Invertebrates facing environmental contamination by endocrine disruptors: Novel evidences and recent insights

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Abstract

The crisis of biodiversity we currently experience raises the question of the impact of anthropogenic chemicals on wild life health. Endocrine disruptors are notably incriminated because of their possible effects on development and reproduction, including at very low doses. As commonly recorded in the field, the burden they impose on wild species also concerns invertebrates, with possible specificities linked with the specific physiology of these animals. A better understanding of chemically-mediated endocrine disruption in these species has clearly gained from knowledge accumulated on vertebrate models. But the molecular pathways specific to invertebrates also need to be reckoned, which implies dedicated research efforts to decipher their basic functioning in order to be able to assess its possible disruption. The recent rising of omics technologies opens the way to an intensification of these efforts on both aspects, even in species almost uninvestigated so far.

Keywords: endocrine disruption; endocrine disrupting chemicals (EDCs); nuclear receptors; oestrogeno-mimetics; ecdysteroids ; Juvenile hormone; omics; mollusks; annelids; arthropods

1. Introduction

Anthropogenic pollutants are seen as one of the main causes concurring to the ongoing collapse of global biodiversity. Among them endocrine disrupting chemicals (EDCs) pervade virtually all ecosystems on Earth. They are largely reported in marine as well as freshwater environments, notably since actual treatment systems fail to completely remove them from wastewater (*e.g.* Aris et al., 2014; Huang et al., 2019; Salgueiro-González et al., 2015). They also complex to sediments where they can accumulate and persist, sometimes leading to permanent release in the milieu following soil erosion. At last, many EDCs (notably phthalates, polychlorinated biphenyl (PCBs), polycyclic aromatic hydrocarbons (PAHs), brominated flame retardants, dioxins, alkylphenols, perfluorinated chemicals (PFCs) and also some pesticides) have been shown to be present in the atmosphere at worrying concentrations (Annamalai and Namasivayam, 2015), and maybe conveyed by the microplastic particles recently detected in the atmospheric compartment (Dris et al., 2016). Although they belong to various classes of synthetic and natural compounds, the common feature of EDCs lays in that they can interfere at different levels of the endocrine system of animals, disrupting physiological, biochemical and /or molecular processes that control development, growth or reproduction; recent works add neurological and immune processes to the list (Jones et al., 2017). Toxicological studies have uncovered multiple effects of EDCs, many of which are hard to predict due to non-monotonic dose-response, frequent cocktail effects and transgenerational implications (Flint et al., 2012; Skinner, 2014; Vandenberg et al., 2012; Xin et al., 2015; Z. Xu et al., 2017). Beyond actual concerns as regards human health, the large spread of these molecules, through all ecosystems, from

Arctic ices to primitive rainforests, also raises concern about the effects of such contamination on animal health and, ultimately, on worldwide biodiversity (Lenoir et al., 2016; Lyons, 2006; Sonne, 2010). In this respect, studying endocrine disruption in invertebrate species is meaningful. First, from an anthropocentric point of view, invertebrates are tremendous tools to detect and quantify the presence of EDCs in natural substrates, and thus may serve as useful bioindicators. They also allow us to gain knowledge about EDC toxicological properties at the scale of the individual's lifetime, which is much more difficult with vertebrate models (Hutchinson, 2007). Besides, due to their small size which make them easy to handle and stock in the laboratory, and to the large quantity of material they provide when rearing is possible, they represent a convenient and quick option to set up multigenerational assays to investigate potential transgenerational effects of EDCs. Second and most importantly, from an ecological point of view, invertebrates represent a huge part of worldwide biodiversity and fulfill many crucial ecological roles in every ecosystem (Wilson, 2006); this implies that their present decline is a clear worrying issue that requires to be handled urgently.

Because of their specific physiology (that diverged from that of vertebrates more than 600 MA ago) it appears necessary to develop and use invertebrate models allowing to detect and predict the whole set of effects of environmental EDCs on invertebrates wild populations. Contamination routes are likely multiple, with proportions depending on species' life history, and often different from that of vertebrates. In particular, because of their small size and thus relatively high surface/volume ratio compared to vertebrates, invertebrates suffer from higher relative exposure levels, which can imply a higher bioaccumulation, or the need to develop greater excretion capacities. In the aquatic environment, absorption coefficients may be higher compared to vertebrate species due to the properties of invertebrates body wall, which is often a functional surface of exchange, for respiratory gases for instance. As regards terrestrial invertebrates, direct contact and oral route are so far considered as the most probable ways of contamination. However, the possible role of arthropods cuticle as a trap for atmospheric pollutants just begins to be studied. It could constitute an effective way of contamination disregarded so far (Lenoir et al., 2014, 2012). Noteworthy, invertebrates generally lack the blood-gonad barrier that provide some protection to reproductive organs in mammals. At last, as for fishes and amphibians, mature gametes are in most cases directly released in the environment, which implies an exposure of organisms all along the life cycle – from gametogenesis to adulthood – including the highly vulnerable first stages of development. In short, apprehending the consequences of invertebrates exposure to EDC implies to take into account many of their peculiarities, and specific researches are required in this field, which is reflected by the growing body of literature dedicated to the subject.

In this review, we will first try to gauge the burden environmental EDCs place on invertebrate populations in nature, focusing as much as possible on studies that evaluate disruption in wild caught animals, exposed to EDCs in real situations. Then, we will produce an overview of the most recent molecular insights that may enlighten our comprehension of endocrine disruption in invertebrates. Finally, we will discuss the contribution of the recent approaches that rely on omics, to get a wider view of EDCs impact on invertebrate physiology. Note that the vast majority of our current knowledge relates to protostome species (mainly arthropods, mollusks and annelids), which explains that the present review will mainly focus on this taxonomic group.

2. Delineating the burden of endocrine disruption on wild invertebrates

Risk assessment procedures have been using invertebrate models (mainly aquatic species) for long, both to detect potential EDC activities in mixtures, and to gain a basic understanding of the ecological effects of known EDCs. Conversely, field studies demonstrating EDCs effects on invertebrate wild populations and communities are scarcer, in aquatic as well as in terrestrial ecosystems (Amiard and Amiard-Triquet, 2015).

2.1 In aquatic ecosystems

In aquatic ecosystems, the most relevant case certainly remains the imposex phenomenon that affects gastropod mollusks living in areas contaminated by organotins: tributyltin [TBT] and triphenyltin [TPT], at levels as low as few ng per liter (DeFur, 1999). These organometallic compounds have been used as antifouling agents

for several decades until their progressive ban by most countries, in the 80s. However, incomplete observation of worldwide legislation, as well as organotins desorption from contaminated sediments explain that organotins contamination is still a major toxicological concern (Laranjeiro et al., 2018). At the individual level, endocrine disruptive effects in contaminated areas result in the imposition of male sex characteristics on female snails, with a clear relationship between the extent of the masculinization process and the dose of organotins detected in the environment (Graceli et al., 2013). At some point, affected females suffer from impaired reproduction ending up to sterility and even to death (DeFur, 1999). Individual defects due to environmental contamination thus translate into population-level concerns, with a demographic fall and acute risk of local extinction in the worst polluted areas (Bryan et al., 1986; Langston et al., 2015; Roach and Wilson, 2009). In the field, TBT was also shown to affect other invertebrates, disturbing shell calcification and reproduction in bivalve mollusks, and affecting crustacean growth, carbohydrate and lipid metabolism and sexual maturation (Graceli et al., 2013; Vogt et al., 2018). More recently, concerns were raised over the toxicity of TBT in terrestrial ecosystems that can be contaminated notably through soil enrichment with marine sediments or sewage sludge. Silva and colleagues (2014) assessed TBT toxicity in terrestrial invertebrates and observed negative effects on the food consumption and assimilation capabilities in isopods (*Porcellionides pruinosus*), and on juvenile production and/or mortality in collembolans (*Folsomia candida*). But additional studies in field conditions are still required, notably in terms of TBT doses, especially in soils continuously enriched with sewage sludge.

In line with the vertebrate (and especially fish) situation, field exposure cases to estrogenic compounds (e.g. xeno-estrogens such as 17α-ethinylestradiol (EE2), bisphenol A (BPA), nonylphenols and octylphenols) provide another illustration of endocrine disruption in wild aquatic invertebrates (Amiard and Amiard-Triquet, 2015; Jin et al., 2012), with effects including sex-ratio modification, delayed sexual maturity and intersexuality. Numerous studies have reported disruptive effects of estrogens in invertebrate models, including at concentrations compatible with doses measured in the field (ng/l) (Aris et al., 2014; Bovier et al., 2018; Flint et al., 2012; Herrero et al., 2015; Leonard et al., 2017; Liu et al., 2012; Morales et al., 2018; Oehlmann et al., 2009, 2007; Oetken et al., 2004; Wright-Walters et al., 2011). Experimental studies have also demonstrated that the reproduction of the *Potamopyrgus antipodarum* snail (a freshwater mollusk quite abundant in Europe) is impaired by the exposure to a mixture of environmental estrogens. This response was found to be comparable to that of four species of freshwater fishes, with a similar sensitivity to these molecules (Jobling et al., 2004), suggesting that EDC concerns extend to wild invertebrate populations exposed to estrogenic effluents, especially as regards sessile species. In good agreement with this study, when caged downstream of an effluent discharge, the same snail species was found to bioaccumulate more alkylphenols, BPA, Estradiol and Testosterone than when animals were located upstream. These experiments also demonstrated a sharp decrease of reproductive parameters in these animals (reduction in the number of embryos produced) after 6 weeks of exposure (Gust et al., 2014). Concomitant measures of egg proteins and mRNA levels allowed authors to demonstrate that reproductive disruption did not affect oocyte development, but rather targeted early embryonic development, suggesting the disruption of estrogen signaling pathways (Gust et al., 2014). Recent *in situ* surveys on the marine mussel *Mytilus trossulus* also evidenced some reproductive impairment in bivalve wild populations exposed to presumed estrogenic water wastes. Individuals living in the vicinity of a sewage purification plant outlet showed an increased frequency of gonadal regression and atresia and malformations, which were successfully mimicked by an experimental exposure to 50 or 500 ng/dl of EE2 (Smolarz et al., 2017). Such experimental exposure in natural conditions is a powerful way to evidence local contamination by EDCs, but requires a careful choice of the biomarkers recorded. The level of yolk protein vitellogenin, most often estimated by the Alkali Labile Phosphate method, has been commonly used as a biomarker of feminization in several aquatic species. Both the method (Sánchez-Marín et al., 2017) and its results (Boulangé-Lecomte et al., 2017; Short et al., 2014) have recently been questioned by proteomic and transcriptomic approaches, respectively.

In freshwater ecosystems, chironomids are increasingly imposing themselves as a convenient and suited model of study. Studies carried out in *Chironomus riparius* recently contributed to reveal the endocrine disruptive effects of complex mixtures of heavy metals in experimental settings in which larvae were reared in sediments (more or less contaminated) originating from abandoned mines, polluted shores or reference sites. Among other negative effects recorded as regards biomass and respiration rate, exposed individuals showed disrupted

expression of ecdysone-related genes, impaired reproduction and growth, both at the larval and adult stages (Arambourou et al., 2020, 2019).

2.2 In terrestrial ecosystems

In terrestrial ecosystems, the main cases for which endocrine disruption effects are well identified under field conditions relate to insect growth disruptors (IGD, formerly named insect growth regulators or IGR). Such substances have been designed by purpose to affect insect growth and development through a specific disruption of their endocrine regulatory pathways, including juvenile hormone analogues (JHA), and steroidal ecdysone agonists (Pener and Dhadialla, 2012). For these molecules, the drop of target insect populations is the hallmark of their efficacy. Unfortunately, although the design of these molecules was intent to produce more selective pesticides (they are often sold as "reduced-risk insecticides"), non-target species sharing similar hormone systems can also be affected. Pener and Dhadialla (2012) reviewed several studies demonstrating lethal or sublethal effects of IGD on non-target species, especially predators or parasitoids of the target species, proving the diffusion of IGD in food webs. Lawler (2017) recently reviewed the environmental effects of methoprene use for mosquito control and pinpointed some negative issues at realistic environmental levels, mainly on aquatic invertebrate species: lobster larvae, mysid shrimp embryos, small diptera and zooplankton-sized crustacean. Although often disregarded, these species, and especially planktonic species which occupy key positions in aquatic food webs, for sure deserve further attention on the subject of endocrine disruption in field context. Regarding IGD's use for agronomic purposes, the potential threatening of pollinators and other beneficial insects' survival is a big subject of concern, due to the huge ecosystemic services they fulfill. This concerns domestic honeybees but also Lepidoptera, parasitic wasps, Coleoptera and numerous wild bees that play crucial roles in plant-insect mutualistic networks (Quesada and Sadof, 2019; Steffan-Dewenter et al., 2005). Beyond direct mortality provoked by many pesticides, some IGD are responsible of sublethal effects that may contribute to the actual decline of (wild and managed) pollinators and beneficial insects populations (Johnson et al., 2010; Mommaerts and Smagghe, 2011). Bees illustrate how complex and multiple the routes of exposure can be: in addition to direct contact associated with spraying as well as the interaction with treated plants, foragers collect nectar and pollen susceptible to be contaminated. They also bring it back to the nest to feed all the members of the colony (social bees) or their larvae (solitary bees). For brood as well as for hive bees, contamination thus probably mainly arise through food intake, which may strongly lead to specific toxic effects (Sanchez-Bayo and Goka, 2014; Stanley and Preetha, 2016). Recent studies evidenced a clear contamination of the pollen stored into beehives, by many pesticides and herbicides at sublethal concentrations, among which ecdysone analogues (tebufenozide, methoxyfenozide) and JHA (fenoxycarb, pyriproxyfen, methoprene) (Böhme et al., 2018; Calatayud-Vernich et al., 2018; Hakme et al., 2017). Experimental work had previously demonstrated that exposure of micro-colonies of *Bombus terrestris* to JHA (pyriproxifen or kinoprene), through pollen contamination (e.g. not through sugar water contamination) at the maximum field recommended concentration, has no acute toxicity on workers but enhances larval mortality. This effect was hypothesized to be linked with a lethal blocking of development before metamorphosis (Mommaerts et al., 2006). In the same study, authors evidenced a stimulatory effect of a very low dose of kinetoprene (0.0650 mg/l) on the development of the female reproductive system, together with a higher reproductive output, strongly arguing in favor of an endocrine nature of the disruption. Even though neurotoxic pesticides – and in the first place all neonicotinoids – are the most incriminated molecules in the complex interactions that lead to bee CCD (colony collapse disorder; Sanchez-Bayo and Goka, 2014), IGD chronic exposure should not be disregarded, as regards its potential sublethal effects and possible involvement in synergistic effects. Furthermore, neonicotinoids themselves were recently shown to exert EDC-like effects in bees, generating sublethal effects suspected to largely contribute to population extinctions over a long time scale (Woodcock et al., 2016; Baines et al., 2017). More field studies are now needed to assess and cross-reference several key parameters of pollinators' potential poisoning by pesticides. These include (1) the dynamics of pollen contamination in relation with its species of origin and the nature of the contaminants; (2) the persistency of chemicals under specific conditions inside real hives and along seasons; (3) the link between external exposure (direct contact and/or chronic ingestion) and potential risk; (4) the possible interaction between contaminants, which needs to be addressed by testing

realistic pesticide mixture (see Böhme et al., 2017); (5) the direct effects on bee survival and activity but also sublethal effects (fertility, abnormal larval development) that may affect colony productivity; (6) the side-effects that may arise through the disturbance of other functions such as olfactive performance (see e.g. Chakrabarti et al., 2015), orientation and learning capacities, which are essential to foraging process and feeding, flight activity and foraging efficiency (see Prado et al., 2019), pheromonal communication (notably blurring of nestmate identification) and social interactions (see e.g. Fourrier et al., 2015); and (7) the indirect adverse effects that may ensue from microbiota modifications.

More generally, all herbivorous invertebrates may come across EDCs through the contamination of their food: some well identified or emerging organic contaminants including BPA, nonylphenol or triclosan are largely distributed in the water cycle and end up in reclaimed water eventually used for food crop irrigation. This can lead to their accumulation in leafy vegetables (Dodgen et al., 2013) and to a direct exposure of herbivorous species. In the insect crop pest *Spodoptera littoralis*, Maria and colleagues (2019) evidenced a higher mortality at pupal stage and an increase in some larval instar duration associated with modifications of ecdysteroid titers and nuclear receptor expression, after consumption of food contaminated with BPA concentrations similar to that found in plants. At last, atmospheric contamination also potentially concerns all epigeic species. Although this route of exposure has barely been considered so far, we recently evidenced a chronic contamination of several species of aerial insects (mainly ants but also crickets and honeybees) by phthalates, which are potent EDCs (Oehlmann et al., 2009; e.g. Mankidy et al., 2013). These phthalates impregnate the cuticle and mix with the cuticular components (Lenoir et al., 2012). In these studies, all tested individuals were found to be contaminated, with varying contamination levels depending on the species. Phthalates accounted for 0.11 to 2.66% of the cuticular compounds in the different ant species tested, 0.73% in honey bees and 2.76% in the wood cricket *Nemobius sylvestris*. This contamination was clearly established to originate from the atmosphere. It seems that, due to its biochemical properties, insects cuticle can traps the phthalates present in both the vapor phase and the particulate phase (adsorbed to atmospheric particles; Lenoir et al., 2014; Teil et al., 2006). The insect cuticle keeps trace of this contamination in the form of a basal level of phthalates mixed to cuticular compounds, which can be measured by GC-MS. In addition, phthalates were also clearly demonstrated to be able to cross the epidermal barrier in these insects, as they were detected inside the body of animals, mainly in fat. Experimental contaminations by environmental doses spiked onto the cuticle induced a reduction of queens' eggs output and disturbed immune gene expression in workers of the black garden ant (*Lasius niger*), suggesting possible endocrine disruptive effects in natural populations (Cuvillier-Hot et al., 2014). Considering that these observations may potentially apply to all invertebrate species possessing a cuticle or a lipophilic integument, it appears necessary to pursue investigations on other species, in order to examine whether it is necessary to include this mode of contamination in risk assessment protocols for terrestrial invertebrates.

3. New insights in invertebrate molecular disruption

As an emerging science, EDC ecotoxicology first based on a vertebrate-centered approach, taking advantage of our better knowledge of vertebrates physiology, to detect and identify disturbances linked with environmental exposure to contaminants. Then, knowledge acquired from vertebrate models was transposed to invertebrates and researches have focused on susceptible endocrine pathways shared between invertebrates and vertebrates, and in a first place on the possible disruption of the estrogen receptor (ER)-associated cascades. But, as more than 600 Million years have elapsed since the split between protostomes and deuterostomes, the former acquired endocrine systems of regulation unique to them (e.g. ecdysteroids, Juvenile hormones), which are potential targets for environmental endocrine disruption and which call for dedicated researches. Even nowadays, while substantive knowledge has been accumulated for some groups (e.g. insects), a main gap of our current knowledge over endocrine disruption in invertebrates remains our lack of detailed comprehension of endocrine signaling pathways in many disregarded groups of invertebrates (Castro and Santos, 2014; Hutchinson, 2007). This concerns receptors and ligands, associated cascades and regulation, but also the dynamics of bioaccumulation and elimination of potential disruptors. This basic knowledge is necessary to ascertain that an adverse effect observed in the field is indeed the result of an endocrine disruption mechanism

rather than a more general direct or indirect toxic effect (Barata et al., 2004; Hutchinson, 2007; Lagadic et al., 2007; Weltje and Schulte-Oehlmann, 2007). In the past years, some attempts have been made to develop arthropods-specific *in vitro* assays dedicated to screen environmental contaminants for ecdysteroid agonist/antagonist activities (Dinan et al., 2001; Kontogiannatos et al., 2015; Soin et al., 2009; Swevers et al., 2004; Yokota et al., 2011; Zotti et al., 2013). However, it appears that ecdysteroid receptors have ligand-binding properties that can substantially vary between invertebrate species, even among members of a same order (Graham et al., 2007). This suggests that the results of such molecular assays can hardly be generalized among species on a large scale and involve the need to develop taxa-specific tests (Santos et al., 2018; Yokota et al., 2011).

EDCs can interfere with the endocrine pathways of animals in many ways. They can affect endogenous hormonal levels, or those of hormone receptors by disturbing their specific synthesis and/or catabolism; they can also disturb hormonal receptor functions by spatially mimicking endogenous hormones, thus affecting receptors availability or leading to unexpected signalization (agonist or antagonist). For decades, physiological and developmental endpoints have been used to detect and characterize EDCs in invertebrates, all the more that several model species, easy to breed and possessing a short generation time are available in several taxonomic groups. Many reviews have reported about lists of suspected EDCs and proposed modes of action based on the examined studies (e.g. Hutchinson, 2002; Lagadic et al., 2007; LeBlanc, 2007; Oehlmann et al., 2007; Oetken et al., 2004; Rodríguez et al., 2007; Soin and Smagghe, 2007; Zou, 2005). More recently, molecular approaches allowed a deeper investigation of the mode of action of these EDCs in invertebrates, which we will focus on in the next chapter. Figure 1 sums-up the main molecular regulatory pathways hypothesized or demonstrated to be affected by chemicals in protostomes.

3.1 Molecular pathways susceptible to endocrine disruption and shared with vertebrates

3.1.1. Estrogen receptors and relatives

In vertebrates, many EDCs exert their action as agonists of estrogen receptors (ER) or/and antagonists of androgen receptors (AR). The invertebrate orthologs of these receptors are thus potential targets for environmental endocrine disruption. To date, the only steroid receptor evidenced in protostomes belongs to the ER family and shares a common ancestor with vertebrate steroid receptors (SR) named AncSR, which would have arose before protostome and deuterostome cleavage (Castro and Santos, 2014; Jones et al., 2017; Markov et al., 2009). Absent from the Ecdysozoa, ERs orthologs have been characterized in mollusks (Hultin et al., 2014; e.g. Keay et al., 2006; Matsumoto et al., 2007; Raingeard et al., 2013; Thornton, 2003; Zhang et al., 2012), annelids (Keay and Thornton, 2009; Lv et al., 2017) and recently in rotifers (Jones et al., 2017). As in vertebrates, these receptors have genomic signaling pathways that activate gene transcription through the direct binding of the nuclear ER complex to ERE (estrogen-response elements) sites (or indirectly through AP-1 or Sp-1 binding sites) upstream of target genes (Hamilton et al., 2017). However, the modalities of ER commitment clearly differ among invertebrate groups. Indeed, the Mollusca receptor was shown to have been vestigialized through some decisive substitutions that froze the ER in an active conformation, resulting in its constitutive activity. Subsequent mutations then filled the ligand pocket with bulky residues precluding any ligand binding and rendering unlikely any reversal to the ancestral function (Bridgham et al., 2014). ERs in mollusks are thus ligand-independent transcriptional activators, which implies that mechanisms of endocrine disruption by estrogenic environmental contaminants in these species are necessarily independent of any nuclear ER activation (Canesi and Fabbri, 2015). On the contrary, annelids and rotifers ERs display the classic properties of vertebrates ER, including apparent sensitivity to estrogens (Castro and Santos, 2014; Jones et al., 2017; Keay and Thornton, 2009). However, the exact nature of their endogenous ligand is still unknown. Steroidogenesis exists in protostomes but has evolved independently from vertebrate cascades, partly through the recruitment of cytochrome P450 enzymes formerly involved in xenobiotic detoxification (Markov et al., 2009). Recent work by Blalock and collaborators (2018) evidenced, for the first time in a protostome (the mussel *Mytilus edulis*), a partial protein equivalent to vertebrate CYP11A enzyme, responsible for the side-chain cleavage of cholesterol to pregnenolone. Hence, within the whole steroid biosynthesis pathway, only the last step of aromatization of testosterone into estradiol would still be missing in lophotrochozoa, the aromatase CYP19A appearing restricted

to chordates (Goldstone et al., 2016; Markov et al., 2009). For this reason and also because unbiased methods to detect steroids in invertebrate tissues so far failed to find estrogens, some authors refute the idea of an estrogen-like endogenous ligand for protostome ERs (Holzer et al., 2017a; Scott, 2013). But whatever their origin – endogenous or exogenous – estrogens clearly affect the physiology of many invertebrates, including mollusks, paving the way to possible endocrine disruption by œstrogeno-mimetics. In vertebrates, ERs can be activated by several estrogenic endogenous ligands (estradiol, Δ^5-androstenediol, 5α-androstanediol, and 27-hydroxycholesterol). They also are responsive to several plant-derived compounds with estrogenic activity such as genistein, coumestrol, and resveratrol (Baker and Lathe, 2018). This promiscuous responsiveness is proposed to explain their response to synthetic chemicals such as BPA and phthalates (Engel et al., 2017; Flint et al., 2012). Similar cross-binding may be considered for ligand-sensitive ER described in protostomes. Keay and Thornton (2009) for example report an agonist effect of genistein and an antagonist effect of BPA on *Platynereis dumerilii* ER.

In vertebrate models, it is well established that ER signaling can be modulated by estrogen-related receptors (ERRs). These are also nuclear receptors sharing sequence similarity with ERs, but that respond very weakly to endogenous estrogens while being stimulated by some xenoestrogens. They may thus interfere with ER signaling upon environmental EDC exposure, along several modalities: (1) by directly interacting with ERs, (2) by competing with ERs for ERE site binding or (3) by targeting similar genes as ERs via the presence of ERR-response elements in their promoter region, alongside with ERE (Xu et al., 2017). As a consequence, influence of ERR activation on ER signaling will clearly depend on the level of expression and tissue distribution of both receptors. Sequences homologous to ERRs have been described in annelids (the marine worm *Capitella* and the leech *Helobdella*, Baker, 2008) and in mollusks (the snail Lottia, Baker, 2008; the snail Physa, Morales et al., 2018; two Mytilus species, Nagasawa et al., 2015). Quite expectedly, mRNA of ER homologues were abundantly detected in reproductive tissues (ovary but also pedal ganglion) in the mussels *Mytilus edulis* and *M. galloprovincialis*, while mRNA of ERR homologues, otherwise involved in metabolism regulation and mitochondrial function (Hubbard et al., 2015), had high levels of expression in the gill and digestive gland ; however, both receptors had basal levels of expression in all tissues examined and may thus interfere with each other (Nagasawa et al., 2015). In *M. edulis*, ER expression in ovary appeared regulated by *in vitro* exposure to 17β-estradiol, while that of ERR did not (Nagasawa et al., 2015). In the snail *Marisa cornuarietis*, various vertebrate-like estrogens were tested for their possible ER and ERR gene transcription modulation, but gave negative results for both genes. Only genistein and BPA up-regulated gene expression of ER and ERR orthologs respectively (Bannister et al., 2013). In this species, BPA exposure at environmentally relevant concentrations had clear disruptive effects, leading to a superfeminization phenomenon characterized by enlargement and malformations of female organs, and higher egg mass production (Oehlmann et al., 2006). Also in the snail *Physa acuta*, BPA exposure led to a significant increase in the mRNA levels of both ER and ERR, suggesting that these receptors could be involved in molecular events that regulate the endocrine disruptor activity of this chemical in Gastropods (Morales et al., 2018). Interestingly, ERR gene homologues have also been characterized in insects and their expression appeared modulated by EE2 (Bovier et al., 2018) and ethylparaben (Liu et al., 2014) in *Drosophila melanogaster*, and by Bisphenol S (BPS), Triclosan, BPA, nonylphenol or diethylhexyl phthalate (DEHP) in *Chironomus riparius* larvae (Herrero et al., 2018, 2015; Martínez-Paz et al., 2017; Park and Kwak, 2010). In these cases, disruption is proposed regarding the 20-Hydroxyecdysone pathway (see § 3.2), highlighting the multi-target character of many EDCs.

Alongside and apart from direct interactions of estrogeno-mimetics with nuclear estrogen receptors, many studies in vertebrates pointed out more rapid effects of these molecules (within seconds to minutes) through non-genomic signaling pathways (Xu et al., 2017). They may be initiated by estrogen-responsive non-nuclear receptors and may lead to local effects such as modification of ion fluxes or to activation of cytosolic kinase cascades that ultimately affect gene transcription. Several studies reviewed by Janer & Porte (2007) suggested the involvement of such non-genomic processes in endocrine regulation also in invertebrates. Recent contributions from Omics studies brought further support: individuals of the mudsnail *Potamopyrgus antipodarum* exposed to an effluent discharge, while accumulating alkylphenols, BPA and vertebrate-like sex-steroid hormones, showed an inhibition of the expression of genes involved in non-genomic signaling pathways,

together with an induction of the repressors of the genomic pathway; this led to a drastic decrease in embryo production (Gust et al., 2014). Similarly, a transcriptomic approach of mussels exposed to EE2 or 4-nonylphenol (leading notably to female-skewed sex-ratios) revealed similar patterns of gene dysregulation with main targets belonging to the non-genomic estrogen signaling pathway (Blalock et al., 2018).

3.1.2. Other nuclear receptors

At the molecular level as at the individual and population scales, the case of endocrine disruption by organotins was much investigated and led to many relevant trails. In the imposex phenomenon, endocrine disruption is highly suspected at different levels. High levels of testosterone observed in TBT treated prosobranch females could ensue from an inhibition of enzymes involved in steroid metabolism (reviewed in Graceli et al., 2013, Lafont and Mathieu, 2007). TBT may also induce adverse neuromodulation through the production of APGWamide which was shown to induce imposex in mud snails (Ilyanassa obsoleta, Oberdörster and McClellan-Green, 2002). Finally Pascoal and colleagues (2013) strengthened the hypothesis of a disruption of the retinoid X receptor (RXR) – peroxisome proliferator-activated receptor-γ (PPARγ) pathway by evidencing a strong transcriptional response of the retinoid receptors and of putative members of this signaling pathway after TBT treatment in *Nucella lapillus*. They also showed that activating this pathway with a vertebrate ligand of PPARγ led to imposex in this species. As additional support to this third presumed mechanism, it had been previously established that TBT was able to bind and activate RXR–PPAR vertebrate heterodimers, by mimicking 9*cis*-retinoic acid interaction with RXR (le Maire et al., 2009). All these clues suggest a strong involvement of this signaling pathway, quite shared between vertebrates and invertebrates, in imposex physiopathology (André et al., 2014; Iguchi and Katsu, 2008).

RXRs are nuclear receptors involved in numerous signaling pathways and biological functions since they can signal as homodimers, but also form heterodimers with many partners, such as PPAR (as exemplified above), retinoic acid receptor (RAR) or thyroid hormone receptor (TR). In Ecdysozoans, RXRs homologues (known as ultraspiracle (USP) in insects) are the obligate heterodimeric partners of ecdysteroid receptors (EcR, see §3.2). RXRs are widely distributed among metazoans and show a great degree of homology between taxa. Furthermore, their sensitivity to 9-cis-retinoic acid (RA) appears as an ancestral character already present in the common ancestor of Cnidaria and Bilateria, widely shared among metazoans and secondarily lost in ecdysozoans (André et al., 2017). Their functional partner in RA sensing, RARs, have also been detected in some annelids and mollusks. In both cases, the essential of the signaling machinery is present so that functional RA signaling pathways is expected, but according to specific processes, given that the molluscan RAR were proved insensitive to retinoids (Gutierrez-Mazariegos et al., 2014; Handberg-Thorsager et al., 2018). Finally, genome data mining recently identified key players possibly involved in retinoid metabolism and storage process in some protostomes, providing new potential targets for disruption. All this lead André et al. (2017, 2014) to suggest that RXR-dependent modulation by organotins might be evolutionary conserved among lophotrochozoans and vertebrates, and foresee that many more metazoan species might be potential targets for EDCs through disruption of the retinoid system. The identification of the physiologic effects of such disruptions clearly deserves future research in all invertebrate groups.

Over the last years, the endocrine disruption of vertebrate biological processes controlled by thyroid hormone receptors (TRs) has been of growing concern, mainly because we progressively uncover the importance of this endocrine system on embryogenesis and early neurogenesis. Several EDCs including PCBs, the pesticide chlorpyrifos, BPA, poly-fluorinated surfactants and polybrominated diethyl ethers (PBDEs), are suspected to disrupt TH regulated pathways (Préau et al., 2015). TRs are also nuclear receptors functioning as ligand-dependent transcription factors; they mediate thyroid hormone (TH) effects by genomic and non-genomic mechanisms. If the genome of *Drosophila melanogaster* and *Caenorhabditis elegans* appeared deprived of TR-like sequences, lophotrochozoans on the contrary clearly possess TR orthologs (Orozco et al., 2017; Sainath et al., 2019). Note that putative TR orthologs have been proposed from *Daphnia pulex* and *D. magna* genomes, but their high sequence divergence poses question about their real origin (Sainath et al., 2019). Activating ligands are clearly different for protostome and deuterostome TRs, in agreement with the poor degree of conservation of their respective ligand-binding domains; and so far, endogenous ligands of protostome TRs remain unknown.

Still, endogenous synthesis of TH by the sea hare *Aplysia californica* is supported by its enzymatic equipment (Heyland et al., 2006), T4 and T3 have been extracted from *Crassostrea gigas* hemolymph (Huang et al., 2015), and TH were shown to induce settlement and metamorphosis in several mollusk species suggesting that TH signaling is possible in Lophotrochozoa (Holzer et al., 2017b; Taylor and Heyland, 2017). Although up to now no clear link has been established between TH and activation of TR in this group, the question remains open about the possible effect of environmental thyroid disruptors on invertebrates development and physiology, and urgently requires fundamental scientific work to understand the role of the thyroid endocrine system in protostomes.

3.2 Molecular pathways susceptible to endocrine disruption and specific to Protostomes

Protostomes have developed specific endocrine regulatory pathways that can also be affected by environmental EDCs. In arthropods, there are three main classes of hormones: peptide hormones, ecdysteroids (mainly 20-hydroxyecdysone (20E) in insects and crustaceans, together with ponasterone A in chelicerate species), and terpenoids (mainly juvenile hormones (JH) in insects, methyl farnesoate (MF) in other arthropods). All three systems interact to regulate notably growth, development and reproduction. The hormonal action of 20E exerts via the activation of the ecdysone receptor (EcR), again a member of the superfamily of nuclear hormone receptors, that regulates gene transcription through heterodimerization with RXR (called USP in insects) and genomic binding to EcRE (ecdysone responsive element). It is now clear that the developmental events that lead to prepupal and pupal molt depend on a tightly timed dynamic of hemolymphatic 20E titer. A sharp rise followed by a clear decrease in the circulating levels of 20E are both important to regulate the expression of the different waves of transcription factors that coordinate the different molting processes, trigger apolysis, timely induce the expression of chitin synthase genes, regulate the production of the molting fluid needed to degrade the old cuticle and provoke its shedding (Guittard et al., 2011; Song et al., 2017). Numerous EDCs, beginning with IGDs that have been purposely developed to disrupt molting in insect pests (see §2.2), are able to negatively affect synthesis or secretion of 20E, or to interfere with EcR (Soin and Smagghe, 2007). As a consequence, it deregulates the fine-tuned signalization by 20E, causing notably molting failure and death, or impairing gametogenesis or embryogenesis. All the adverse consequences of chemically mediated disruption of molting in arthropods have recently been reviewed and illustrated by Song and colleagues (2017), highlighting the multiplicity of potential EDCs targets in the process that can lead to mortality by ecdysis failure. In many of the investigated cases, EDCs exert 20E agonistic effects, evidenced notably by an up-regulation of EcR or other genes involved in the ecdysone cascade (e.g. four oestrogeno-mimetics in an amphipod, Gismondi, 2018; BPA in the Lepidoptera Sesamia nonagrioides, Kontogiannatos et al., 2015; the insecticide fipronil in a copepod, Gaertner et al., 2012; Triclosan, Martínez-Paz et al., 2017; Nonylphenol, Nair and Choi, 2012; several commonly used UV-filters, Ozáez et al., 2014, 2013; the phthalate BBP, Planelló et al., 2011; BPA, Planelló et al., 2008; and Bisphenol S, a subtitute of BPA, in the Chironome aquatic larvae, Herrero et al., 2018). For most of these chemicals, gene expression changes came along with affected developmental endpoints such as molting malformations, increased pupation time and reduced emergence success in *S. nonagrioides* (Kontogiannatos et al., 2015), emergence failures (Nonylphenol, Lee and Choi, 2006), delay in embryo hatching (Ozáez et al., 2014) and reduced survival (Planelló et al., 2008) in *Chironomus riparius*. Noteworthy, a cell-based reporter assay in *Bombyx mori* Bm5 cell line confirmed the agonistic effect of BPA in Lepidoptera (Kontogiannatos et al., 2015). Less frequently, EDCs act as anti-ecdysteroids (e.g. 5-chloro-1H-benzotriazole, tamoxifen or testosterone in *Daphnia magna*; resp. Giraudo et al., 2017a; Jo et al., 2018; Mu and LeBlanc, 2002). It is clearly the case for the phthalate DEHP, which was shown to induce a significant drop in EcR transcription both at short exposures to high doses or at long exposures to low doses (i.e. environmental concentrations and below) in the Chironome larvae (Herrero et al., 2017; Planelló et al., 2011). Similar antagonist effects have been observed in the lepidopteran *Spodoptera littoralis*, also manifest through longer larval / pupal stages and global delay in adult emergence (Aviles et al., 2019). On the other hand, very few is known over ecdysteroid function and possible disruption outside of the arthropod group. EcR orthologs have been detected in the genome of mollusks, annelids and nematods and *in silico* analyses predict possible binding to an ecdysone-related steroid (Laguerre

and Veenstra, 2010). Interestingly, while the exact role of such molecules in lophotrochozoa physiology is so far unknown, the expression of EcR in the earthworm *Eisenia fetida* have been shown to be down-regulated in male reproductive tissues after exposure to low doses of BPA (Novo et al., 2018), and up-regulated in full body extracts after exposure to sublethal doses of 4-hydroxybenzophenone, a main product of degradation of benzophenone-3 that is currently used as UV-filter in sunscreens (Novo et al., 2019). The endogenous roles of such receptors in mollusks and annelids need clarification.

The sesquiterpenoid hormones, JH and MF, are also key regulators in arthropods, regulating various developmental and reproductive processes such as molting, growth, metamorphosis, and gonad maturation. The elucidation of the signaling pathway of sesquiterpenoid hormones in the last decade evidenced Methoprene-tolerant (Met; germ-cell expressed, Gce, in Drosophila) as their main receptor. The Met/Gce-hormone complexes are translocated to the nucleus, where they bind, together with the co-activator SRC (Tai in Drosophila), the specific JH response elements (JHRE) to activate transcription of the downstream target genes (Qu et al., 2018). Easy screening of putative EDCs with JH-like activity is now possible with the OECD TG211 ANNEX 7 assay that use daphnids. Daphnid species are parthenogenetic species that produce only females in controlled conditions, but may generate male offspring in response to JHs. The validated assay takes advantage of this specificity to specifically detect JH analogs. Recently, Abe and colleagues (2015b) developed a derivative short-term screening assay using adult *Daphnia magna* and with a chemical exposure of only 7 days. This test notably ascertained the JH agonist effect of the IGD Diofenolan. In parallel, a two-hybrid assay evidenced that diofenolan induced heterodimerization of Met and SRC, suggesting its direct binding to the JH receptor; microarray analyses confirmed the dysregulation of many gene markers of JH action, evidencing this chemical as a strong JH agonist (Abe et al., 2015a). The same study evidenced a concomitant disruption of the ecdysteroid signaling pathway, reinforcing the hypothesis of a cross-talk between JH and ecdysteroid signaling pathways in arthropod developmental processes, as previously suggested with other JH agonists such as fenoxycarb, pyriproxyfen or Tris(2-butoxyethyl) phosphate, a flame retardant (Giraudo et al., 2017b; Tuberty, 2005). Sesquiterpenoid hormones had long been thought restricted to arthropods. However, Schenk and colleagues (2016) recently established in *Platynereis dumerilii* that the hormone called nereidin, produced by head ganglia and known to regulate growth and sexual maturation in annelids by inhibiting the switch to reproductive state, corresponds to MF. They also evidenced that eleocytes, the cells that produce vitellogenin then captured by maturing oocytes, express a Met ortholog, whose transcription is up-regulated both by nereidin and by exogenous MF. Interestingly, the JHA methoprene and pyriproxyfen mimicked the effect of nereidin at similar concentration (10nM), reducing vitellogenin expression in cultured eleocytes. Recent insights over EcR and JH presence in annelids raise clear concerns about this group, notably as crucial members of the soil infauna. Their endogenous way of life makes them largely exposed to IGD sprayed on fields and cultures. We need to acquire background knowledge over the role of such putative regulatory pathways in this group so as to assess their possible disruption in natural conditions.

4. The promises of the high-throughput omics era

Over the last decades, omics techniques became within reach of researchers to decipher how expressed genes (transcriptomics), proteins (proteomics) and metabolites (metabolomics) are altered by exposure to environmental pollutants (Colin et al., 2016). This gave rise to a new research field called ecotoxicogenomics that contributes to an ongoing mutation of our comprehension of the effect of endocrine disruption. Indeed, these recent techniques now make possible to compare different taxa on a large scale, to consider the multiplicity of the endogenous targets of individual toxics, and to monitor endocrine disruption in natural conditions much more precisely (Oliveira et al., 2016). Proteomics and metabolomics for instance enable to investigate how entire pathways react to toxic exposure, giving more weight to subtle changes that may have gone unnoticed if only few markers had been considered. Thereby, metabolomics profiling suggested that DEHP has negative effects on energy production and more particularly on TCA cycle, as in vertebrates, in the crop pest *Spodoptera littoralis* (Aviles et al., 2019). Tests on *Daphnia magna* metabolome confirmed that dissolved organic matter specifically affects the bioavailability of some drugs present in waste water (Kovacevic et al., 2019), and revealed convergent metabolic effects of some psychiatric drugs, despite different modes of action (Garreta-

Lara et al., 2018). Lastly, metabolomics approaches highlighted alterations in energy metabolism, amino acids metabolism and glycerophospholipid metabolism in mussels exposed to a synthetic progestin while no changes in gonad maturation and in steroids level were recorded (Cappello et al., 2017). For their part, coupled to NGS technologies, genomics and transcriptomics offer via *de novo* approaches an unprecedented power of description and analyses of gene sequences whose expression is altered after exposure, even in environmentally-relevant species for which physiological and genomic knowledge had been historically poor. It can reveal so far undetected disruption, and identify new biomarkers for the monitoring of given species in specific environmental conditions. For example, the transcriptomic analysis of the response to mercury exposure of two copepod species recently revealed an endocrine-disrupting potential for this metal, evidencing a dysregulation of the estrogen signaling pathway in both species (Wang et al., 2017). Similarly, microarray analyses of oysters exposed to microplastic particles revealed a molecular signature of endocrine disruption, in the form of differential expression of hormone receptors or transcripts involved in hormonal pathways (Sussarellu et al., 2016). Interestingly no EDC could have been detected in the biological samples of this study, illustrating the pre-eminence of bio-assays over chemical quantification when incriminated substances are active at very low doses and possibly below detection limits. Even more remarkable, omics approaches can unveil endocrine disruption even in cases where compensatory mechanisms suppress any noticeable physiological effects. For instance, in *Daphnia magna*, several studies screened the gene regulation changes after exposition to different potent EDC (Giraudo et al., 2015; Houde et al., 2016). Contrary to other benzotriazoles, 1H-benzotriazole (BTR) has no effect on molting frequency but RNAseq analyses revealed the up-regulation of cuticular proteins after BTR exposure, suggesting the latters could have compensated a possible molting disruption (Giraudo et al., 2017a).

We seemingly are at the beginning of a new era of research possibilities, with much more tools to apprehend complex toxic effects and intricate organism responses. This is especially true for invertebrates, so far neglected with respect to the huge biodiversity they represent. Furthermore, constant progresses in high-throughput sequencing techniques, both in terms of rapidity and prices, enhance the possibilities to compare multiple species, populations, life-stages or tissues, expanding the scope of experimental possibilities (e.g. An et al., 2014 for a comparative study of testis and digestive gland response to estrogen exposure in *Chlamys farreri*). Finally, combining genetic, proteic or metabolic changes detected through omics approaches with the description of altered apical endpoints (e.g. body condition indexes, lipid content, fertility measures…) will undoubtedly provide useful insights into the genes, proteins and metabolites possibly implicated in physiological disorders, unveiling unknown molecular mechanisms of the physiological pathways affected (see Ciocan et al., 2012, and Grilo and Rosa, 2017 for examples about intersex in invertebrates). A good example is probably that of the dog whelk (*Nucella lapillus)*, for which transcriptomics coupled to *in vitro* testing confirmed the alteration of some processes already evoked (steroid metabolism, neuroendocrine regulation and retinoid mechanisms) following TBT exposure, but also revealed the involvement in imposex of a regulatory pathway (peroxisome proliferator-activated receptor or PPAR), not reported until then in invertebrates (Pascoal et al., 2013). The next challenge is now to integrate omics in routine environmental monitoring studies notably to help regulatory agencies in their management of environmental risks (Gouveia et al., 2019; Oliveira et al., 2016; Piña et al., 2018).

5. Conclusion

A growing research effort coupled with the use of ever more accurate and efficient methods and approaches now allow us to better identify EDCs and refine our knowledge of their mode of action, even in non-model animal species for which basic knowledge is limited. The disruption of hormonal regulatory pathways is of course the hallmark of these harmful chemicals, but additional toxicological modes of action may also be involved in sub-lethal effects (Flint et al., 2012). In addition to classical endpoints related to reproduction and development, more and more studies pinpoint additional disturbed processes and pathways in invertebrates exposed to EDCs, including immune deficiencies that may hamper the ability of individuals to fight their natural pathogens and parasites (Canesi et al., 2007; Gagné et al., 2008; Lu et al., 2013) and behavioral changes that

may negatively affect food finding, habitat choice or sexual partner selection (Baglan et al., 2018; Clotfelter et al., 2004; Lam et al., 2010; McCallum et al., 2013), with possible transgenerational effects (Giraudo et al., 2017b; Li et al., 2018; Oliveira-Filho et al., 2009). Epigenetic modifications are notably evoked as possible mechanism entailed (Novo et al., 2018; Schwindt, 2015) but, as in vertebrates, the identification of the precise mechanisms involved, as well as their implication in invertebrates require deeper investigations. Among future challenges, it is necessary to accurately identify all involved mechanisms, in order to better characterize organisms as well as populations response to multiple environmental stressors.

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Figure 1. Main molecular regulatory pathways susceptible to endocrine disruption in protosomes; some are shared with deuterostomes (dark blue background), while others are specific to invertebrates (light blue background). Outer colored rings indicate the groups in which corresponding orthologs have been evidenced. Hatched display points out non-functional receptors (insensitivity to ligand), and a question mark signifies that an ortholog has been described but its functional activity needs further confirmation. In italic font below each molecular pathway, are listed examples of EDCs strongly suspected to interfere with the corresponding molecular pathway in protostomes (IGD, insect growth disruptors; BPA, bisphenol A; BPS, bisphenol S; NP, nonylphenol; DEHP, di-ethylhexyl phthalate; BBP, benzyl butyl phthalate; E2, 17β-estradiol; EE2, 17α-ethinylestradiol; TBT, tributyltin; TPT, triphenyltin).