Filling the gaps: resources and perspectives on the use of Nuclear Receptor based-assays to improve risk assessment of emerging contaminants

Santos M.M.[1, 2*+], Ruivo R.[1+], Capitão A.[1, 2], Fonseca E. [1, 2], Castro L.F.C.[1, 2,*]

[1]CIMAR/CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Av. General Norton de Matos s/n, 4450-208 Porto, Portugal

[2] FCUP - Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal

*corresponding author:

e-mail: santos@ciimar.up.pt; filipe.castro@ciimar.up.pt

+ Equal contribution

Abstract

Biological control of key processes, such as development, reproduction, and metabolism, is largely ascribed to a superfamily of ligand-dependent and independent transcription factors named Nuclear Receptors (NRs). Given their ability to accommodate ligands, NRs are prime targets of man-made compounds that mimic or antagonise the action of endogenous ligands. Accordingly, NRs occupy a prominent role in OECD and EPA guidelines for testing and assessment of Endocrine disruptors. Numerous cases of NR-mediated endocrine disruption have been reported, mostly in vertebrates. The best-studied examples include the feminization of teleost fish by Estrogen Receptor (ER) modulators; the imposex phenomenon in gastropods associated with retinoid X receptor (RXR) agonists; and, more recently, the obesogenic effect of different classes of anthropogenic chemicals acting through the Peroxisome Proliferator Activated Receptor (PPAR) and RXR. Although NR assays are already a key instrument in the OECD Conceptual Framework for Testing and Assessment of Endocrine Disruptors, the focus is mostly on vertebrate NRs. Hence, if we aim to improve risk assessment of EDCs and emerging pollutants at an ecosystems scale, and understand their mode of action (MOA), we must establish a framework to include a broad phylogenetic sampling of Metazoans. Here, we address the chief knowledge gaps in the field and set research priorities.

Keywords: EDC, Risk Assessment, Emerging pollutant, Mode of action, Biodiversity

1. Introduction

Currently over 100,000 chemicals are continuously produced. Many of these chemicals will ultimately reach aquatic ecosystems, thus threatening the integrity and health of non-target organisms (Sumpter and Johnson, 2005). 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 commonly designated as endocrine disrupting chemicals (EDCs) display a wide array of structures and are bioactive at rather low levels, particularly in sensitive time-windows. Importantly, several EDCs are known to display a non-monotonic dose-response (Lagarde *et al*., 2015). This brings additional challenges to risk assessment and toxicity testing.

In addition to EDCs, there is also increased concern that emerging pollutants (EPs) may pose a considerable health and environmental risk (Noguera-Oviedo, 2016; Ahmed *et al*., 2017). 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 environmental leakages are likely to produce physiological disruptions in non-target organisms.

Considering the limitations of classical toxicity testing outputs for wildlife protection from EDCs and PPCPs, environmental agencies such as EPA and OECD 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). In effect, a vast collection of EDCs act as high-affinity ligands of NRs. This ability to be ligand-activated is of crucial ecological relevance and several studies have revealed NR exploitation (agonist/antagonist) by EDCs, with dire endocrine outcomes (Castro and Santos, 2014). In this context, both the EPA and OECD guidelines suggest a key role for NR screening in this framework. Examples of NR disruption by environmental chemicals include teleost fish feminization by Estrogen Receptor (ER) modulators (Liney *et al*., 2007; Ferreira *et al*., 2009); the imposex phenomenon in gastropods directly associated with retinoid X receptor (RXR) agonists (Nishikawa *et al*., 2004; Castro *et al*., 2007); and the obesogenic effect of different classes of anthropogenic chemicals acting through the Peroxisome Proliferator Activated Receptor (PPAR) and RXR (Chamorro-Garcia *et al*., 2013; Lyssimachou *et al*., 2015;). Although NRs are present in all Metazoans (Figure 1), the OECD guidelines for testing and assessment of EDCs is at present strongly skewed towards mammalian models due to the lack of validated i*n 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) 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.

Here, we compile and systematize basal information that is key to implement risk assessment of EDCs and EPs in a large sample of metazoans; this includes data on available genomes/transcriptomes, functional characterized NR, laboratory-established life cycles of representative *taxa*, OECD toxicity testing protocols. The development of these tools across metazoans will allow the implementation of this step-wise procedure to prioritize chemicals to assess their potential risk as EDCs. Finally, we also address the major gaps of knowledge in the field and we set research priorities.

2. Material and Methods

Genome and transcriptome projects, as well as publication records, were retrieved from National Center for Biotechnology Information (NCBI) databases. To compile information on nuclear receptors (NRs) in the selected taxonomic groups, an extensive search was carried out in PubMed using relevant keywords: taxonomic group, species name and NR. Articles containing information about NRs characterization, by means of binding assays, *in situ* hybridization or transactivation assays, were selected. Breeding and maintenance protocols were also retrieved using relevant keywords using the Science Direct repository: "life cycle", "development", "breeding", "embryonic development", "laboratory" as well as taxonomic group and species name. Publications describing the establishment of life cycles or use of established animals' cultures were selected. Organization for Economic Co-operation and Development (OECD) and Environmental Protection Agency (EPA) protocols and guidelines were retrieved from the respective on-line repositories: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-2-effects-on-biotic-systems_20745761, https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-850-ecological-effects-test-guidelines, https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-890-endocrine-disruptor-screening-program. The overall findings are presented in Figure 1. Annex 1 displays the extended version of all compiled and systematized data.

3 Results and Discussion

Several examples indisputably show that the genomic make up of a given species is a key aspect that rules the response towards chemical insults. Therefore, it is not surprising that disclosing the molecular kernel of a given species is fundamental to understand the MOA 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 EPs) targeting conserved signaling pathways. Therefore, we first performed a detailed analysis using online databases and literature surveys to identify the number and phylogenetic position of available metazoan genomes/transcriptomes. 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. It is also essential as a proxy to identify the affected signaling pathways. Our search shows that 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 (Figure 1, Annex 1). Likewise, genomes are also available for most Metazoan lineages. We expect this background information to boost the implementation of risk assessment of EDCs and EPs 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

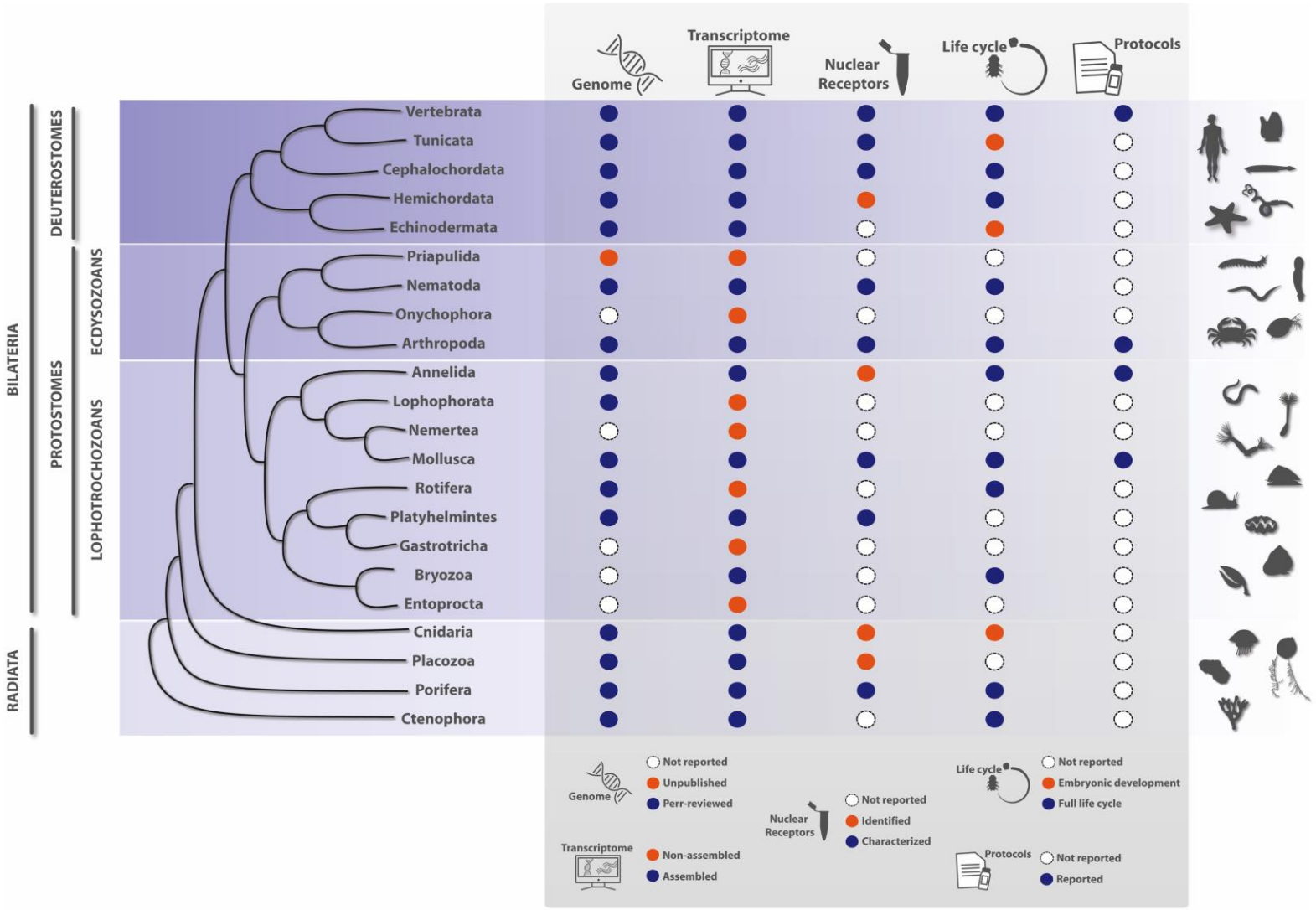
Genome
Transcriptome
Nuclear Receptors
Life cycle
Protocols
BILATERIA
DEUTEROSTOMES
PROTOSTOMES
ECDYSOZOANS
LOPHOTROCHOZOANS
RADIATA
Vertebrata
Tunicata
Cephalochordata
Hemichordata
Echinodermata
Priapulida
Nematoda
Onychophora
Arthropoda
Annelida
Lophophorata
Nemertea
Mollusca
Rotifera
Platyhelmintes
Gastrotricha
Bryozoa
Entoprocta
Cnidaria
Placozoa
Porifera
Ctenophora
Not reported
Unpublished
Perr-reviewed
Non-assembled
Assembled
Not reported
Identified
Characterized
Not reported
Embryonic development
Full life cycle
Not reported
Reported

Figure 1. Summary of resources publically available for implementing a mechanistic-based toxicity testing and assessment of EDCs and EPs across Metazoans

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. Since NRs are present in all major metazoan clades (Bridgham *et al*., 2010), we next carried out a detail analyze to identify the presence of functionally characterized NRs (Figure 1, Annex 1). In contrast to the background genomic data, 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.

Following in *vitro assays*, those chemicals suspected to modulate NRs or NRs-independent pathways should go through more detail testing involving *in vivo* assay. Hence, we also revise and compile here the presence of laboratory-established life cycles with representatives of all metazoans, and the availability of OECD/EPA toxicity testing protocols with these organisms (Figure 1, Annex 1). Our search shows that for 11 major taxonomic groups, life cycles are available for at least one representative species. This allows to link the effects of environmental chemicals at molecular and biochemical level with adverse effect endpoints, improving risk assessment. Although challenging, an effort should be done to establish life-cycle tests with additional *taxa*. When analyzing OECD/EPA toxicity testing protocols the scenario is somehow different; we could only hint toxicity testing protocols for Vertebrates, Arthropods, Molluscs and Annelids. This is a major drawback since it represents only a small fraction of Metazoan diversity. Hence, given the availability of many more *taxa* with laboratory-established life cycles, we believe that it should be a priority to use this background information to validate new partial and full life-cycle toxicity protocols that can integrate tier 4 and 5 of the OECD Conceptual Framework for Testing and Assessment of Endocrine Disruptors.

4. Conclusion

Overall, the systematization of information provided here shows that a large body of resources is already publically available for implementing a mechanistic-based toxicity testing and assessment of EDCs and EPs across Metazoans. This includes genomes/transcriptomes, functionally characterized NRs, and laboratory-established life cycles of representative *taxa*. Priority should be given to the functional characterization of NRs from additional metazoans and to the validation of new partial/life-cycle toxicity test protocols with non-vertebrate.

Acknowledgments

This work was supported by Norte2020 and FEDER (Coral—Sustainable Ocean Exploitation—Norte-01-0145-FEDER-000036). Grants SFRH/BD/100262/2014, Elza Fonseca, SFRH/BPD/72519/2010, Raquel Ruivo, SFRH/BD/90664/2012, Ana Capitão, from Foundation for Science and Technology (FCT), Portugal.

References

Ahmed M.B., Zhou J.L., Ngo H.H., Guo W., Thomaidis N.S., Xu J. (2017). Progress in the biological and chemical treatment technologies for emerging contaminant removal from wastewater: A critical review. *Journal of Hazardous Materials*, 323, 274-298.

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

Castro L.F.C., Santos M.M. (2014), To bind or not to bind: The taxonomic scope of nuclear receptor mediated endocrine disruption in invertebrate phyla. *Environmental Science and Technology*, **48**, 5361–5363.

Castro L.F.C., Lima D., Machado A., Melo C., Hiromori Y., Nishikawa J., Nakan- ishi T., Reis-Henriques M.A., Santos M.M., (2007), Imposex induction is mediated through the Retinoid X Receptor signalling pathway in the neogastropod *Nucella lapillus*. *Aquatic Toxicology*, **85**, 57–66.

Celander M.C., Goldstone J.V., Denslow N.D., Iguchi T., Kille P., Meyerhoff R.D., Smith B.A., Hutchinson T.H., Wheeler J.R. (2011), Species extrapolation for the 21st century. *Environmental Toxicology and Chemistry*, **30**, 52–63.

Chamorro-Garcia R., Sahu M., Abbey R.J., Laude J., Pham N., Blumberg B. (2013), Transgenerational inheritance of increased fat depot size, stem cell reprogramming, and hepatic steatosis elicited by prenatal expo- sure to the obesogen tributyltin in mice. *Environmental Health Perspectives*, **121**, 359–66.

Cruzeiro C., Lopes-Marques M., Ruivo R., Rodrigues-Oliveira N., Santos M.M., Rocha M.J., Rocha E., Castro L.F.C. (2016), A mollusk VDR/PXR/CAR-like (NR1J) nuclear receptor provides insight into ancient detoxification mechanisms. *Aquatic Toxicology*,**174**, 61-69.

Ferreira F., Santos M.M., Castro L.F., Reis-Henriques M.A., Lima D., Vieira M.N., Monteiro N.M. (2009), Vitellogenin gene expression in the intertidal blenny Lipophrys pholis: a new sentinel species for estrogenic chemical pollution monitoring in the European Atlantic coast?. *Comparative Biochemistry and Physiology C Toxicololgy and Pharmacology*,**149**, 58-64.

Fonseca E., Ruivo R., Lopes-Marques M., Zhang H., Santos M.M., Venkatesh B, Castro L.F.C. (2017), LXRα and LXRβ nuclear receptors evolved in the common ancestor of gnathostomes. *Genome Biology and Evolution*, pii: evw305.

Lagarde F., Beausoleil C., Belcher S.M., Belzunces L.P., Emond C., Guerbet M., Rousselle C. (2015), Non-monotonic dose-response relationships and endocrine disruptors: a qualitative method of assessment. *Environmental Health*, **11**,14-13.

Liney K.E., Hagger J.A., Tyler C.R., Depledge M.H., Galloway T.S., Jobling S. (2007), Health effects in fish of long-term exposure to effluents from wastewater treatment works. *Environmental Health Perspectives*,**114**, 81-89.

Lyssimachou A., Santos J.G., André A., Soares J., Lima D., Guimarães L., Almeida C.M., Teixeira C., Castro L.F., Santos M.M. (2015), The Mammalian "Obesogen" Tributyltin Targets Hepatic Triglyceride Accumulation and the Transcriptional Regulation of Lipid Metabolism in the Liver and Brain of Zebrafish. *PLoS One*, **10(12)**:e0143911.

Noguera-Oviedo K, Aga D.S. (2016), Lessons learned from more than two decades of research on emerging contaminants in the environment. *Journal of Hazardous Materials*, **316**, 242-251.

Nishikawa J.-I., Mamiya S., Kanayama T., Nishikawa T., Shiraishi F., Horiguchi T. (2004), Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. *Environmental Science and Technology*, **38**, 6271–6276.

Santos M.M., Ruivo R., Lopes-Marques M., Torres T., Carmen B., Castro L.F.C. and Neuparth T. (2016), Statins: An undesirable class of aquatic contaminants?. *Aquatic Toxicology*, **174**, 1-9.

Sumpter J. P., Johnson A. C. (2005), Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. *Environmental Science and Technology*, **39**, 4321−4332.

Vogeler S., Galloway T.S., Lyons B.P., Bean T.P. (2014), The nuclear receptor gene family in the Pacific oyster, Crassostrea gigas, contains a novel subfamily group. *BMC Genomics*,**15**, 369.