	P.lividus_PPAR_RT_R	ATTCATCCCGGTCAGGTGATAC	Reverse			
RXR	P.lividus_RXR_RT_F	GGCCATCGTGCTCTTTAACC	Forward	60ºC	96%	164 bp
RXR	P.lividus_RXR_RT_R	GAAGGGCAGGCAGTCTAAGG	Reverse			
CPT I	P.lividus_CPT1_RT_F2	ACGATGTCGAACCATTCCGA	Forward	62ºC	81%	128 bp
CPT I	P.lividus_CPT1_RT_R2	ATGAAGCAGAGAGGCAGCAT	Reverse			
ACC	P.lividus_ACC_RT_F	TGCAGCCACCATGCTTCAT	Forward	62ºC	86%	151 bp
ACC	P.lividus_ACC_RT_R	GGCCACGGATACCATTACGA	Reverse			
ACSL	P.lividus_ACSL_RT_F	AGCTCAAGCATTCGTCTATGGT	Forward	62ºC	89%	172 bp
ACSL	P.lividus_ACSL_RT_R	TTTGACGGCATCTCGTACCG	Reverse			
Tuba1a	P.lividus_Tuba1a_RT_F3	CGTTCCCCGTGCCGTTTT	Forward	60ºC	90%	141 bp
Tuba1a	P.lividus_Tuba1a_RT_R3	CACCGAAGCTGTGGAAGATAAG	Reverse			

P. lividus isolated sequences and sequence fragments

RXR (ORF)

ATGGATACTATGCCAGATTTGGACGCTATGAAGTCCATGCCTCTCGGTAGCATGCAGTTACCGACTAGTACCATGG
GTTCTCCTTCAATGGTGAACCAAGGCCTCAGCTCCCCTACACTCTCATCACCTGGTCTACACTCAGGGATCACAGA
TTACCGTCTGCACTCGCCACCCATGCAGTCACCTACACTGAGCAGTCAGGGATCCATGGAAGACATCAAGCCAATC
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CTATCAGAAGTGTCTGGGAATGGGCATGCGCAGGGAAGCTGTACAAGAGGAGAGACAACGCAAGGACAAACCCAC
CGGAGACAGTCAGGTAGAGAGTACAAGTGGGGCAAGTAATGACATGCCAGTGGAGAAGATCCTGGATGCTGAACT
AGCCGTAGAACCCAACCACGGGCCGTACGTAGACACTCCGCGCGATCCAGTGACAAACATCTGCCAAGCTGCCGA
TAAACAACTCTTCACATTGGTTGAGTGGGCTAAGCGAATCCCACACTTTACAGAACTGCCACTGGATGATCAGGTCA
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CCTCACTGAGCTGGTTGCTAAGATGAGAGAGATGAAGATGGATAAGACGGAACTGGGGTGCCTCAGGGCCATCGT
GCTCTTTAACCCAGATGCTAAGAACTTGACCTCGGTACAAAAGGTGGAAGAGCTTCGGGAGAAGGTCTATGCATCC
CTTGAGGAATACTGCCGCAACCAGTACACAGAAGAACCTGGCCGCTTTGCCAAGCTGCTCCTTAGACTGCCTGCC
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CCTCATGGAGATGCTGGAAGCGCCGAATAACAGTTGA

PPAR (partial ORF)

TTATGTCACACGATGCCTCCAGGTTCGGTCGCATGCCACGGGAGGAGAGGCTGCGGCTCCTGGAGGAGCTGAAG
GAGGAGGAGACGGAGGCGACGGAGGAGGAGAAACATCGGGCAGAGATCAGGGAGCTGACGGATAGGATACACA
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CAGATGGAACTGAGAACCAATCACAGTAACCATCGCTTGTTACTCCCTAGACTACTGTCCCTGCTGGTAGACCTCA
GGCAACTAGTCCTGGACCATATTAAACAGGTCCAGCAACTAATGCTGATGGACGACCCTTCGTATTCGGGTCCACC
GCCTCTTATCAAGGAGATATTTGGACTCTATTGA

ACC

1º Fragment

ATTACATCACCTCCGTGCTGTGTCAGTTCCCAAGTCAACAGATTGCCAATGTGTTAGATCGCTATGCAGCCACCATG
CTTCATAAAAAAGCAGAACGTGATACCTTCTTCATGCATACTCAGGGAATCGTGCAACTTGTACAACGATATCGTAA
TGGTATCCGTGGCCACATGAAGGCCTGCGTGCTAATC

2º Fragment

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GAGTGCTCGTCATTTAGAAGTGCAGCTGCTTGCAGACAAGTACGGCAATGCCATCTCCATATTTGGTCGTGACTGC
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TGGAGAAAGCCGCCGTCACCCTGGCAAT

3ºFragment

ACTAGTGATTTCCGGCGGCATGAAAGATATGTATGACCAGGTGCTGAAATACGGGTCCTACATCGTGGATGCTCTG
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ACS

GATTCCAAACGGCACGCTGAAGATTATTGATCGTAAGAAGCATATCTACAAGCTTGCTCAGGGTGAATACGTAGCA
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FAS

1st Fragment

GCCGCGGGAATTCGATTCAGGCCGACTGCATGGATCCTCAGCTGAGAATTCTACTGGAGGTAGCCTATGAGGCTA
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TGGCCAACCGGATGTCCTACTTCTTCGAATCACTAGTGAAT

2nd Fragment

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ACTTGATGTGAGTGTTACATCTACTGGAGATTTTGAAATCTGTGAAAGTGGAAACCTGTCTGTTAGTGGAAATATCC
AAATAATGGAAGACCCCAATCAAAACTCTGAATACTTTGATAGTGAAGCTGCAGAAGATACTAAAGTTGGGATAGAT
NTTCCAAATCAGACGTATATAAA

CPT I

1st Fragment

TGGTCGTCTTGGATGAAGAGGAGCACGAGTACGATGTCGAACCATTCCGAGACTTTATCAAGAATCAAGCTTTACC
TGCTTGGTACAAAGGAGATCCTTTTGCTAACAAGGACCAGGGGGATGGAAAGTTGGATAAGTATGCTGCCTCTCTG
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2nd Fragment

TTCGATTCGTGACCGACTGGTGGGAAGAATACGTCTACCTTAGGGGGCGCTCTCCAATCATGGTCAATAGCAACTA
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CAATCACATTGTAGTCATGTCTAAAGGTCGCTATTACAAGCTCATCATCCAGTCTAATGGTAGTCTCATCAAACCCT
GCGAGCTGGAGAAGCAATACCAGGACATCTTGGATAGCCCTGAGGAAGCCGAGGGTGAGAGACGTCTAGCTGTAC
TCACCGCAGGAGACAGAATTCCTTGGGCTGCCGCTCGTCAACGATTCTTTATGTCCGGTGTGAACAAGACCTCTCT
GGCTGCCATAGAAACTGCTGCCTTCGTGGTCGTCTTGGATGAAGAGGAGCACGAGTACGATGTCGAACCATTCCG
AGACTTTATCAAGAATCAAGCTTTACCTGCTTGGTACAAAGGAGATCCTTTTGCTAACAAGGACCAGGGGGATGGA
AAGTTGGATAAGTATGCTGCCTCTCTGCTTCATGGCAAATGCTACAACCGTTGGTTTGATAAA

Table 6 - Accession numbers of the PPAR sequences used in the phylogenetic analysis * indicates sequences isolated in the current work see primer table 4 for more details.

	PPARα	PPARβ	PPARγ
Homo sapiens	Q07869	Q03181	P37231
Mus musculus	P23204	P35396	P37238
Gallus gallus	NP_001001464.1	NP_990059.1	NP_001001460.1
Anolis carolinesis	XP_003221452.1	*	XP_003220387.1
Xenopus tropicalis	XP_002940784.2	XP_012826243.1	*
Danio rerio	ABI30003.1	NP_571543	NP_571542.1
Pantodon buchholzi	Ctg24603	Ctg21483	Ctg18143
Lepisosteus oculatus	W5NK92*	ENSLACG00000015749	XP_006631094.2*
Latimeria chalumnae	XP_005991369.1	nf	XP_006004475.1
Callorhinchus milii	SINCAMG00000010348	SINCAMP00000016425	XP_007901520
Leucoraja erinacea	*	*	*
	PPAR		
Ciona intestinalis	NP_001071801.1		
Branchiostoma floridae	jgi|Brafl1|174611		
Branchiostoma lanceolatum	*		
Branchiostoma belcheri	XP_019618227.1_		
Biomphalaria glabrata	XP_013069840.1, XP_013069841.1		
Patella depressa	*		
Lottia gigantea	jgi1|Lotgi1|174409, jgi2|Lotgi1|238472		
Strongylocentrotus purpuratos	XP_781750.1 XP_784429.3		
Saccoglossus kowalevskii	XP_006819446.1, NP_001164713		
Crassostrea gigas	XP_011412920.1		
Paracentrotus lividus	*		
	RXR		
Homo sapiens	NP_002948.1		
Xenopus laevis	P51128.1		
Danio rerio	AAC59720.1		
Branchiostoma floridae	AAM46151.1		
Ciona intestinalis	NP_001071809.1		
Polyandrocarpa misakiensis	BAM66778.1		
Nucella lapillus	ABS70715.1		
Lymnaea stagnalis	Q5I7G2.1		
Thais clavigera	BAJ76722.1		
Biomphalaria glabrata	NP_001298239.1		
Daphnia magna	ABF74729.1		
Locusta migratoria	AAQ55293.1		
Uca pugilator	AAC32789.3		
Marsupenaeus japonicas	BAF75376.1		
Drosophila melanogaster	NP_476781.1		
Tribolium castaneum	NP_001107766.2		
Bombyx mori	NP_001037470.1		
Tripedalia cystophora	AAC80008.1		
Aurelia aurita	AGT42223.1		
Mesocentrotus nudus	AEL87703.1		
Acanthaster planci	XP_022085088.1		
Saccoglossus kowalevskii	XP_002731697.1		
Strongylocentrotus purpuratos	ABA41640.1		
Lingula anatina	XP_013412668.1		
Crassostrea gigas	XP_011434498.1		

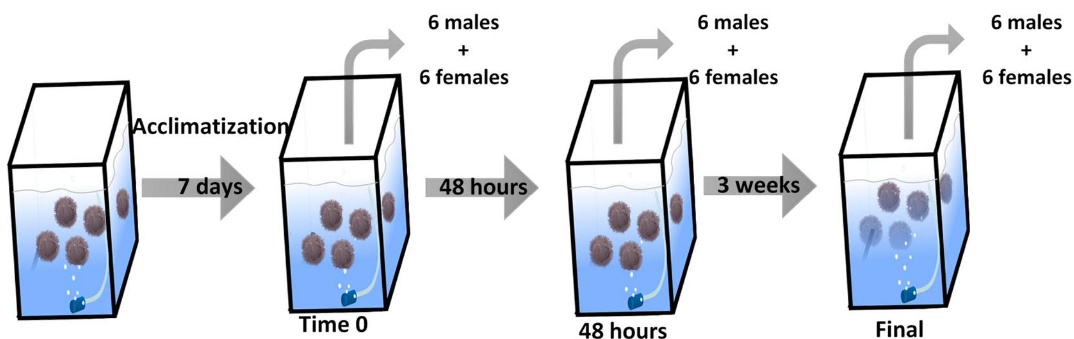

Fig. 2– In vivo experiment schematic representation.

Table 7 – Fatty acids limits of quantification with the methodology used.

Fatty acid		LOQ (μg/ml)
Butiric	4:0	65,840
Hexanoic	6:0	53,890
Octanoic	8:0	36,207
Decanoic	10:0	35,948
Undecanoic	11:0	20,067
Lauric	12:0	48,223
Tridecanoic	13:0	28,980
Miristic	14:0	62,327
Myristoleic acid	**14:1**	30,236
Petadecanoic	15:0	35,570
Pentadecanoic cis	15:1	35,512
Palmitic	16:0	122,593
Palmitoleic acid	**16:1**	40,004
Heptadecanoic	17:0	46,833
Heptadecanoic	17:1	46,454
Stearic	18:0	96,904
Oleic Acid	**18:1 n9 c**	144,446
Elaidic	**18:1 n9 t**	
Linolelaidic	**18:2 n6 t**	44,549
Linoleic acid	**18:2 n6 c**	53,191
gamma- linoleic	18:3n6	43,696

Linolenic acid	**18:3 n3**	45,512
Arachidic	20:0	95,937
Eicosanoic acid	**20:1**	49,438
11,14-Eicosadienoic acid	20:2	50,209
11,14,17-eicotrienoic	20:3 n3	48,835
8,11,14- eicosatrienoic	20:3 n7	46,591
Arachidonic	**20:4 n6**	46,811
EPA	20:5 n3	46,327
Heneicosanoic	21:00	43,687
Docosanoic	22:0	177,982
Erucic acid	**22:1 n9**	77,250
13,16-docosadienoic	22:2	48,964
Tricosanoic	23:0	45,077
Lignoceric	24:0	94,636
DHA	**22:6 n3**	51,653
Nervoic acid	**24:1**	48,986

Chapter 4 - The transphyletic exploitation of *PPAR* orthologues by organotins in gnathostome lineages

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The transphyletic exploitation of *PPAR* orthologues by organotins in gnathostome lineages

Abstract

Background:

Global obesity is an escalating pandemic in western societies. Extensively triggered by nutritional changes and overconsumption, this complex condition is, nonetheless, also influenced by individual and environmental cues. Of note are the globally persistent man-made chemicals, with ever-growing ecosystemic consequences, a hallmark of the Anthropocene epoch. A striking example highlights the role of organotins as "*obesogenic drivers*" in mammalian species, *via* the high affinity binding and activation of the PPARγ nuclear receptor.

Objective: We sought to investigate the evolutionary conservation of the obesogenic impacts through the exploitation of PPARγ by tributyltin (TBT), across the chordate phylum.

Methods: We combined extensive database mining with comparative genomics and phylogenetics to determine the evolutionary history and sequence diversity of *PPARs* in several chordate species. Identified *PPAR*α, β and γ from selected species were isolated and characterized with transactivation assays. Additionally, we employed site-directed-mutagenesis to further clarify the structural determinants of TBT affinity towards vertebrate PPARs.

Results and Conclusions: We show that the emergence of multiple PPARs (α, β and γ) in gnathostome ancestry coincides with the "*acquisition*" of TBT binding affinity to the chondrichthyan PPARγ, since the amphioxus single copy PPAR is irresponsive to TBT. Strikingly, teleost PPARγ is not activated by TBT, a probable consequence of a mutational event at a cysteine residue in the teleost ancestor. Our findings endorse the modulatory ability of environmentally occurring man-made chemicals and suggest an evolutionary complex obesogenic response to TBT.

Introduction

Obesity in human populations is a thriving health condition particularly in the western hemisphere (Flegal et al., 2010). In the past, obesity was mostly linked to unbalanced and excessive dietary intakes. Strikingly, the last decade brought to light a direct link between this epidemic and the exposure to an ample group of chemicals, which were named obesogens (Baillie-hamilton and Phil, 2002; Grün and Blumberg, 2006). The list of confirmed and suspected obesogens is extensive and includes a wide variety of compounds including phthalates, bisphenol A and organotins (Holtcamp, 2012). Importantly, a string of studies using mice has clearly established that the obesity phenotype, induced by obesogens such as tributyltin (TBT), results from the activation of a master control gene of adipogenesis: the peroxisome proliferator–activated receptor gamma (PPARγ) (Evans et al., 2004; Grün and Blumberg, 2006). In addition to mammals, this class of endocrine disruptors has also been shown to induce similar lipid alterations in other phyla (Capitão et al., 2017; Grün et al., 2006; Janer et al., 2007; Jordão et al., 2015; Lyssimachou et al., 2015; Ouadah-Boussouf and Babin, 2016), with several lipid metabolic modifications described upon obesogen exposure in teleosts and amphibians (Grün et al., 2006; Lyssimachou et al., 2015; Ouadah-Boussouf and Babin, 2016; Riu et al., 2014; Zhang et al., 2013, 2009). Yet, while the toxicological impact has been extensively described in numerous *taxa*, is it mostly unknown whether these effects underpin conserved (or divergent) evolutionary mechanisms of action, exploited by compounds such as TBT and acting via PPARs (Capitão et al., 2017; Castro and Santos, 2014; Santos et al., 2012). For instance, while in mammals TBT was shown to act as a PPARγ agonist (Grün et al., 2006; Yanik et al., 2017), in teleosts a reporter gene assay revealed that TBT is a potent repressor of PPARα and β but has no effect on PPARγ (Colliar et al., 2011). In effect, animals exhibit conserved and divergent sensitivities to external stimuli yet, the role of PPARγ as a direct target of obesogens has been addressed in a minute number of lineages (Grün et al., 2006; Grün and Blumberg, 2006; Janesick and Blumberg, 2011; Pereira-Fernandes et al., 2013). Also, the extent to which genetic variation, underlies such dissimilar responses is far from fully assessed; nor are the contribution of evolutionary processes such gene duplication or loss, and mutation in the promotion of disparate responses.

PPARs display a very unique taxonomic distribution. Initially considered a chordate novelty, with the emergence of numerous genome projets *PPAR*-like sequences were found in invertebrate deuterostomes but also in the protostome molluscs (Kaur et al., 2015; Vogeler et al., 2017, 2014; this thesis). Besides mammals

and teleosts, with clearly established *PPAR* genes, (Bertrand et al., 2007), several vertebrate lineages remain uncharacterized with respect to the diversity of *PPAR* gene repertoire or their exploitation by obsesogens. Therefore, we have carried out an extensive examination of *PPAR* gene diversity in the main chordate lineages, including cephalochordates (amphioxus), chondrichthyans (sharks, skates and chimaeras) and actinopterigii (fish). We surveyed a phylogenetic blueprint to address the impact of TBT acting *via* PPARγ signalling pathways, a critical regulator of adipogenesis. By investigating representative species of the chordate phylum, we provide a detailed map of TBT activation/repression, as proxy for obesogenic disturbance, over an evolutionary scale. Our study shows the central importance of comparative approaches to unravel the full ecosystem impact of endocrine disruptors.

Results

PPAR duplicated before gnathostome radiation to originate *PPAR*α, β and γ paralogues

To examine the precise *PPAR* gene collection across an ample set of chordate lineages including Cephalochordata, Tunicata, Sarcopterigii, Actinopterigii and Chondricthyans, we deployed an exhaustive search into various genome databases. *PPAR*-like sequences covering all major chordate lineages were recovered through tblastn and blastp searches from available genome databases. A total of 36 sequences were recovered from 16 species. The number of *PPAR*-like sequences identified in the examined species varied between one in invertebrate chordates (sea squirt and amphioxus) to 3 in most gnathostomes (sequence accession numbers in supplementary table 8). In teleosts, a higher number has been described as a consequence of genome duplications (Betrand et al., 2007; Madureira et al., 2017). We next investigated the phylogenetic relationships of the isolated sequences by employing a Maximum likelihood analysis (Fig.22). Our approach uncovered three well-supported monophyletic groups corresponding to the described α, β and γ gene lineages (Fig.22). Each of these clades is composed uniquely of gnathostome sequences, including species from previously uncharacterized lineages such as the chondrichthyans (e.g. elephant shark and little skate), basal actinopterygii (e.g. spotted gar) and early-diverging teleosts such as the osteoglossomorpha (e.g. butterfly fish). Moreover, to validate the orthology of the newly identified sequences in the chimaera *C. milii* and the holostean *L. oculatus*, we examined the *ppar* gene *loci* composition (synteny) in these species (supplementary figure 3A). This analysis clearly indicates that these are *bona fide PPAR* gene orthologues. Finally, the single copy invertebrate chordate *ppar* sequences from Tunicates and Cephalochordates were found to out-group the totality of the gnathostome gene paralogues (Fig.22). Therefore, our phylogenetic analysis strongly indicates that the expansion of the *PPAR*$\alpha/\beta/\gamma$ gene family is coincident with the evolution of the vertebrate lineage approximately 500 million years ago, predating gnathostome radiation.

This timing of *PPAR* diversification is consistent with the involvement of genome duplications, the so-called 2R (Putnam et al., 2008). We further perused this hypothesis by analyzing the genomic *locus* of the *PPAR* in invertebrates and in human. We find that human *PPAR*$\alpha/\beta/\gamma$ map to human chromosomes 22, 6, and 3 respectively (Supplementary Fig. 3 A). The analysis genomic location of *ppar* and neighboring genes in *B. floridae* and *B. belcheri*, reveals that the corresponding orthologous of the

neighboring genes map to human chromosomes 2 (2.6), 3 (3.2), 17 (17.6), and 12 (12.5) in regions linked to a four-fold paralogy namely LG 16 (Putnam et al., 2008) or ancestral chromosome E (Nakatani et al., 2007), originating from genome duplication. Although only *PPAR*γ falls within the linked regions (chr3-3.2), the paralogy linkage in anfioxus together phylogenetic analysis suggests that vertebrate *PPAR*$\alpha/\beta/\gamma$ originated through genome duplications, while *PPAR*α/β, are most probably located in regions that have translocated in early vertebrate evolution (Supplementary Fig. 3B and C).

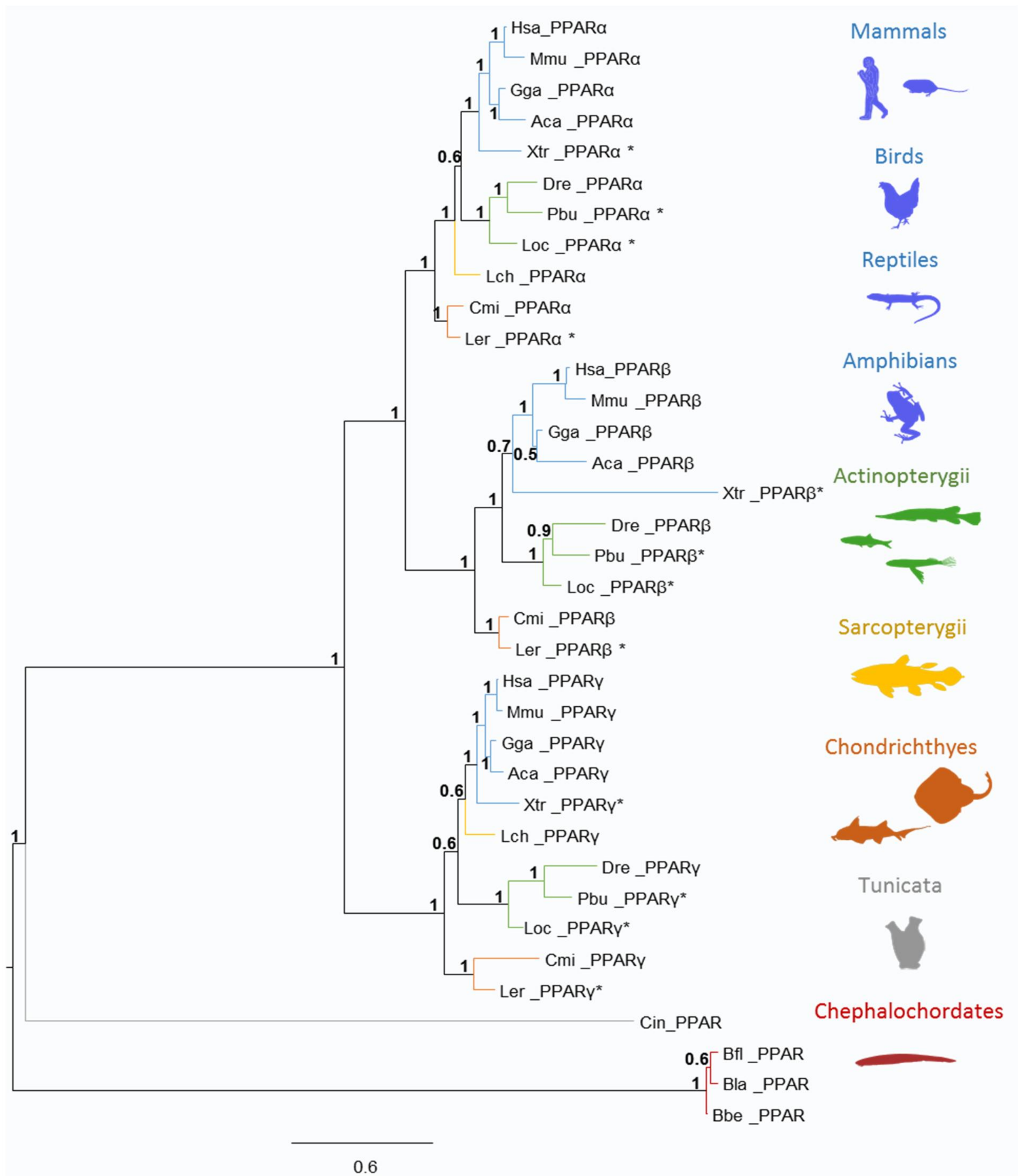

Fig. 22 - Bayesian phylogenetic analysis of *PPAR* amino acid sequences, values at nodes indicate posterior probabilities. Hsa: *Homo sapiens*; Mmu: *Mus musculus*; Gga: *Gallus gallus*; Aca: *Anolis carolinesis*; Xtr: *Xenopus tropicalis*; Lch: *Latimeria chalumnae*; Dre: *Danio rerio*; Pbu: *Pantodon buchholzi*; Loc: *Lepisosteus oculatus*; Cmi: *Callorhincus milii*; Ler: *Leucoraja erinacea*; Bbe: *Branchiostoma belcheri*; Bla: *Branchiostoma lanceolatum*; Bfl: *Branchiostoma floridae*. Sequences indicated with * correspond to sequences tested during this work.

Chordate PPAR transactivate gene expression in the presence of fatty acids

To investigate the evolution PPAR ligand binding properties, we next isolated the full repertoire of *ppar* genes in the European amphioxus (Cephalochordata) and in the little skate (Chondrichthyans), and addressed their capacity to transactivate gene expression in the presence of presumed physiological PPAR ligands (Kliewer et al., 1997; Krey et al., 1997; Xu et al., 1999): arachidonic acid (ARA), eicosapentaenoic acid (EPA) and a mixture of lipids comprising ARA, EPA, oleic acid, linoleic acid, γ-linolenic acid and palmitic acid (Fig.23 and Fig.24). Human PPARs were used as a positive control. We found a consistent and statistically significant activation of gene transcription with all the tested PPARs and the respective ligands. In the case ARA, significant activations were observed only at the highest concentration (200µM), for both the human and the little skate PPAR (Fig.23). In effect, in the majority of the cases the *L. raja* PPAR orthologues showed higher activations compared with the human receptors. In amphioxus, ARA elicited transcription at the lowest tested concentration (50 µM) but displayed a lower fold activation when compared to the gnathostome PPARs (Fig.23). The overall pattern found with EPA was similar. However, this fatty acid failed to transactivate, within the tested concentration range the human PPARα, while it did so at 200µM for little skate (Fig.23). A strong and consistent transactivation was observed for the little skate PPARγ, in contrast to the lower sensitivity of the human orthologue, which was only activated at 200µM (Fig.23). In contrast to the ARA assay, EPA only activated the amphioxus PPAR at maximum concentration (Fig.23). Finally, when a mixture of lipid molecules was examined for their capacity to modulate reporter gene expression, similar responses were recovered (Fig.24). Overall, our results suggest that the tested fatty acids are *bona fide* ligands of PPARs in chordates.

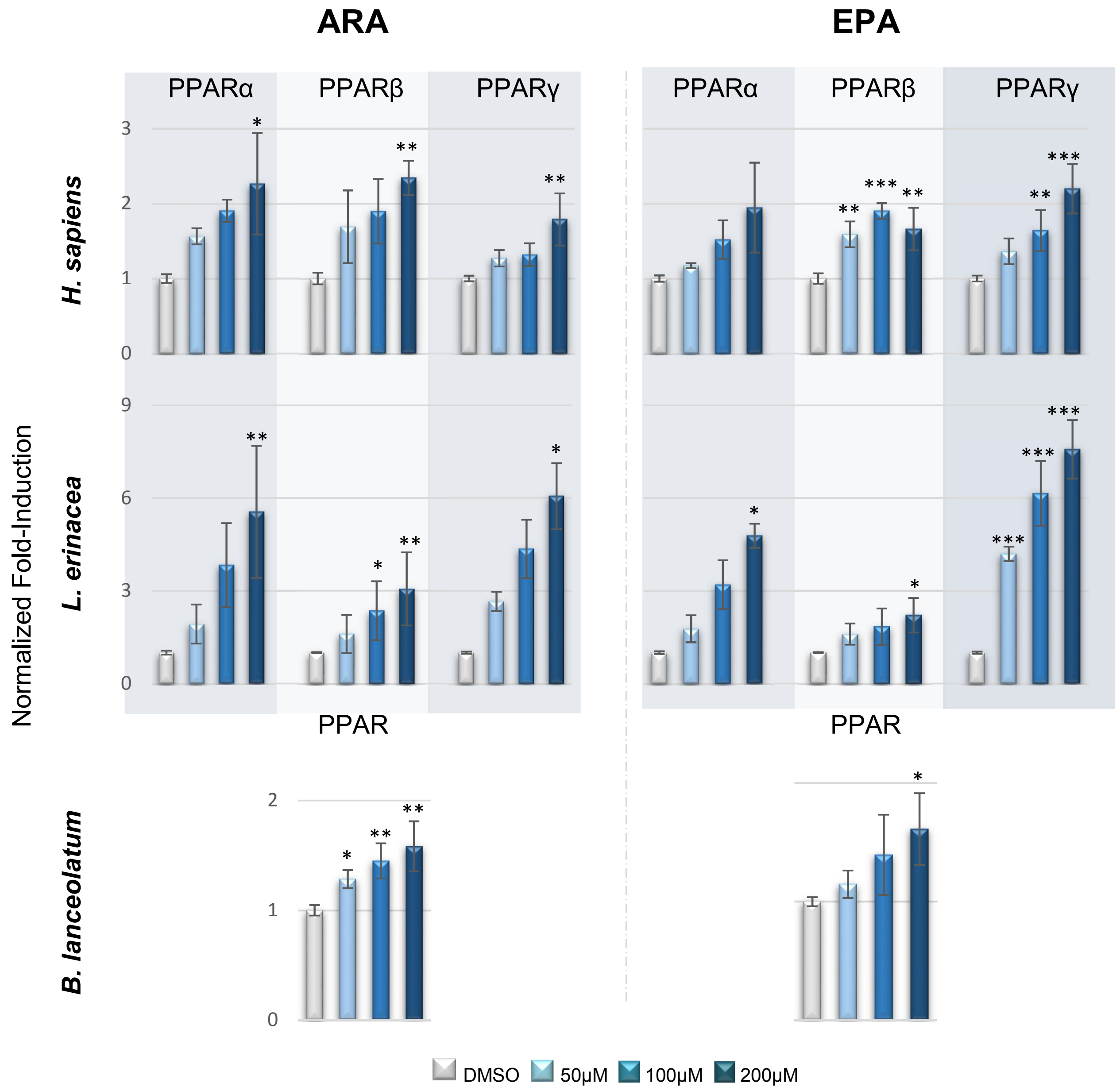

Fig. 23– Transcriptional activation in vitro of PPARs in the presence of the fatty acids ARA and EPA. Normalized fold-induction of *H. sapiens* PPARα, β and γ, *L. erinacea* PPAR α, β and γ and *B. lanceolatum* PPAR in the presence of 50µM, 100 µM and 200 µM of ARA/EPA. Data are shown as mean± standard deviation of the normalized fold-induction (*- p<0.05; ** - p<0.01; *** - p<0.001).

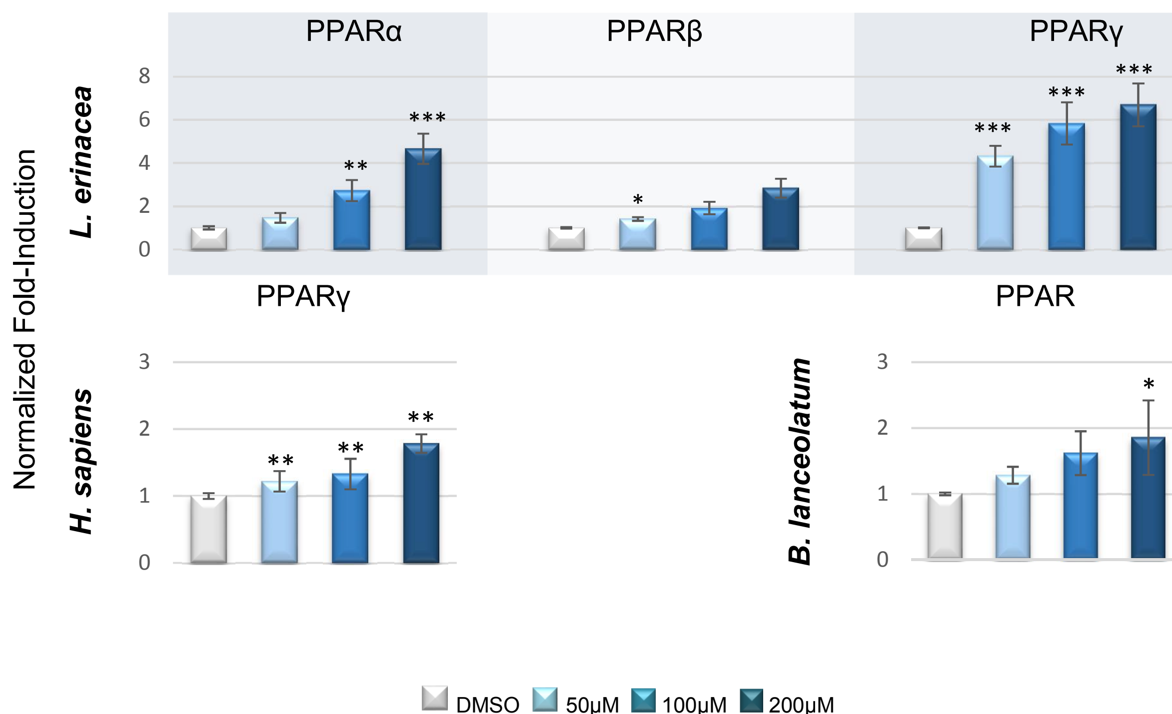

Fig. 24- Transcriptional activation in vitro of PPARs in the presence of the lipid mixture containing 50 µM of ARA, 50 µM of EPA, 50 µM of oleic acid, 50 µM of linoleic acid, 50 µM of γ-linolenic acid and 13.3µM of palmitic acid. Normalized fold-induction of *H. sapiens* PPARγ, *L. erinacea* PPARα, β and γ and *B. lanceolatum* PPAR. Data are shown as mean± standard deviation of the normalized fold-induction (*-p<0.05; ** - p<0.01; *** - p<0.001).

TBT elicits a complex spectrum of response profiles in Chordate Ppar

We next assayed the ability of various chordate PPARs to activate transcription in the presence of a model obesogenic ligand, TBT (Grün et al., 2006). To warrant a wide phylogenetic coverage we isolated and tested, using a reporter gene assay, the *PPAR* gene collection from human (mammal), western clawed frog (amphibian), butterfly fish (osteoglossomorpha – post-3R Actinopterygii), spotted gar (pre-3R Actinopterygii), little skate (chondrichthyans) and the European amphioxus (cephalochordate). As expected the human PPARγ, a significant fold induction was observed at two of the tested concentrations – 100 and 250 nM, with values of 1,8 and 2,2 respectively (Fig.25). Similar profiles were observed for the amphibian and skate PPARγ orthologue; although the lowest tested concentration (10 nM) was able to induce significant transcription for the skate PPARγ (Fig.25). In contrast, both PPARγ from the butterfly fish and the spotted gar revealed no significant differences from the control. Hence, our results and those of others (Colliar et al., 2011) clearly suggest that the obesogenic capacity of TBT as a PPARγ agonist is not fully conserved across gnathostomes.

Using the same reporter system, we next examined the capacity of TBT to induce PPARα/β-mediated transcription from the same set of gnathostome species. The human PPARs displayed a statistically supported suppression, which in the case of PPARα was observed at two of the tested concentrations (100 nM and 250nM) (Fig.25). In the case of *Xenopus*, a statistically supported suppression was also evident at 100 nM and 250 nM for PPARα, while the same was detected at 250 nM for PPARβ (Fig.25). In the Actinopterygii species (butterfly fish and spotted gar), a consistent repression was also determined for both PPARs in the two species, with the exception of PPARα from spotted gar (Fig.25). In contrast, the PPARα/β orthologues from little skate exhibited a fold increase in transcription (~1.5), at the highest tested concentration (250 nM) (Fig.25). Finally, in the case of the amphioxus PPAR, no significant differences were observed in comparison with the control treatment (Fig.25). In summary, we find a strikingly diverse set of ligand potencies and affinities of TBT that vary according to chordate lineages, ranging from no transcriptional activation (e.g. amphioxus), to novel activation/suppression activities acting *via* PPARα/β/γ gnathostome orthologues.

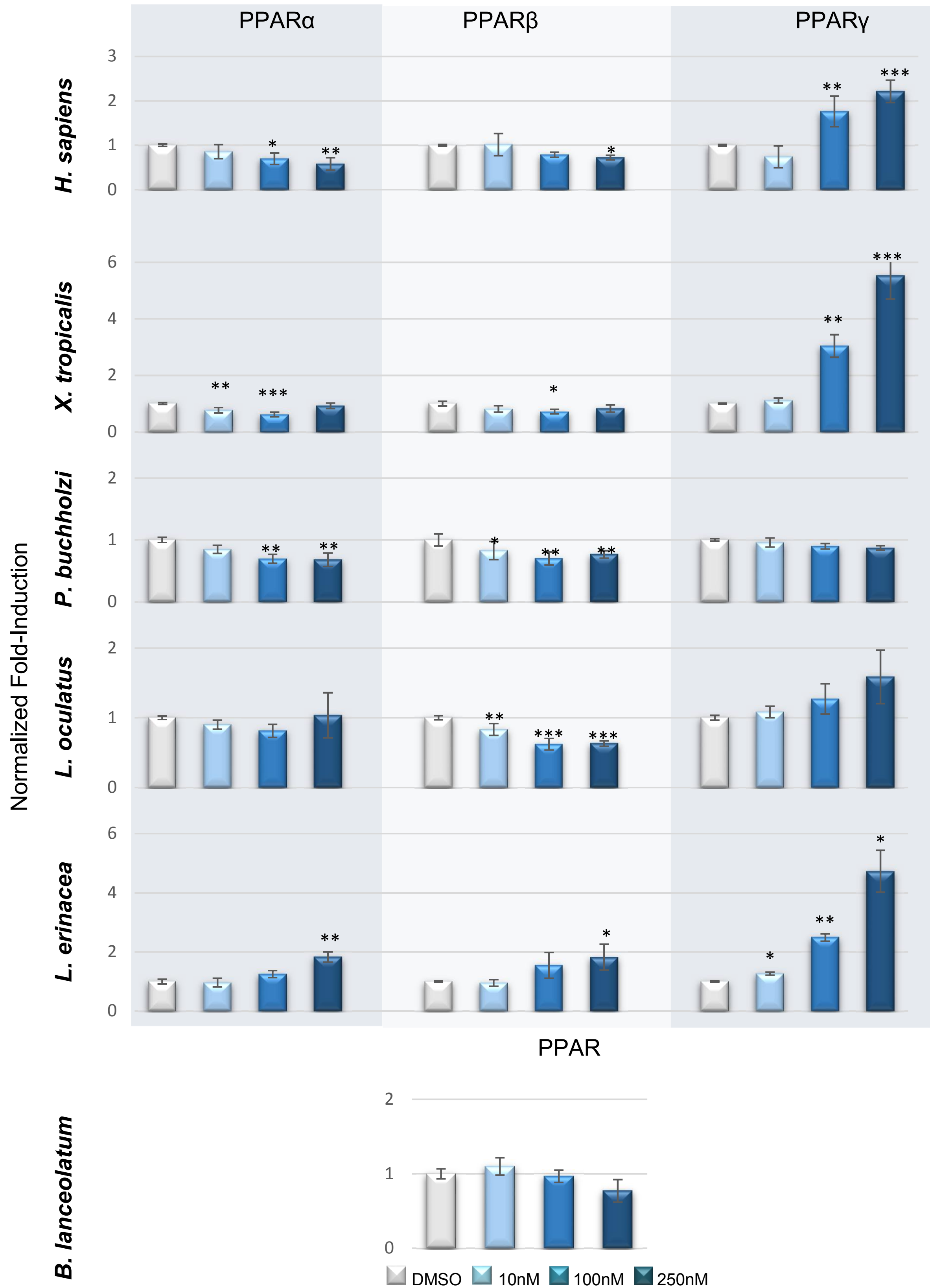

Fig. 25- Transcriptional activation in vitro of PPARs in the presence of TBT. Normalized fold-induction of *H. sapiens* PPARα, β and γ, *X. tropicalis* PPARα, β and γ, *P. buchholzi* PPARα, β and γ, *L. oculatus* PPARα, β and γ, *L. erinacea* PPAR α, β and γ and *B. lanceolatum* PPAR in the presence of 10nM, 100 nM and 250 nM of TBT. Data are shown as mean± standard deviation of the normalized fold-induction (*-p<0.05; ** - p<0.01; *** - p<0.001).

TPT activates and suppresses transcription via human and little skate Ppar orthologues

We next investigated a second organotin compound with a well-known capacity to produce obesogenic responses in mammals, TPT (Kanayama et al., 2005). The human α and β orthologues displayed a repression profile, opposing the fold increase observed for the γ gene (Fig. 26). In contrast, regarding little skate, TPT was only able to induce a low, albeit significant fold increase with the PPARβ orthologue, while a repression was observed with the PPARγ (Fig.26). In both these cases, the observed effects were determined at the highest tested concentration. The amphioxus PPAR was repressed transactivation at 250 nM (Fig.26).

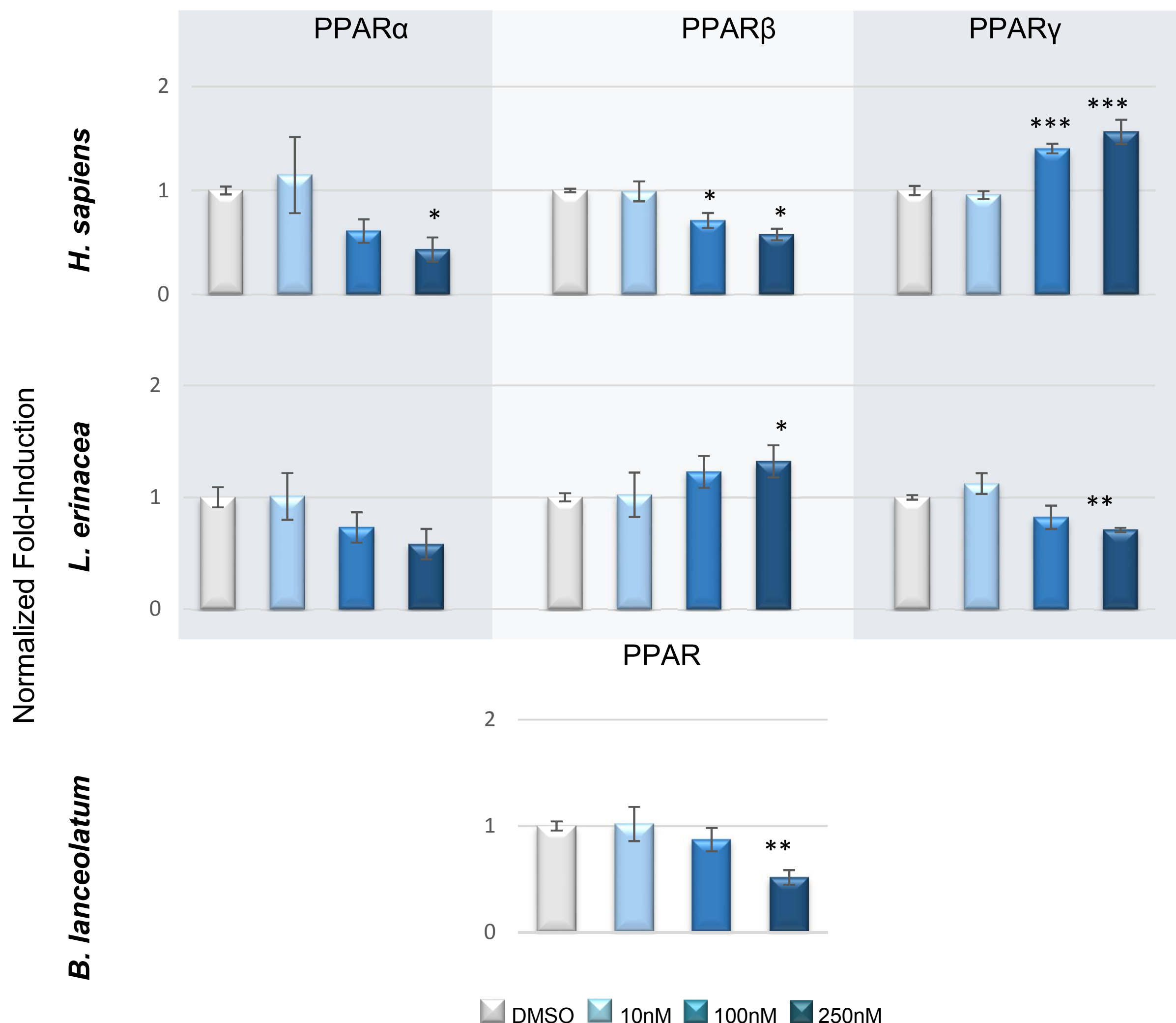

Fig. 26- Transcriptional activation in vitro of PPARs in the presence of TPT. Normalized fold-induction of *H. sapiens* PPARα, β and γ, *L. erinacea* PPAR α, β and γ and *B. lanceolatum* PPAR in the presence of 10nM, 100 nM and 250 nM of TPT. Data are shown as mean± standard deviation of the normalized fold-induction (*-p<0.05; ** - p<0.01; *** - p<0.001).

Cysteine 285 is key for TBT binding in mammals and chondrichthyans

Previous reports have shown that the cysteine 285 in the human PPARγ is essential in the transactivation of this receptor by organotins (Harada et al., 2015). The cysteine 285 is transversal in vertebrates PPARγ, apart from some teleosts (e.g. zebrafish and butterfly fish) which have a tyrosine at the corresponding position and are unresponsive to TBT (Riu et al., 2014) (Fig. 4 in the supplementary material). To assess if the role of the cysteine 285, in the response produced by TBT, is conserved through vertebrates we created a PPARγ cysteine>alanine mutant, using the vertebrate little skate. Also, this position as changed to a tyrosine to mimic the teleost phenotype. In agreement, our results show that when the cysteine 285 is replaced by a tyrosine the transactivation observed in the wild type is abolished and, at the highest concentration tested (250nM), the transcriptional activity is significantly repressed. When replaced by an alanine, the repression is more pronounced spanning the three concentrations of TBT tested (Fig.27).

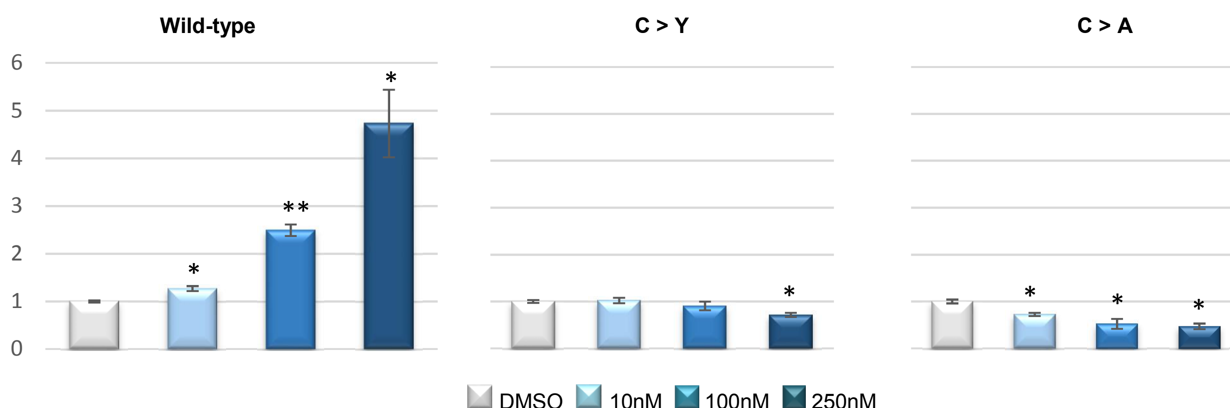

Fig. 27– *L. erinacea* PPARγ (Wild-type), C > A PPAR and Y > A PPAR transactivation in the presence of TBT. C > Y: *L. erinacea* PPARγ with the cysteine correspondente to the amino acid 285 in human PPARγ mutated to a tyrosine; C > A: *L. erinacea* PPARγ with the cysteine correspondente to the amino acid 285 in human PPARγ mutated to a alanine. Data are presented as mean± standard deviation of the normalized fold-induction (*-p<0.05; ** - p<0.01; *** - p<0.001).

In vivo experiment exposure to TBT does not entail lipid changes in lesser spotted catshark

Given that the chondrichthyan PPARs are targeted by organotins, we next examined the *in vivo* impact of TBT exposure in the lesser spotted catshark, an elasmobranch. Several morphological parameters that indicate the physiological state of the animals were recorded or calculated during and in the end of the experiment (Fig.28). The gonad somatic index (GSI) decreased in the animals injected with TBT. No significant alterations were observed in the hepatosomatic index (HIS), condition factor (K) or the quantity of food ingestion during the course of the experiment (Fig.28).

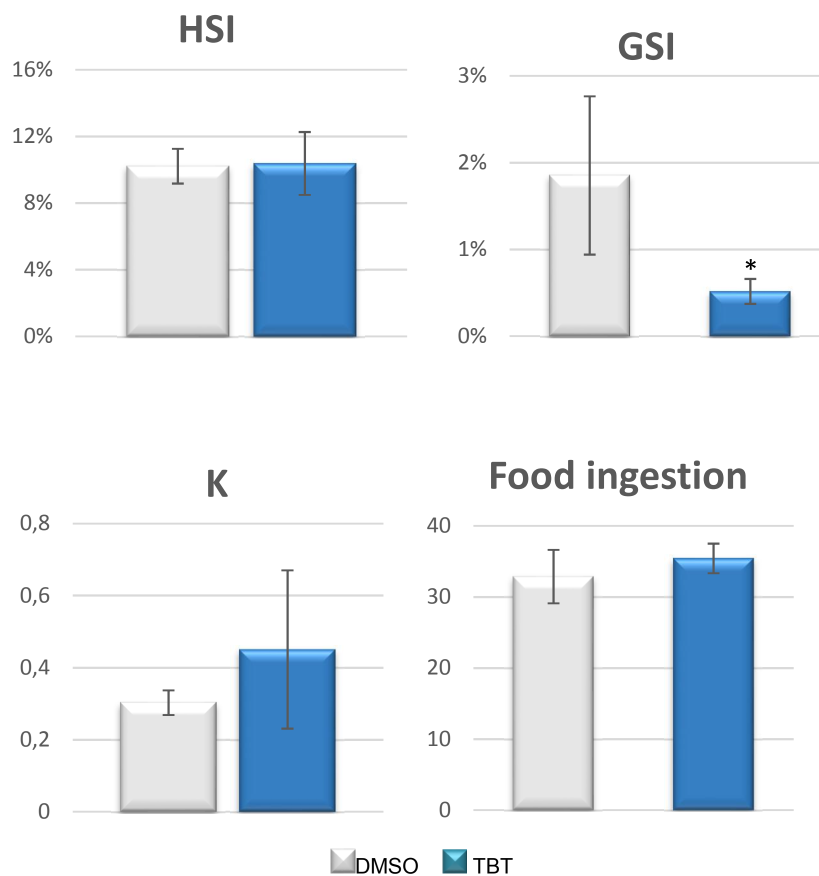

Fig. 28 –TBT effects in the physiological parameters of *S. canicula*. HIS: Hepatosomatic index (%): GSI: Gonad somatic index (%); K: Condition factor; Food ingestion: quantity of food (gr) ingested by 1 animal/week. Data is present as mean± standard deviation (*-p<0.05; ** - p<0.01; *** - p<0.001).

Next, we examined the fatty acid composition of liver samples from female *S. canicula* specimens (table13: quantitative data is provided for the final sampling). From the 36 fatty acids analysed 23 were detected. Fatty acids with more than 22 carbon atoms were not detected. 63% of the detected fatty acids were unsaturated; from those,

the polyunsaturated fatty acids (PUFA) were present in higher amounts, 32%, with monounsaturated fatty acids (MUFA) representing 29%. Yet, the single most abundant fatty acid in females was DHA followed by palmitic and oleic acid. No significant variations in the fatty acid profile were found between the organisms injected with DMSO and TBT.

Table 13- Fatty acid profile of the liver of *S. canicula* in the end of the experiment of the animals injected with DMSO and TBT. Values are expressed as µg/mg of tissue. Data is present as mean± standard deviation (*-p<0.05; ** - p<0.01; *** - p<0.001).

Fatty acid	**DMSO**		**TBT**	
	Mean	**STD**	**Mean**	**STD**
Lauric (12:0)	0.041	±0.0177	0.038	±0.0335
Tridecanoic (13:0)	0.501	±0.1432	0.427	±0.0704
Miristic (14:0)	13.209	±5.2063	10.849	±2.7414
Petadecanoic (15:0)	2.057	±0.6159	1.782	±0.2181
Palmitic (16:0)	77.332	±14.5091	73.642	±5.5258
Heptadecanoic (17:0)	3.009	±0.7349	2.644	±0.1806
Stearic (18:0)	18.288	±4.6427	17.079	±4.6562
Arachidic (20:0)	0.439	±0.0545	0.491	±0.0424
Heneicosanoic (21:0)	41.365	±8.5085	35.210	±10.7703
Σ SFA	**114.875**	**±32.8514**	**106.953**	**±20.27023**
Myristoleic acid (14:1)	0.149	±0.0645	0.237	±0.0539
Palmitoleic acid (16:1)	28.215	±4.1878	28.746	±2.4493
Heptadecanoic (17:1)	2.043	±0.1041	2.207	±0.1366
Oleic Acid (18:1 n9c)	75.455	±5.2569	80.954	±2.9394
Eicosanoic acid (20:1)	17.474	±6.1063	15.013	±3.9672
Erucic acid (22:1n9)	1.277	±0.4795	1.129	±0.1100
Σ MUFA	**120**	**±14.9587**	**130**	**±9.3540**
Linolelaidic (18:2n6t)	4.511	±0.9935	4.113	±0.4828
Linolenic acid (18:3n3)	3.073	±0.5828	2.819	±0.4833
11,14-Eicosadienoic acid (20:2)	1.616	±0.2473	1.550	±0.0604
11,14,17-eicotrienoic (20:3 n3)	0.437	±0.1000	0.371	±0.0385
8,11,14- eicosatrienoic (20:3 n7)	0.133	±0.1346	0.244	±0.2302
Arachidonic (20:4 n6)	4.853	±0.5276	4.614	±0.4365
EPA (20:5 n3)	0.711	±0.0610	0.719	±0.0895
DHA (22:6 n3)	117.058	±28.0624	104.321	±4.2853
Σ PUFA	**130**	**±30.4143**	**120**	**±5.3894**
Σ UFA	**260**	**±44.5716**	**250**	**±14.6272**
Σ FA	**410**	**±76.9642**	**390**	**±36.5871**

We also examined whether any alteration in the triglyceride and cholesterol content occurred after TBT injection. No alterations were observed in any of these parameters (Fig.29).

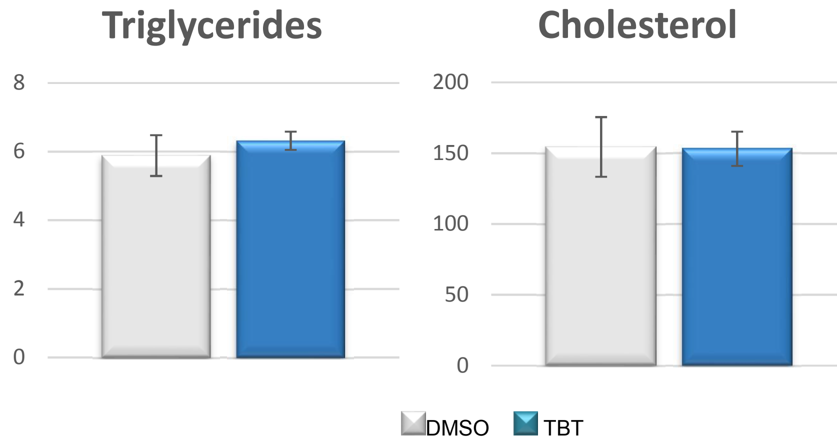

Fig. 29 - TBT effects on hepatic triglycerides and cholesterol of *S. canicula* females. The effect of TBT on hepatic triglyceride and cholesterol levels 8 weeks after being injected with DMSO and 400ng Sn/L TBT. Data is present as mean± standard deviation (*-p<0.05; ** - p<0.01; *** - p<0.001).

Discussion

Metazoan endocrine systems encompass an intricate network of signaling cascades, that when disturbed lead to episodes of disruption with developmental and physiological impairment (Kabir et al., 2015). In this context, a critical and complex question is that of how man-made chemicals "exploit" the genetic and physiological animal biodiversity and influence homeostasis. In recent years, the concept of obesogens has gathered increase attention as the obesity epidemic gained pace in western societies. The overall feature of these compounds is their capacity to activate the mammalian PPARγ orthologue; yet in some teleost orthologues seem irresponsive (Colliar et al., 2011; Harada et al., 2015; Janesick and Blumberg, 2011). Our analysis of various genomes assemblies and transcriptomes of chordate species provides a solid indication that *PPAR* duplicated before gnathostome radiation and after tunicate divergence. Moreover, we were able to deduce that three gene paralogues corresponding to *PPAR*α, *PPAR*β and *PPAR*γ genes are present in the full range of lineages including the chondrichthyans, holosteans, osteoglossomorpha, amphibians and mammals. In teleosts, *ppar*α and *ppar*β underwent additional episodes of duplication (Bertrand et al., 2007; Madureira et al., 2017). This is in contrast to the single true orthologue *ppar* gene found in invertebrates such as amphioxus, the sea squirt and molluscs (Kaur et al., 2015; Vogeler et al., 2017; this thesis). As with many other NR gene families, the increase in the number of *PPAR* genes is most likely a consequence of the genome duplications in vertebrate evolution (2R, 3R and 4R) (Braasch et al., 2016; Jaillon et al., 2004; Putnam et al., 2008).

PPARs are key regulators of fatty acid metabolism (Berkenstam and Gustafsson, 2005; Desvergne et al., 2006). In accordance, a range of fatty acids such as ARA and EPA are known activators of PPAR paralogues in mammals, thus controlling lipid anabolism and catabolism (Forman et al., 1997; Kliewer et al., 1997; Xu et al., 1999). By profiling the PPARs from basal chordate lineages, the cephalochordates and the chondrichthyans, we show that this function extends beyond the mammalian order. ARA, EPA and many other long chain fatty acids are known to be endogenously produced in addition to the dietary uptake in vertebrates (Castro et al., 2016). In effect, the molecular module responsible for elongation and desaturation of fatty acids underwent a complex upgrade upon gnathostome radiation (Castro et al., 2016; Monroig et al., 2016). Despite the significant shift in endogenous fatty acid at the invertebrate/vertebrate transition (Monroig et al., 2016), our findings suggest that lipid metabolism control under PPAR signaling is much older than previously anticipated. Yet, it remains to be investigated

whether this duplication episode, dating to the origin of vertebrates, entailed processes of *PPAR* gene neo or sub-functionalization and with what consequences in lipid physiology.

In mice PPARγ is a direct target of organotins, namely TBT (Grün et al., 2006), with the morpho-physiological effects including a range of obesogenic phenotypic features – increases in the white adipocyte tissue and lipid accumulation in liver (Chamorro-García et al., 2013; Grün et al., 2006; Wang et al., 2016). PPARα and PPARβ, on the other hand, are mostly associated with fatty acid oxidation (Mello, 2010). Critically, at the structural level this exploitation of the mammalian PPARγ is known to be mediated by a specific amino acid, cysteine 285, via a non-covalent ionic bound with the tin atom (Harada et al., 2015). Sequence analysis across a broad range of chordate species shows that cysteine 285 is conserved in most gnathostomes but not in teleosts or cephalochordates. In effect, in zebrafish and butterfly fish TBT acts as an inhibitor of PPARα and PPARβ but yields no effect on PPARγ (Leaver; this work), similarly to the amphioxus PPAR. In contrast, cysteine-285-bearing sequences from other analyzed lineages showed a clear fold induction in the transactivation assays. Moreover, the mutagenesis of the little skate cysteine residue results in the abolishment of the activation response (cysteine->tyrosine) or in an inhibitory behavior (cysteine->alanine). Thus, this cysteine residue is key to the anchoring of TBT and the induction of transcription, both in mammals and chondrichthyans. Considering this scenario, we propose that the gain of TBT activation emerged in the ancestor of gnathostomes (and possibly vertebrates) concomitant with the diversification of PPARs. In this context, the transformation of an activation profile to that of inhibition observed in some teleosts represents a secondary event, possible determined by changes in the ligand pocket. These observations raise a paradoxical scenario. While both mammals and teleosts experience obesogenic outcomes upon organotin exposure (Grün et al., 2006; Lyssimachou et al., 2015; Ouadah-Boussouf and Babin, 2016; Tingaud-Sequeira et al., 2011), the exact molecular disruptive mechanisms are apparently different, since the teleost PPARγ does not activate transcription in the presence of TBT (Colliar et al., 2011; this work). This would involve an apparent absence of obesogenic impacts in fish. Yet, TBT inhibits the teleost PPARα/β (Colliar et al., 2011), eventually suppressing lipid oxidation in vivo, which could potentially cause fat accumulation (Lyssimachou et al., 2015). Other hypothesis is the activation of the heterodimer PPARγ/RXR by TBT through the RXR monomer (Ouadah-Boussouf and Babin, 2016). Whether these different molecular mechanisms are responsible for similar phenotypic outcomes remains to be

established. Also, of crucial relevance will be to disclose the structural sequence events responsible for the “diversification” of PPAR exploitation by obesogens in different species. Though, the role of cysteine 285 emerges as conserved and ancestral element in organotin binding, our results anticipate the importance of yet unknown critical amino acid residues. When comparing the amino acids that were identified to interact with TBT in the human PPARγ, we observed a lower conservation in the teleosts tested when compared with the other vertebrates.

Several groups of vertebrates have already been investigated for obesogenic responses due to exposure to TBT, yet an important basal group is still missing, the chondrichthyans (Capitão et al., 2017). To fill this gap we performed an *in vivo* exposure of the small-spotted catshark (*Scyliorhinus* canicula) to TBT through injection. During the eight weeks after the injection no significant observations were detected in the feeding behavior between control and exposed animals. After the eight weeks, an effect in the ovaries of the animals exposed to TBT was observed. The previous observation is in line with the reported effects of TBT in the fecundity of teleosts (Lima et al., 2015). Looking into the fatty acid profile, triglyceride and cholesterol content, no alterations were observed between the DMSO and TBT animals. The lack of alterations is probably related to the distinct functions of the three PPARs in the lipid homeostasis. PPARα and PPARβ are mostly associated with fatty acid oxidation while PPARγ is associated with the lipid accumulation (Mello, 2010). In bony fish, amphibians and humans the TBT-dependent transactivation of the PPARγ was accompanied by the repression of PPARα and/or β. Accordingly, and assuming conserved roles for the three PPARs across vertebrates, TBT exposure should yield a higher accumulation of lipids associated with a lower oxidation rate. In chondrichthyans, however, TBT transactivates the three PPARs, which possibly causes an increase in the oxidation of fatty acids that outweigh the increase in the lipid accumulation.

In conclusion, the conceptual framework explored in this study puts into context the role of PPARs in “obesity” disruption processes across the chordate evolutionary scale, which would be impossible to reveal with model species.

Methods

Sequence investigation

Tblastn and blastp searches using human *PPARα* (Q07869), *PPARβ* (Q03181) and *PPARγ* (P37231) as query were performed in the available databases NCBI Ensemble and JGI. *Ppar* sequences were retrieved for the major vertebrate lineages: mammals (*Homo sapiens*, *Mus musculus*); birds (*Gallus gallus*) reptiles (*Anolis carolinesis*); amphibians (*Xenopus tropicalis*); coelacanths (*Latimeria chalumnae*); Lepisosteiformes (*Lepisosteus oculatus*) Osteoglossomorpha (*Pantodon buchholzi*) Cyprinipforme (*Danio rerio*), Chondrichthyes (*Callorhinchus milii*) and for the following invertebrates, Tunicates (*Ciona intestinalis*) cephalochordate (*Branchiostoma floridae, Branchiostoma lanceolatum, Branchiostoma belcheri*) (See supplementary table 8 for accession number).

Phylogenetic analysis

Phylogenetic analysis was performed using the set of sequences through database search as well as the isolated sequences. A total of 36 sequences were aligned in MAFFT (Katoh et al., 2005; Katoh and Toh, 2008) with L-INS-I method. The resulting sequence alignment was stripped of all columns containing 90% gaps leaving a total of 515 positions for phylogenetic analysis. Maximum likelihood phylogenetic analysis was performed in PhyML V3.0 (Guindon et al., 2010) and the evolutionary model was determined using the smart model selection (SMS) option resulting in a JTT +G. The branch support was calculated using aBayes. The resulting tree was analyzed in Fig Tree V1.3.1 available at http://tree.bio.ed.ac.uk/software/figtree/ and rooted with the invertebrate sequences.

Synteny maps

Comparative synteny maps were constructed using NCBI Gene database, using as reference the latest available genome assemblies for the following species: *H. sapiens* (GCF_000001405.33), *L. oculatus* (GCF_000242695.1), *C. milii* (GCF_000165045.1), *B. floridae* (GG666565.1) and *B. belcheri* (AYSS01004225.)1. For each species, with the exception of the cephalochordates that present a sole *PPAR*, we analysed the genomic location of *PPARα, PPARβ* and *PPARγ* gene. The genomic locus of each *PPAR* gene was retrieved, as well as, the five contiguous flanking genes to each side, when possible. Following the assembly of the synteny maps, we proceeded to

identify and localize the corresponding human orthologues of non-conserved neighbouring genes in the *B. floridae* and *B belcheri*. Orthology was determined through the Ensembl orthologue-paralogue pipeline and our own phylogenetic analysis (not shown). Finally, synteny maps and annotated orthologues were then used to infer the localization of the ancestral *PPAR* gene in the reconstructed genome of the vertebrate ancestor using as reference the reconstruction presented by Nakatani and colleagues (Nakatani et al., 2007) and the reconstruction presented by Putnam and colleagues (Putnam et al., 2008).

Chemicals and solutions

All chemicals and reagents were obtained from Sigma-Aldrich unless stated otherwise in the text. Three TBT-Cl and three TPT-Cl (Triphenyltin chloride) stock solutions were prepared in DMSO for the *in vitro* experiment: 250 μM, 100 μM and 10 μM. Three ARA (Arachidonic acid) and three EPA (cis-5,8,11,14,17-Eicosapentaenoic acid) stock solutions were prepared in DMSO with the concentrations of 200 mM, 100 mM and 50 mM. For the *in vivo* experiment a TBT solution 6.9mg/ml was prepared in DMSO.

Gene isolation and cloning

Hinge and DBD of *PPARα*, *β* and *γ* genes from *Homo sapiens, Xenopus tropicalis, Pantodon buchholzi, Lepisosteus oculatus, Leucoraja erinacea* and *Branchiostoma lanceolatum* (accession numbers in supplementary material table 9) were isolated using a PCR approach (primer sequences in supplementary material table 2). The hinge and ligand-binding domains were then cloned into the pBIND vector to produce an NR LBD-Gal4 hybrid protein. This hybrid protein, contain the DNA binding domain of Gal4 and acts on a UAS response element. Plasmid sequences were confirmed using Sanger sequencing (GATC).

Cell culture and *in vitro* assays

COS-1 cells were grown in Dulbecco's modified Eagles medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37ºC in a humidified chamber containing 5% CO_2.

Cells were seeded in 24-well culture plates at a density of 2 x 10^5 live cells/ well. After 24 hours cells were transfected using lipofectamine 2000 reagent (Invitrogen), in Opti-MEM reduced serum medium (Gibco, Thermo Fisher), according manufacturer's indications and 0.5 µg of the respective pBind PPAR LBD-Gal4 and 1µg of pGL4.31 luciferase reporter vector, containing five UAS elements upstream the firefly luciferase reporter gene. After 5 hours of incubation the medium was replaced with phenol red-free DMEM supplemented with 10% dextran-coated charcoal-treated serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) containing the test compounds (TBT, TPT, ARA and EPA) dissolved in DMSO (0.1%). 24 hours later cells were lysed and the luminescent activities of Firefly luciferase (pGL4.31) and Renilla luciferase (pBIND) 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). Renilla Luciferase, co-expressed with the NR LBD-Gal4 hybrid proteins, served as an internal control for transfection efficiency. All assays were performed, independently, three times and each time two technical replicates per condition were performed to validate the results.

Animal maintenance, experimental setup and sampling

To evaluate the ability of TBT to alter the lipid homeostasis in Chondrichthyes we performed an *in vivo* exposure of *Scyliorhinus canicular*. Six *S. canicula* females were bought to a local fisherman and transported to CIIMAR's (Interdisciplinary Centre of Marine and Environmental Research) aquatic animal facilities. In CIIMAR the animals were divided in two aquaria (3 animals/aquarium) containing approximately 300L of filtrated salt water at 17 ± 2°C, a natural light/dark cycle through a window and continuous aeration. During all acclimation and experimental period the animals were feed with *Trachurus trachurus* (bought in a local store) *ad libitum* 3 times a week and the amount of food ingested was recorded**.** Temperature was monitored daily and the water physicochemical parameters (35‰ of salinity, 0.37 ± 0.29 mg/L of ammonium and 0.35 ± 0.37 mg/L of nitrite) were monitored weekly. After six weeks of acclimatization the animals were anesthetized in salt water containing 100mg/L of MS-222 and 100mg/L sodium hydrogen carbonate and injected with DMSO (0.2µl/gr) or TBT dissolved in DMSO (400ng Sn/g). Eight weeks after the injection the animals were sacrificed in salt water with 200mg/L of MS-222 and 200mg/L sodium hydrogen carbonate. Length, whole weight, gonad and liver weight was recorded. The tissues were separated and the ones kept to biochemical analyzes were frozen in liquid nitrogen and subsequently stored at -

80°C until further analysis, while the one kept for gene expression were stored in RNA later (Sigma) according to manufacturer's recommendations. Gonad index (GSI), hepatosomatic index (HSI) and condition factor (K) were calculated using:

$$GSI = \frac{Wet\ weight\ of\ gonads}{Wet\ weight\ of\ whole\ animal} \times 100$$

$$HSI = \frac{Wet\ weight\ of\ liver}{Wet\ weight\ of\ whole\ animal} \times 100$$

(Sadekarpawar and Parikh, 2013)

$$K = \frac{Wet\ weight\ of\ whole\ animal}{Length\ of\ whole\ animal\ \times 3} \times 100$$

(Datta et al., 2013)

Fatty acid, Triglycerides and Cholesterol quantification

For fatty acid quantification, a sample of liver with approximately 250mg of each animal was extracted and analysed. Lipid extraction was performed according to the Folch method with minor adjustments (FOLCH et al., 1957). Briefly, the tissue was homogenized with chloroform/methanol (2:1) to a final volume of 20ml/gr of tissue. After homogenization tricosanoic acid was added as internal standard the mixture was agitated during 3 hours in an orbital shaker at room temperature protected from the light. The sample was then centrifuged for 30 minutes at 2000 rpms. The supernatant was recovered for a new tube and vortexed for 1 minute with 0.25 volume of a NaCl (9%) solution. The mixture was then centrifuged for 10 min at 2000 rpm for phase separation, the upper phase was discarded. A final wash with methanol/water (1:1) was performed previous to the centrifugation. The upper phase was discarded and the lower phase was evaporated under a nitrogen stream. The samples were kept at -20ºC until derivatization. Fatty acid derivatization was performed as described by Cohen et al., 1988 using acetyl chloride as catalyst and the detection was performed according to Guedes et al., 2011. Fatty acids were quantified in µg/mg of tissue as done in Morais et al., 2003 (quantification limits in the supplementary table 10).

To measure the triglycerides and cholesterol the dry extracts were re-suspended in Hexane. Triglyceride and cholesterol content was measured using Infinity

Triglycerides Liquid Stable Reagent (Thermo Scientific) and Cholesterol Liquid Stable Reagent (Thermo Scientific), following the manufacture indications. Samples were measured in duplicates and absorbance was determined at 540 nm using a microplate reader (PowerWave 340, Bio-Tek). Triglycerides and cholesterol concentration was calculated against a standard curve generated from triolein and cholesterol standards, respectively.

Statistical analysis

Transactivation data were normalized with DMSO and the mean of the technical replicates was used in the statistical analysis. One-way ANOVA followed by Fisher LSD post hoc test was used to analyze differences when parametric criteria was achieved, otherwise a non-parametric Kruskal-wallis ANOVA followed by a Games-Howel test was used. All analyses were performed using IBM SPSS Statistics 24. $P < 0.05$ was considered statistically significant in all analyses.

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Supplementary material

Table 8 – Accession numbers

Vertebrates	PPARα	PPARβ	PPARγ
Homo sapiens	Q07869	Q03181	P37231
Mus musculus	P23204	P35396	P37238
Gallus gallus	NP_001001464.1	NP_990059.1	NP_001001460.1
Anolis carolinesis	XP_003221452.1	*	XP_003220387.1
Xenopus tropicalis	XP_002940784.2	XP_012826243.1	*
Danio rerio	ABI30003.1	NP_571543	NP_571542.1
Pantodon buchholzi	Ctg24603	Ctg21483	Ctg18143
Lepisosteus oculatus	W5NK92*	ENSLACG00000015749	XP_006631094.2*
Latimeria chalumnae	XP_005991369.1	Nf	XP_006004475.1
Callorhinchus milii	SINCAMG00000010348	SINCAMP00000016425	XP_007901520
Leucoraja erinacea	*	*	*
Invertebrates	***Accession number***		
Ciona intestinalis	NP_001071801.1		
Branchiostoma floridae	jgi|Brafl1|174611		
Branchiostoma lanceolatum	*		
Branchiostoma belcheri	XP_019618227.1_		

Table 9 - Primer list

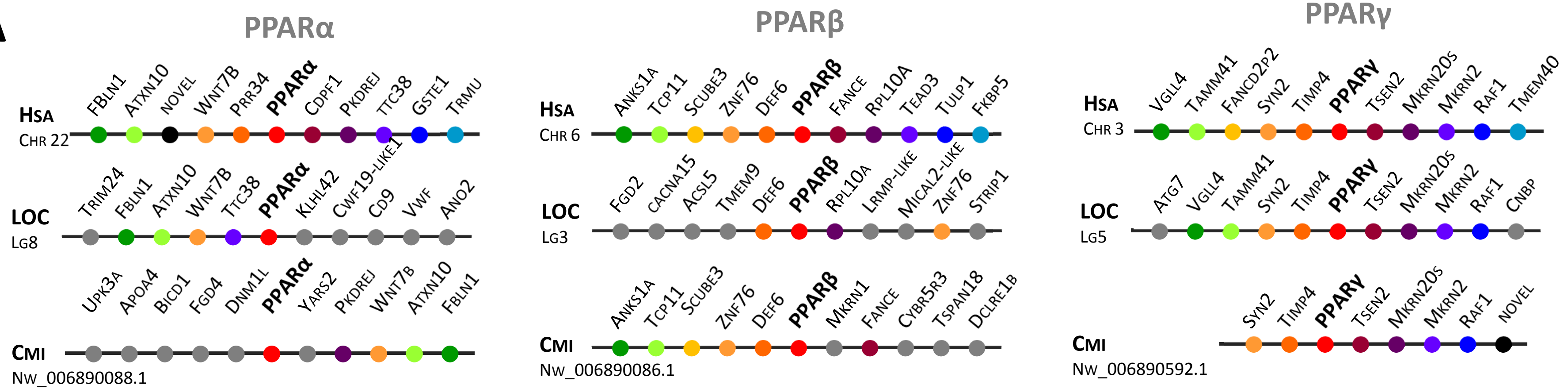
Target	Organism	Primer name	Primer sequence	Direction
pparα	H. sapiens	HsPPARalphaXBAI_F	CCCTCTAGAATGTCACACAACGCGATT	Foward
pparα	H. sapiens	HsPPARalphaKPNI_R	ATAGGTACCTCAGTACATGTCCCTGTAGA	Reverse
pparβ	H. sapiens	HsPPARdeltaXBAI_F	CCCTCTAGAATGTCACACAACGCTATC	Foward
pparβ	H. sapiens	HsPPARdeltaKPNI_R	ATAGGTACCTTAGTACATGTCCTTGTAGATC	Reverse
pparγ	H. sapiens	hsPPARGpBINDBamHiF	GCTGCTGGATCCGAATGCCACAGGCC	Foward
pparγ	H. sapiens	hsPPARGpBINDKpnIR	ATAGGTACCCTAGTACAAGTCCTTGTAGATCTCC	Reverse
pparγ	X. tropicalis	BamhI_PPARa_xtropicalis_F	TAGCGGATCCTTaaggcggaagtgcagacgtg	Foward
pparα	X.tropicalis	KpnI_PPARa_xtropicalis_R	GTCAggtaccatcagtacatgtccctgtaga	Reverse
pparβ	X. tropicalis	BamhI_PPARb_xtropicalis_F	TAGCGGATCCTTGTGCAGGCACCAGTAACGGA	Foward
pparβ	X.tropicalis	KpnI_PPARb_xtropicalis_R	GTCAggtaccaTCAATACATGTCTCTGTAAA	Reverse
pparγ	X. tropicalis	BamhI_PPARg_xtropicalis_F	TAGCGGATCCTTCTATTGGCTGAGATCTCCAG	Foward
pparγ	X.tropicalis	KpnI_PPARg_xtropicalis_R	GTCAggtaccatcagtataagtctttgtaga	Reverse
pparα	L. oculatus	BamhI_PPARa_Loculatus_F	TAGCGGATCCTTAAGGCGGAGATTCTGACGGG	Foward
pparα	L. oculatus	KpnI_PPARa_Loculatus_R	GTCCggtacctcagtacatgtccctgtaga	Reverse
pparβ	L. oculatus	BamhI_PPARb_Loculatus_F	TAATGGATCCTTGTGGCCGGGTTGCTGGCTGG	Foward
pparβ	L. oculatus	KpnI_PPARb_Loculatus_R	GTCCggtaccggCTAGTACATATCTTTGTAGA	Reverse
pparγ	L. oculatus	BamhI_PPARg_Loculatus_F	TCGTGGATCCTTTTGGCTGAATTCTCTTCAGA	Foward
pparγ	L. oculatus	KpnI_PPARg_Loculatus_R	GTCCggtaccggCTAATACAAGTCCTTTATTA	Reverse
pparα	P. buchholzi	PbuPPARAlpha_XBAI_F	TCTAGAATGTCCCACAACGCCATCCG	Foward
pparα	P. buchholzi	PbuPPARAlpha_KPNI_R	GGTACCTTAGTACATATCCTTGTAGATCTCTTG	Reverse
pparβ	P. buchholzi	PbuPPARDelta_XBAI_F	TCTAGAATGTCCCACGATGCGATTCGTT	Foward
pparβ	P. buchholzi	PbuPPARDelta_KPNI_R	GGTACCCTAGTACATATCCTTGTAGATCTCC	Reverse
pparγ	P. buchholzi	PbuPPARg_XBAI_F	TCTAGAATGTCCCACAACGCCATCCGCT	Foward
pparγ	P. buchholzi	PbuPPARg_KPNI_R	GGTACCCTAATACAAGTCCTTCATGATCTCCTG	Reverse
ppar	B. lanceolatum	Bl_PPAR_pbind_F	GCAAGGATCCTAATCCTGGAGCTGCAGAATCA	Foward

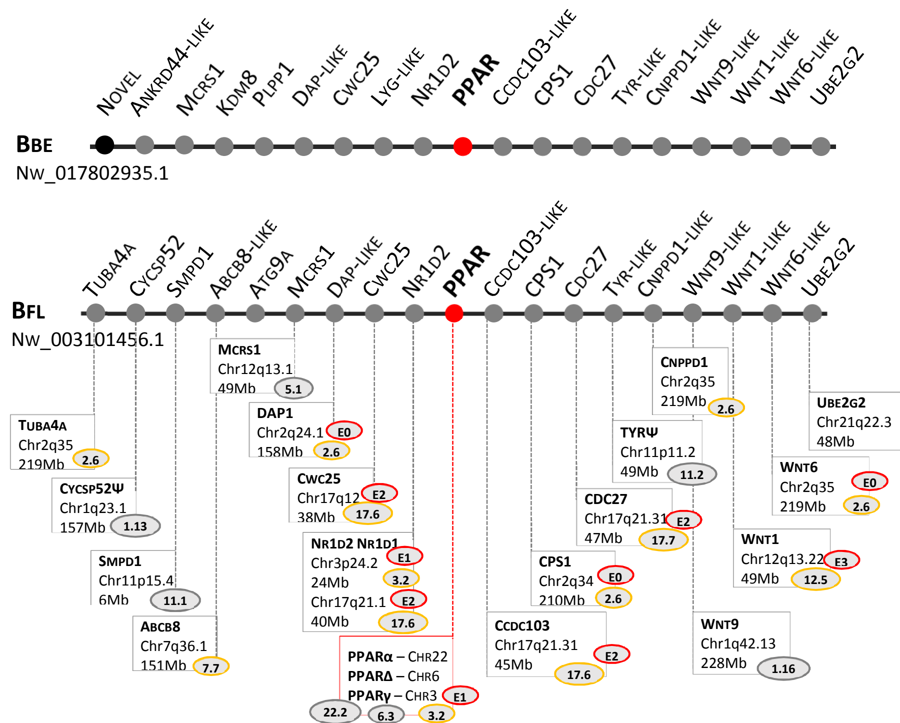
ppar	B. lanceolatum	Bl_PPAR_pbind_R	GCAATCTAGAATCCTAATGCACTTCGTGGAT	Reverse
pparα	P. buchholzi	Pbu_PPARAlpha_Orf_F	GTATACGTGCGTGCATTCGT	Foward
pparα	P. buchholzi	Pbu_PPARAlpha_Orf_R	CCAGCTGGATCCCAGTAAGA	Reverse
pparα	L. erinacea	LSK-PPARa-XBAI-F	AATTTCTAGACCACAATCTGAGAAGGAGAAATTAAAAG	Foward
pparα	L. erinácea	LSK-PPARa-KPNI-R	AATTGGTACCTCAGTACATATCCCTGTAAATTTCCTG	Reverse
pparβ	L. erinácea	LSK-PPARd-XBAI-F	AATTTCTAGACCTGAAGCAGAGAAGAAAAAGCTAG	Foward
pparβ	L. erinácea	LSK-PPARd-KPNI-R	AATTGGTACCTCAATACATGTCCTTGTAGATCTCTTG	Reverse
pparγ	L. erinácea	LSKxbaIPPAR_F	ACTGtctagaCCACAGGCTGAGAAGGAG	Foward
pparγ	L. erinácea	LSKkpnIPPAR_R	ATAggtaccCAGGGTGAGGATCTCTAA	Reverse

Table 10 – Fatty acid quantification limits

Fatty acid		LOQ (µg/ml)
Butiric	4:0	65,840
Hexanoic	6:0	53,890
Octanoic	8:0	36,207
Decanoic	10:0	35,948
Undecanoic	11:0	20,067
Lauric	12:0	48,223
Tridecanoic	13:0	28,980
Miristic	14:0	62,327
Myristoleic acid	**14:1**	30,236
Petadecanoic	15:0	35,570
Pentadecanoic cis	15:1	35,512
Palmitic	16:0	122,593
Palmitoleic acid	**16:1**	40,004
Heptadecanoic	17:0	46,833
Heptadecanoic	17:1	46,454
Stearic	18:0	96,904
Oleic Acid	**18:1 n9 c**	144,446
Elaidic	**18:1 n9 t**	
Linolelaidic	**18:2 n6 t**	44,549
Linoleic acid	**18:2 n6 c**	53,191
gamma- linoleic	18:3n6	43,696
Linolenic acid	**18:3 n3**	45,512
Arachidic	20:0	95,937
Eicosanoic acid	**20:1**	49,438
11,14-Eicosadienoic acid	20:2	50,209

11,14,17-eicotrienoic	20:3 n3	48,835
8,11,14- eicosatrienoic	20:3 n7	46,591
Arachidonic	**20:4 n6**	46,811
EPA	20:5 n3	46,327
Heneicosanoic	21:00	43,687
Docosanoic	22:0	177,982
Erucic acid	**22:1 n9**	77,250
13,16-docosadienoic	22:2	48,964
Tricosanoic	23:0	45,077
Lignoceric	24:0	94,636
DHA	**22:6 n3**	51,653
Nervoic acid	**24:1**	48,986





C

Putnam 2008
LG16
A- 10.2
B-12.5
C- 17.6 /17.9
D-3.2 /7.2 /7.4 /7.7
E- 2.6
Nakatani 2007
Ancestral chromosome E
E0- Chr2
E1- Chr3/Chr7/Chr10
E2- Chr17
E3- Chr12

Fig. 3- **A**: Synteny maps of the genomic location of *PPARα, PPARβ* and *PPARγ* in HSA- *Homo sapiens*, LOC- *Lepisosteus oculatus,* CMI- *Callorhinchus milii.* **B**: Synteny maps of the genomic location of *PPAR* in BFL-*Branchiostoma floridae* and BBE- *Branchiostoma belcheri*, localization of the orthologous genes in *H. sapiens* is indicated below the synteny map of BFL, as well as the localization to the corresponding ancestral linkage groups or chromosomes in circles. **C** – Reconstruction of linkage group 16 and ancestral chromosome E according to Nakatani et al. 2007 and Putnam et al. 2008.

Fig. 4 - PPAR sequence alignment. Human PPARγ (PPARg_Hsa), Xenopus tropicalis pparγ (PPARg_Xtr), Pantodon buchholzi pparγ (PPAR_Pbu), Lepisosteus oculatus Pparγ (PPAR g_Loc), Leucoraja erinacea pparγ (PPARg_Ler) and Branchiostoma lanceolatum ppar (PPAR_Bla) amino acid sequences. Red- anchor; Blue- interact with TBT. Based on (Harada et al., 2015)

Chapter 5 - General discussion and conclusions

General discussion and conclusions

The disruption of lipid homeostasis by EDCs has been largely explored in mammals, mostly associated with the rising of obesity rates among human populations (Casals-Casas et al., 2008; Chamorro-García et al., 2013; Grün et al., 2006; Grün and Blumberg, 2009). Several studies reported effects that ranged from alterations in the expression of genes and proteins involved in the lipid metabolism to lipid accumulation and weight increase in organisms exposed to a group of environmental chemicals, known as obesogens (Grün et al., 2006; Grün and Blumberg, 2009; Holtcamp, 2012; Lyssimachou et al., 2015; Riu et al., 2014; Wang et al., 2016). Recent studies have enlarged the focus of the research related to obesogens to a wider range of taxonomic groups. This has been driven by reports of alterations in the lipid levels and composition or metabolism in several non-vertebrates, such as mollusks (Janer et al., 2007; Lazzara et al., 2012; Lyssimachou et al., 2009; Pascoal et al., 2013), tunicates (Puccia et al., 2005) and arthropods (Jordão et al., 2016a, 2016b, 2015), which raises concern on the global ecological consequences of these findings.

In this thesis, we approached the obesogenic effect and the mode of action of environmental chemicals in poorly investigated groups such as mollusks, echinoderms and chondrichthyes. To explore the susceptibility of other animals, beyond mammals, to obesogens we used a combination of *in vivo* and *in vitro* approaches focusing in key metazoan groups. The main aim was to contribute for a better understanding of the obesogen risk in a large sampling of metazoans. TBT was chosen as the model obesogen because the mechanism of action in mammalls has been extensively explored and documented, i.e., agonist of PPARγ and RXR (Grün et al., 2006; Harada et al., 2015; Kanayama et al., 2005; le Maire et al., 2009). Additionally, although its use as antifoulant has been regulated (Antizar-Ladislao, 2008; Gipperth, 2009), the levels in the aquatic environment, especially in the vicinity of harbors, are still of concern (Chen et al., 2017).

In this thesis we focused our attention in the NRs, PPARs and RXR, given their pivotal role as key regulators of adipogenesis and their reported modulation by EDCs, including the obesogen, TBT (Grün and Blumberg, 2006; Kanayama et al., 2005). Most vertebrates display three distinct PPARs, α, β and γ. The different PPARs regulate distinct lipid pathways, PPARα and β are mostly involved in fatty acid catabolism while

PPARγ is essential for adipogenesis (Mello, 2010). In some invertebrates, one or two PPAR copies were found with the precise gene orthology still debatable (Kaur et al., 2015; Vogeler et al., 2017, 2014); yet, to our knowledge no functional characterization of PPARs has been done outside vertebrates, or indeed in the vast majority of vertebrate lineages. Thus, the present work is the first to functionally characterize PPARs in a large set of vertebrate and non-vertebrate *taxa* and discuss the findings in an evolutionary and toxicological context.

The studies carried out in the course of the present work demonstrate that fatty acids, besides transactivating the three PPARs in mammals (Forman et al., 1997; Kliewer et al., 1997; Liberato et al., 2012), Actinopterygii (Colliar et al., 2011; Leaver et al., 2005) and chondrichthyes, are also able to transactivate cephalochordate and mollusc PPARs, suggesting an evolutionary conservation of the putative natural ligands. Although the echinoderm *P. lividus* PPAR did not transactivate in the presence of the selected fatty acids, the activity of the heterodimer PPAR/RXR in the presence of the fatty acid mixture demonstrated that fatty acids also modulate the NR biological unity in this organism. NRs are relevant targets of EDCs and TBT is a demonstrated obesogen acting through the heterodimer PPAR/RXR in mammals (Grün et al., 2006; Harada et al., 2015; le Maire et al., 2009). Evaluating the ability of organotins to modulate PPARs in an evolutionary perspective can help to infer about the possible involvement of these NRs in the lipid disruption by TBT and other environmental chemicals.

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PPAR gamma_Hs extraction   Q S K E V A I R I F Q G C Q F R S V E A V Q E I T E Y A K S
PPAR gamma_Xtr extraction  Q N K E V A I R I F Q R C Q S R S V E A V R E I T E F A K N
PPAR gamma_Pbu extraction  P L D E V Q L R F F H S Y Q Y R S A E A V R E V T E F A K S
PPAR gamma_Loc extraction  P N D E A E L R F F Q R C Q V R S V E A V R E V T E F A K S
PPAR gamma_Ler extraction  Q N A E V E I R I F Q R C Q F R S V E A V R E I T E F A K S
PPAR_Bla extraction        V L S D V Q - R I L E A L Q K E T V R G I E A M A K F A K G
PPAR_Pli extraction        - - - T E K E A L T K F C R G A L L A I I E S L A K F A K K
PPAR_Pde extraction        - - E F F E K G I F T Q Y Q E L I V P V L E A S V K F A K K
```

Fig. 30 – Highlighted in green is the sequence alignment of the amino acids in the correspondent position to the anchor cysteine of TBT and TPT in the human PPARγ (Harada et al., 2015). *Homo sapiens* (Hs), *Xenopus tropicalis* (Xtr), *Pantodon buchholzi* (Pbu), *Lepisosteus oculatus* (Loc), *Leucoraja erinácea* (Ler) PPARγ and *Branchiostoma lanceolatum* (Bla), *Paracentrotus lividus* (Pli) and *Patella depressa* (Pde) PPAR.

Previous works that addressed the modulation of PPARs by organotins were mostly focus in PPARγ (Grün et al., 2006; Harada et al., 2015; Hiromori et al., 2009; le Maire et al., 2009). Although the majority of the key amino acids involved in the interaction of TBT and TPT with PPARγ are conserved, including the anchor amino acid cysteine in the position 285, only TPT can stabilize the helix12. Due to the stabilization of helix 12, TPT acts as a full agonist while TBT behaves as a partial agonist of the human PPARγ (Harada et al., 2015). Our results demonstrate that TBT can modulate

PPARs alone in most taxonomic groups analysed. The repression of PPARα and β in the presence of TBT is conserved between mammals, amphibians and Actinopterygii. Interestingly, an opposite response is observed in the gnathostome basal group, Chondrichthyes. The lack of response of *P. buchholzi* PPARγ to TBT is most probably related with the substitution of the anchor cysteine, that is found in the other vertebrates PPARγ, with a tyrosine. Our results demonstrate that the anchor cysteine in PPARγ is essential for the response to TBT, not only in mammals (Harada et al., 2015), but also in other vertebrates. In the present work, when the anchor cysteine was substituted with an alanine or a tyrosine in *L. erinacea* PPARγ, a loss of transcriptional response is observed when compared to the wild type, corroborating the key role of this amino acid position.

Most non-vertebrate PPARs are repressed in the presence of TBT or TPT. *B. lanceolatum* PPAR was not transactivated in the presence of TBT. Taking together the transactivation results and the effects observed in the lipid metabolism, it is possible to conclude that a disruption of PPAR/RXR seems to be associated with changes in the lipid profile/content of several taxa. In mammals and amphibians the repression of PPARα and β led to a decrease in fatty acid oxidation, favouring the accumulation of lipids caused by the activation of PPARγ, which is in accordance with previous reports that indicate an increase in lipid accumulation of mammals and amphibians when exposed to organotins (Chamorro-García et al., 2013; Grün et al., 2006; Wang et al., 2016). Although, in Actinopterygii, no transactivation of PPARγ was observed in the presence of TBT, several reports indicate that lipid content in this group is also affected by TBT (Lyssimachou et al., 2015; Meador et al., 2011; Ouadah-Boussouf and Babin, 2016; Riu et al., 2014; Tingaud-Sequeira et al., 2011; Zhang et al., 2016, 2013, 2009). This observation can be related with two factors: 1) the repression of PPARα and/or β leading to a lower fatty acid oxidation and consequent increase in the lipid storage (Malandrino et al., 2015); 2) The activation of the permissive heterodimer PPAR/RXR through the RXR (Ouadah-Boussouf and Babin, 2016).

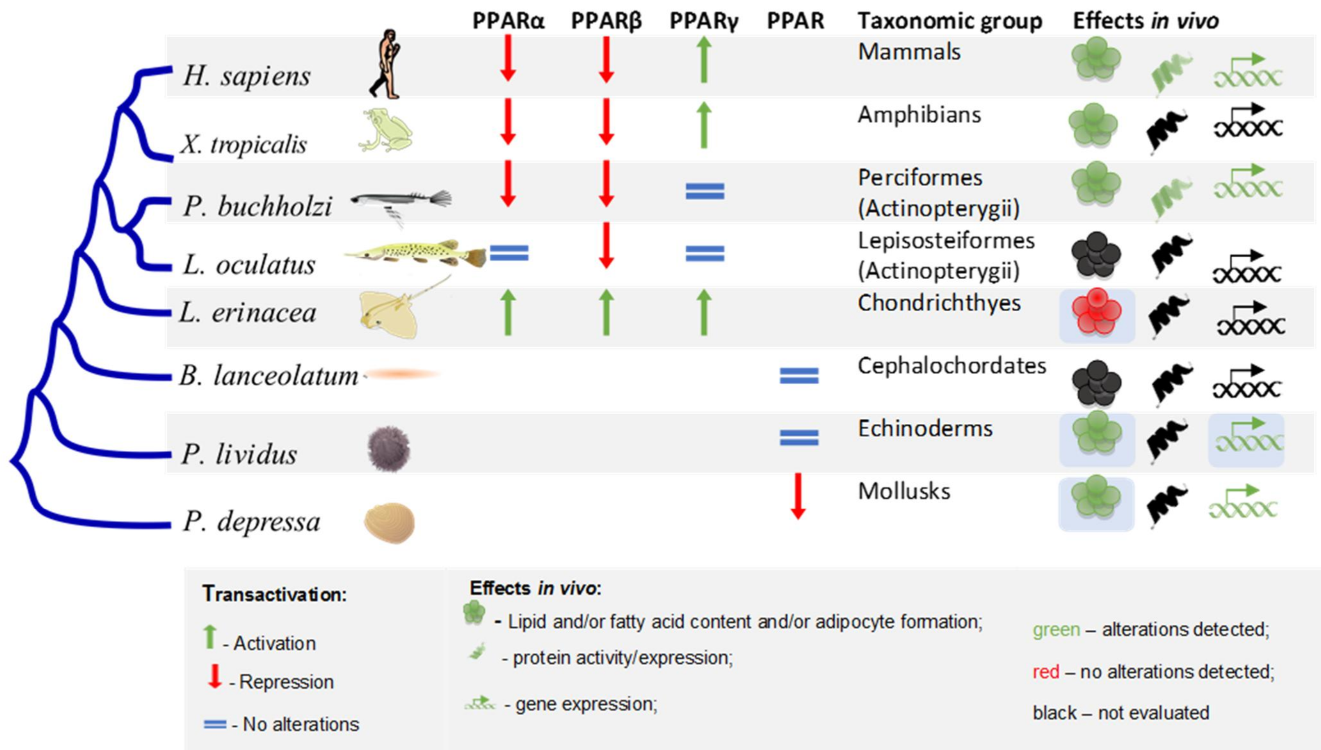

Fig. 31 – Taxonomic scope of TBT effects. PPARs transactivation results acquired during the development of this thesis. Effects of TBT in vivo in the different taxonomic groups (blue background- results acquired during the development of this thesis). Based on the results in this thesis and Bertuloso et al., 2015; Capitão et al., 2017; Chamorro-García et al., 2013; Wang et al., 2016).

Regarding non-vertebrates, until now only mollusks, arthropods and tunicates had been addressed as targets of obesogens (Janer et al., 2007; Jordão et al., 2016b, 2015; Pascoal et al., 2013; Puccia et al., 2005). Our results with the limpet *P. depressa* support the hypothesis that TBT disrupts lipid metabolism in mollusks. Moreover, the potential of TBT to disrupt the lipid metabolism in an echinoderm, *P. lividus,* was also demonstrated for the first time in this work. TBT alters the gene expression of lipid metabolism regulators and enzymes involved in the fatty acid synthesis pathway and change the fatty acid profile of gonads. The alterations observed in the fatty acid profiles of both the mollusk *P. depressa* and the echinoderm *P. lividus* are gender specific; in the limpet, we observed that TBT yielded sex-specific changes in the fatty acid profile, with respect to composition and concentration: decreasing in females and increasing in males. In the echinoderm, the difference between genders was not so clear. The stearic acid was altered in both genders, increased in females and decreased in males, while the other altered fatty acid differ between females and males. The gender specific response in both species is probably related with the different energetic requirements in the gonads of males and females (Martínez-Pita et al., 2010; Morais et al., 2003a, 2003b), and was also reported in zebrafish (Lyssimachou et al., 2015).

Reports regarding obesogen's effects in vertebrates focused on mammals, fishes (only teleosts) and, at a smaller scale, on amphibians (Capitão et al., 2017). A gnathostome basal group was yet unexplored, the Chondrichthyes, which in addition have an unusual energy metabolism (Speers-roesch and Treberg, 2010). With the aim of filling this gap we used an *in vivo* assay using the small-spotted catshark, *S. canicula*. Curiously, animals injected with 400 ng Sn/gr of TBT did not present any detectable alteration in the liver fatty acid profile, triglycerides or cholesterol content, while female gonodosomatic index was repressed. These observations could be explained by the modulation of PPARs by TBT. In fact, our *in vitro* results suggest that TBT transactivates the three Chondrichthyes PPARs. Assuming a similar physiological role as that for mammals, the transactivation of PPARα and β could lead to a higher level of fatty acid oxidation balancing the increase in the lipid accumulation caused by the activation of PPARγ (Mello, 2010). Yet, further studies are necessary to validate this hypothesis. For example, PPARα and β antagonist could be used to inhibit the response of these NRs to TBT, enabling to verify if the exclusive activation of PPARγ disrupts the lipid homeostasis in this group of organisms.

Our results expand the knowledge regarding the taxonomic scope and mode of action of the obesogen TBT. Although we only tested organotins as obesogens, the modulation observed in the tested NRs suggest that other compounds can also interfere with non-vertebrate lipid homeostasis. Moreover, a broader picture of the PPAR evolution was revealed.

Future perspectives

This thesis addresses several key questions in the field; based on the findings, several new questions emerged and should be the focus of future research. For instance, other known obesogens, besides the model obesogen TBT, should also be examined. With this in mind, transactivation assays could be used as preliminary approach to infer the taxonomic scope of such obesogens. Additionally, while the interaction of PPARs and organotins most likely underscores obesogenic phenotypes in an ample set of lineages (e.g. vertebrates), it remains largely unexplored how these compounds cause lipid alterations in PPAR-absent species. In effect, in the vast majority of protostomes, orthologues of this NR sub-family have not been described so far (Bridgham et al., 2010). Importantly, recent studies with the crustacean *D. magna*, have provide solid evidence for lipid metabolic alterations acting via different NR gene modules (Jordão et al., 2016a).

In the wild, organisms can be exposed to obesogens through the full life cycle and even several generations. Thus, the importance of lipids in the production of gametes and survival of the embryos until the feeding stage, should also be addressed in order to monitor the ecological impact of obesogen exposure of aquatic animal populations. Furthermore, it would be also relevant to address the transgenerational effects and the possible role of epigenetic changes upon obesogenic exposure.

Finally, an improved evolutionary perspective is vital to understand the impact of obesogens at an ecosystem scale and address the major challenges raised above. This evolutionary perspective may also assist in the understanding of the etiology of obesity in human populations.

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